UCLA
UCLA Electronic Theses and Dissertations

Title
Mechanisms and Heterogeneity of Stress-Enhanced Fear Learning

Permalink
https://escholarship.org/uc/item/5nz6v3m9

Author
Gonzalez, Sarah Therese

Publication Date
2021

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

UNIVERSITY OF CALIFORNIA

Los Angeles

Mechanisms and Heterogeneity of Stress-Enhanced Fear Learning

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Psychology

by

Sarah Therese Gonzalez

2021

© Copyright by

Sarah Therese Gonzalez

2021

ABSTRACT OF THE DISSERTATION

Mechanisms and Heterogeneity

of Stress-Enhanced Fear Learning

by

Sarah Therese Gonzalez

Doctor of Philosophy in Psychology

University of California, Los Angeles, 2021

Professor Michael S. Fanselow, Chair

Fear is critical for survival but can become harmful when it is disproportionate to the level of threat present. Post-traumatic stress disorder (PTSD) is characterized by such dysregulation of fear and is frequently comorbid with substance use disorders (SUDs), particularly alcohol abuse. However, the factors that mediate susceptibility to these conditions and the biological mechanisms that support these conditions are unclear. Stress-enhanced fear learning (SEFL) is a rodent model of stress exposure that captures the sensitization of fear learning seen in PTSD patients. In this dissertation I used the SEFL model to investigate the factors that govern stress susceptibility as well as the potential role of increased levels of Ca^{2+}-permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in supporting enhanced fear learning following stress.

PTSD is twice as common in women compared to men, yet the vast majority of rodent studies investigating this condition have been conducted exclusively in males. In Chapter 1 I investigated sex differences in responses to an intense, acute stressor (15 footshocks over 90 minutes). I found that male and female rats showed similar changes in fear learning following stress, while some aspects of anxiety-related behavior were differentially impacted by stress. While levels of the GluA1 subunit of the AMPA receptor were altered within the basolateral amygdala (BLA) of male rats, no changes in GluA1 levels were observed in females.

In Chapter 2 I demonstrated that a reduced severity stressor (4 footshocks over 90 minutes) revealed distinct susceptible and resilient groups in both male and female rats. Susceptible male and female rats showed increased fear and anxiety compared to their resilient counterparts. Some aspects of stress susceptibility manifested differently in males compared to females, with susceptibility associated with increased alcohol intake in males and increased baseline anxiety in females.

In Chapter 3 I tested the role of Ca^{2+}-permeable AMPA receptors in supporting changes in fear learning following stress. I found that neither overexpression of GluA1 nor genetic deletion of GluA2 was sufficient to enhance fear learning in the absence of stress. However, GluA2 deletion paired with prior footshock exposure enhanced subsequent fear learning in male mice but not females. Collectively, these findings indicate that while males and females show similar changes in fear learning following stress, these behavioral changes may be supported by different mechanisms. These results provide insight into the differential impacts of stress on behaviors relevant to PTSD symptomology across sexes.

The dissertation of Sarah Therese Gonzalez is approved.

Alicia Izquierdo Edler

Pamela J. Kennedy

Igor Spigelman

Michael S. Fanselow, Committee Chair

University of California, Los Angeles

2021

DEDICATION

This dissertation is dedicated to my Aunt Alison,

Who never failed to make the world around her

A little bit brighter.

TABLE OF CONTENTS

LIST OF TABLES VIII

LIST OF FIGURES IX

ACKNOWLEDGEMENTS XI

VITA XIII

INTRODUCTION 1
References 15

CHAPTER 1 23
Abstract 24
Introduction 25
Experiment 1: Sex differences in fear learning, anxiety and alcohol intake following stress exposure 28
Experiment 2: Effects of stress exposure in the absence of prior alcohol access 44
Experiment 3: Effects of environmental enrichment and stress on alcohol intake 53
Discussion 62
Conclusion 71
References 72

CHAPTER 2 79
Abstract 80
Introduction 81
Experiment 1: Characterization of distinct susceptible and resilient populations following stress exposure 84
Experiment 2: Enhancement of fear to a novel aversive stimulus following extinction of fear generalization 104
Discussion 108
Conclusion 113
Supplementary Materials 114
References 119

CHAPTER 3 125
Abstract 126
Introduction 127

Experiment 1: Effects of GluA1 overexpression on fear learning in the absence of stress. 129
Experiment 2: Stress-enhanced fear learning in floxed GluA2 mice 136
Experiment 3: Impact of BLA GluA2 deletion on fear learning 141
Discussion 152
Conclusion 156
References 157

DISCUSSION 161
References 179

LIST OF TABLES

Chapter 1

Table 1-1:	Timeline for Experiments 1 and 2.	29
Table 1-2:	Timeline for Experiment 3.	54

Chapter 2

Table 2-1:	Timeline for Experiment 1.	86
Table 2-2:	Summary of results indicating distinct Susceptible phenotypes.	109
Table 2-3:	Comparison of Susceptible and Resilient subjects with No Stress subjects run through the same procedures.	118

LIST OF FIGURES

Chapter 1

Figure 1-1	Experiment 1 baseline alcohol intake and anxiety results.	36
Figure 1-2	Experiment 1 fear learning results.	38
Figure 1-3	Experiment 1 open field test results.	40
Figure 1-4	Experiment 1 elevated plus maze and light-dark transition test results.	41
Figure 1-5	Experiment 1 intermittent access 2-bottle choice results	42
Figure 1-6	Experiment 1 Western blot results showing ratio of GluA1 to total protein at 123 KDa within the BLA.	43
Figure 1-7	Experiment 2 pre-stress light-dark transition test results.	46
Figure 1-8	Experiment 2 fear learning results.	48
Figure 1-9	Experiment 2 anxiety test results.	50
Figure 1-10	Experiment 2 intermittent access 2-bottle choice results.	51
Figure 1-11	Experiment 2 Western blot results showing ratio of GluA1 to total protein at 123 KDa within the BLA.	52
Figure 1-12	Experiment 3 SEFL Results.	56
Figure 1-13	Experiment 3 open field test results from subjects in the Enrichment condition	58
Figure 1-14	Experiment 3 results of elevated plus maze and light-dark transition test.	59
Figure 1-15	Experiment 3 acoustic stimulus results.	60
Figure 1-16	Experiment 3 intermittent access 2-bottle choice results	61

Chapter 2

Figure 2-1	Stress-enhanced fear learning following different levels of stress.	93
Figure 2-2	Susceptible and Resilient subjects differ on multiple aspects of fear learning.	95
Figure 2-3	Susceptible subjects show increased anxiety-like behavior following stress exposure.	98
Figure 2-4	Results of intermittent 2-bottle choice following stress exposure	100

Figure 2-5 Baseline levels of alcohol consumption and anxiety-like behavior. 102

Figure 2-6 Experiment 2 results. 107

Figure 2-7 Distribution of SEFL Test scores in males versus females following different levels of stress. 116

Figure 2-8 Performance on light-dark transition test prior to and following stress. 117

Chapter 3

Figure 3-1 Experiment 1 fear conditioning results. 133

Figure 3-2 Experiment 1 histology results. 135

Figure 3-3 Experiment 2 SEFL results. 140

Figure 3-4 Experiment 2 open field test results 141

Figure 3-5 Experiment 3 Context A fear conditioning results 146

Figure 3-6 Experiment 3 Context B fear conditioning results 148

Figure 3-7 Experiment 3 open field test results 149

Figure 3-8 Experiment 3 histology results. 151

ACKNOWLEDGEMENTS

Chapter 2 is a version of:

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*, 100335. https://doi.org/10.1016/j.ynstr.2021.100335.

Vincent Marty contributed to data collection for Western blot and intermittent access two-bottle choice experiments and manuscript revisions, Igor Spigelman contributed to conceptualization and manuscript revisions, Steven P. Reise contributed to data analysis and manuscript revisions, Michael S. Fanselow served as the Principal Investigator. I would also like to thank Chiana Yang, Kareem Ahmed, Sonia Lele, Riley Vo and Inga Yenokian for their contributions to this work.

I would like to thank Dr. Bryce Vissel for the donation of floxed GluA2 mice used in Chapter 3.

Financial support for this research was funded by NIH grants R01-AA026530 (Michael S. Fanselow, Igor Spigelman), NSF Graduate Research Fellowship DGE-1650604 and the Graduate Division Dissertation Year Fellowship (Sarah T. Gonzalez), and the Staglin Center for Brain and Behavioral Health (Michael S. Fanselow).

This work would not have been possible without the incredible support and guidance from my mentor Michael Fanselow. I am forever grateful for the wisdom and guidance you have shared over the years, which has helped me grow as a scientist more than I ever imagined. Thank you to my dissertation committee members Alicia Izquierdo, Pamela Kennedy and Igor Spigelman for their support and insight. Special thank you to Vincent Marty and all the members of the Spigelman Lab who helped make these experiments possible. Lastly, thank you to all of the past and current members of the Fanselow Lab who have been an invaluable source of support, advice and laughter.

VITA

Education

2018	Advanced to Candidacy, Ph.D. in Psychology *University of California, Los Angeles, CA*
2015	Master of Arts, Psychology *University of California, Los Angeles, CA*
2013	Bachelor of Science with Honors, Psychology *Brown University, Providence, RI*

Publications

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

S Gonzalez. (2021). The Cognitive Abilities of Crows. *Psychology in Action*.

AK Rajbhandari, JC Octeau, S Gonzalez, ZT Pennington, F Mohamed, J Trott, ... & MS Fanselow. (2021). A basomedial amygdala to intercalated cells microcircuit expressing PACAP and its receptor PAC1 regulates contextual fear. *Journal of Neuroscience, 41*(15), 3446-3461. https://doi.org/10.1523/JNEUROSCI.2564-20.2021.

S Gonzalez. (2021). Learning Under Pressure: How Stress Alters Memory. *Psychology in Action*.

AN Hoffman, SL Watson, AS Makridis, AY Patel, ST Gonzalez, L Ferguson... & MS Fanselow. (2020). Sex Differences in Behavioral Sensitivities After Traumatic Brain Injury. *Frontiers in neurology, 11*. https://doi.org/10.3389/fneur.2020.553190.

ST Gonzalez, MS Fanselow (2020). The role of the ventromedial prefrontal cortex and context in regulating fear learning and extinction. *Psychology & Neuroscience, 13*(3), 459-472. https://doi.org/10.1037/pne0000207.

E Yavas, S Gonzalez, MS Fanselow. (2019). Interactions between the hippocampus, prefrontal cortex, and amygdala support complex learning and memory. *F1000Research, 8.* https://doi.org/10.12688/f1000research.19317.1.

AK Rajbhandari, ST Gonzalez, MS Fanselow. (2018). Stress-enhanced fear learning, a robust rodent model of post-traumatic stress disorder. *Journal of Visualized Experiments, 140,* e58306, https://doi.org/10.3791/58306.

ST Gonzalez, D Remick, R Creton, RM Colwill. (2016). Effects of embryonic exposure to polychlorinated biphenyls (PCBs) on anxiety-related behaviors in larval zebrafish. *Neurotoxicology, 53,* 93-101. https://doi.org/10.1016/j.neuro.2015.12.018.

ST Gonzalez. (2015). The Neuroscience of Altruism. *Psychology in Action*.

ST Gonzalez, ES Welch, RM Colwill. (2013). Pavlovian contextual and instrumental biconditional discrimination learning in mice. *Behavioural brain research, 256,* 398-404. https://doi.org/10.1016/j.bbr.2013.09.010.

Presentations

ST Gonzalez, V Marty, S Lele, R Vo, I Yenokian, CQ Yang, K Ahmed, I Spigelman, MS Fanselow. "Influences of stress severity and sex on changes in fear learning, anxiety and alcohol consumption following stress exposure"
Society for Neuroscience, October 19-23, 2019, Chicago, Illinois

ST Gonzalez, AK Rajbhandari, J Chavez, E Nguyen, N Keces, MS Fanselow. "Blockade of corticotropin-releasing factor type 1 receptors in the basolateral amygdala decreases post-shock freezing but does not prevent stress-enhanced fear learning"
Society for Neuroscience, November 3-7, 2018, San Diego, California.

AK Rajbhandari, ST Gonzalez, J Perusini, V Makhijani, Y Huang, AN Hoffman, J Waschek, MS Fanselow. "Acute traumatic stress in a rodent model of PTSD (SEFL) produces fear sensitization, increased anxiety and a longlasting increase in GLUA1 protein levels in the basolateral amygdala"
Society for Neuroscience, November 11-15, 2017, Washington, D.C.

ST Gonzalez, MS Fanselow. "The neural mechanisms of safety learning in mice"
Pavlovian Society Meeting, September 17-19, 2015, Portland, Oregon

RM Colwill, S Gonzalez, E Welch. "Biconditional Discrimination Learning in the C57BL/6J Mouse"
Eastern Psychological Association Conference, March 1-3, 2013, New York, New York

S Welch, S Gonzalez, D Remick, R Creton, R Colwill. "Embryonic Exposure to Environmentally Relevant Concentrations of Polychlorinated Biphenyls (PCBs) Affects Startle Response in Zebrafish Larvae"
Eastern Psychological Association Conference, March 1-3, 2013, New York, New York

INTRODUCTION

Fear is critical for survival, as the ability to learn about and recognize threats in the environment allows us to prepare for these threats in the future. However, fear can become harmful when it is disproportionate to the level of threat present, giving rise to maladaptive behaviors such as hypervigilance and avoidance. Exposure to stress or trauma can produce such dysregulation of fear and plays a key role in the development of many psychiatric disorders that are characterized by improper or excessive amounts of fear and anxiety, such as post-traumatic stress disorder (PTSD). Understanding how stress alters fear learning is crucial for treating conditions like PTSD. In this dissertation I used a rodent model of stress exposure to investigate the factors that govern stress susceptibility, as well the biological mechanisms that support changes in fear learning following stress.

Post-traumatic stress disorder (PTSD)

PTSD is an anxiety disorder that develops after exposure to traumatic events such as combat, crime or natural disaster. It has been estimated that approximately one third of the population will experience a trauma during their lifetime, and approximately 10-20% of these individuals will develop PTSD (Brunello et al., 2001). PTSD produces a number of symptoms that can severely impact quality of life. To be diagnosed with PTSD, an individual must show symptoms in the following categories for at least one month: re-experiencing the trauma, avoidance, hyperarousal and changes in mood or cognition (APA, 2013). PTSD is also highly comorbid with substance use disorder (SUDs) with alcohol abuse being the most common (Kessler et al., 1995).

Despite the burden of this disorder, its neural mechanisms and the relationships between PTSD and substance abuse are not fully understood and currently available treatments such as exposure therapy are not always effective (Craske et al., 2008; Hembree et al., 2003). A better understanding of the neural mechanisms of PTSD, the factors that determine susceptibility to the disorder and the relationship between PTSD and substance abuse will enable the development of more effective treatments.

Comorbidity between PTSD and alcohol abuse

PTSD is often co-morbid with substance use disorders (SUDs). Multiple studies have reported a three- to five-fold increase in the occurrence of SUDs among PTSD patients relative to individuals without PTSD, with alcohol abuse being the most common (Mills et al., 2006; Perkonigg et al., 2000).

An important question is the nature of the relationship between PTSD and alcohol abuse. There is some evidence that alcohol abuse increases the risk for experiencing a traumatic event such as physical injury or accident (McFarlane, 1998). However, this seems insufficient to explain the high rates of comorbidity as PTSD is generally more likely to precede rather than follow substance abuse (McFarlane, 1998). Instead, it has long been proposed that the increased rates of alcohol abuse among individuals with PTSD is driven by attempts to self-medicate with drugs such as alcohol to alleviate PTSD symptoms such as hyperarousal and anxiety (Kosten & Krystal, 1988; Leeies et al., 2010; Turner et al., 2018). Alcohol in particular has been reported to suppress stress reactivity and may decrease some aspects of the stress response via its action on the locus coeruleus, a site of norepinephrine release (Brick & Pohorecky, 1983; McFarlane, 1998; Strahlendorf & Strahlendorf, 1983), making it a strong candidate for abuse. In support of

this, a recent study found that optogenetic locus coeruleus stimulation increases alcohol drinking (Deal et al., 2020).

Dysregulation of fear in PTSD

One characteristic of PTSD is an exaggerated response to mild stressors that are reminiscent of the original trauma, leading to inappropriate fear responses (Bremner et al., 1995; Dykman et al., 1997). While the ability to detect and respond to threats in the environment is important for survival, the increased arousal and avoidance of trauma-related stimuli that characterizes PTSD can lead to exhaustion, sleep disturbances, and a constricted lifestyle (Eberly & Engdahl, 1991).

As well as experiencing intrusive symptoms during their daily lives, PTSD patients often show altered fear learning under laboratory conditions. Fear learning in the laboratory is typically studied using Pavlovian fear conditioning, in which an initially neutral conditional stimulus (CS) is paired with an aversive unconditional stimulus (US), such as a mild shock (Maren, 2001). Following this pairing, presentations of the CS alone are sufficient to elicit a fear response, which in human participants can be measured by increases in the skin conductance response (Lovibond, 1992; Orr et al., 2000; Vervliet et al., 2004) or by fear-potentiated startle, which reflects the ability of the CS to enhance a startle response (Falls et al., 1992; Fani et al., 2012; Norrholm et al., 2011). These measures have revealed that PTSD patients show elevated fear learning compared to trauma-exposed individuals that do not experience PTSD (Fani et al., 2012; Gamwell et al., 2015; Norrholm et al., 2011; Orr et al., 2000; Peri et al., 2000), as well as increased generalization of this fear to stimuli that do not signal threat (Morey et al., 2015; Norrholm et al., 2011).

PTSD is also characterized as an impaired ability to suppress fear when no threat is present. One example of this is fear extinction, in which a stimulus previously conditioned to elicit a fear response is repeatedly presented alone until it no longer elicits a fear response (Myers & Davis, 2007). Individuals with PTSD show impaired acquisition of fear extinction as well as impaired retrieval of fear extinction at a later time point (Milad et al., 2008; Norrholm et al., 2011; Orr et al., 2000; Peri et al., 2000). It should be noted that in these studies it is often difficult to disentangle effects of enhanced learning and impaired extinction, as PTSD patients tend to show higher fear levels at the start of extinction, e.g., Norrholm et al (2011). However, collectively these results demonstrate that dysregulation of fear learning is an important component of PTSD.

Heterogeneity within PTSD

Stress susceptibility and resilience

An important question to answer is why some individuals develop PTSD and others do not. While the odds of an individual experiencing a trauma at some point in their lifetime are distressingly high, only a small fraction of those people will go on to develop PTSD (Kessler et al., 2017; Kessler et al., 1995). Furthermore, not all individuals with PTSD display the same types or intensity of symptoms. (Brunello et al., 2001; Hopper et al., 2007). For example, a series of studies by Lanius and colleagues found that approximately 70% of the PTSD patients showed hyperarousal and flashbacks when recalling traumatic experiences, while the other 30% did not show such responses and instead experienced dissociative symptoms such as derealization or "zoning out" (Lanius et al., 2006; Lanius et al., 2002; Lanius et al., 2001). This suggests that

there are underlying factors that render individuals to be susceptible or resilient to the effects of stress, but these factors are poorly understood.

Sex differences in PTSD and SUDs

Substantial differences in the rates of PTSD and SUDs between men and women indicate that gender may be one factor that mediates stress susceptibility, yet the role of sex differences mediating stress susceptibility is severely understudied as the majority of rodent stress research has been conducted exclusively in males (Beery & Zucker, 2011; Shansky, 2015). PTSD is roughly twice as common in women compared to men (Kessler et al., 2017; Kessler et al., 1995). Conversely, SUDs including alcohol abuse are more common in men than in women (Kessler et al., 2005) though this gap may be narrowing (Keyes et al., 2008).

It remains unclear why these disorders occur so differently across genders. One potential explanation is that men and women tend to experience different types of trauma. The traumas most strongly associated with the development of PTSD tend to be combat and physical assault or injury in men and sexual abuse, sexual assault and intimate partner violence in women (Goldstein et al., 2016; Kessler et al., 1995). Sexual assault and intimate partner violence are most strongly associated with the development of PTSD, suggesting that perhaps the gender differences in rates of PTSD are partially due to the types of trauma men and women tend to experience (Goldstein et al., 2016; Kessler et al., 2017). However, higher rates of PTSD are still observed in women after controlling for trauma type (Breslau et al., 1997; Kessler et al., 2017), indicating that additional factors are also at play.

Gender also appears to play an important role in mediating the relationship between PTSD and SUDs. Overall, men show higher rates of SUDs including alcohol abuse compared to

women and a higher proportion of men with PTSD also show comorbid SUDs (Conway et al., 2006; Kessler et al., 2005). However, despite showing overall lower rates of SUDs, women who do experience SUD are more likely to also have comorbid PTSD compared to men with SUD (Kessler et al., 1997). Gender is therefore a likely mediator of both susceptibility to PTSD and SUD and the relationships between the two conditions.

To summarize, the relationships between PTSD and SUDs are complex, and there are many factors that contribute to the development of PTSD. This dissertation will utilize a rodent model of PTSD to address several key questions pertaining to these factors. First, do males and females show different behavioral and biological changes following stress? Second, what are the factors that mediate stress susceptibility? Specifically, are females more likely to show stress-induced behavioral changes compared to males, and do other pre-existing differences such as baseline anxiety levels or alcohol intake predict future stress susceptibility? Lastly, what are the changes within the brain that support behavioral changes following stress and do these differ between males and females? The approaches that will be used to answer such questions are described in the following sections.

The stress response

Understanding how stress contributes to the development of PTSD and SUDs requires understanding how stress affects the body. Stress is commonly defined as a state of real or perceived threat to homeostasis. To prepare for the incoming threat, the body mobilizes a combination of endocrine, nervous, and immune system responses that are collectively known as the stress response. A key mediator of the stress response is the hypothalamus-pituitary-adrenal (HPA) axis, a cascading system of endocrine signals that ultimately promotes a host of

behavioral and biological changes (Smith & Vale, 2006). In response to stress, neurons in the paraventricular nucleus (PVN) of the hypothalamus first release corticotropin-releasing factor (CRF), which stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. ACTH in turn travels to the adrenal gland, where it stimulates the synthesis and release of glucocorticoids (primarily cortisol in humans and corticosterone in rodents). This glucocorticoid release is the main output of the HPA axis and mediates numerous behavioral and physiological changes throughout the body.

The HPA axis has a considerable impact on brain regions involved in learning and memory. The limbic system, which includes the hippocampus and amygdala, is the main anatomical substrate for learning and memory formation as well as emotional responses (Anagnostaras et al., 2001; Fanselow & Kim, 1994; Fendt & Fanselow, 1999; Maren et al., 1994). The hippocampus is particularly important for declarative memory in humans and spatial memory in both humans and rodents (Anagnostaras et al., 2001; Broadbent et al., 2004; Kim et al., 1993). Hippocampal function is highly sensitive to the effects of stress. For example, moderate stress exposure has been shown to enhance spatial learning (Conboy & Sandi, 2010; Luine et al., 1996; Sandi et al., 1997) while chronic stress or exposure to high amounts of glucocorticoids have been shown to impair spatial learning (Conrad et al., 1996; Conrad et al., 1999; Dachir et al., 1993; Luine et al., 1994) and cause atrophy of hippocampal neurons (Conrad et al., 1999; Lakshminarasimhan & Chattarji, 2012; Pavlides et al., 2002). The hippocampus also provides feedback inhibition of the HPA axis, as the binding of glucocorticoids to glucocorticoid receptors within the hippocampus play a role in terminating the stress response (Jacobson & Sapolsky, 1991).

The amygdala is heavily interconnected with the hippocampus and is central to learning about the emotional valence of stimuli. The amygdala is most well-known for its key role in threat detection and fear learning (Fendt & Fanselow, 1999), though it is also important for learning about the value of rewards (Baxter & Murray, 2002; Wassum & Izquierdo, 2015). Frequently referred to as a threat detector, the amygdala is important for triggering the activation of the HPA axis. The amygdala itself is also highly sensitive to stress, as stress exposure has been shown to enhance fear learning (Conrad et al., 1999; Cordero et al., 2003; Rau et al., 2005) and increase the density of dendritic spines within the amygdala (Lakshminarasimhan & Chattarji, 2012; Vyas et al., 2002). These findings indicate that stress-induced changes in amygdala activity may underlie the changes in fear learning observed in PTSD patients.

Stress-enhanced fear learning (SEFL)

The SEFL model

Our laboratory has developed a rodent model of stress exposure termed stress-enhanced fear learning (SEFL) that uses aspects of Pavlovian fear conditioning to capture the exaggerated fear responses observed in PTSD patients. In standard Pavlovian fear conditioning, a neutral conditional stimulus (CS), such as a context, is paired with an aversive unconditional stimulus (US), such as a footshock. Following the formation of a CS-US association, the CS will elicit a fear response. In the SEFL model, animals are exposed to an intense acute stressor (typically 15 unsignaled footshocks for rats, 10 footshocks for mice) in a distinct context, followed by a single footshock in a different context (Rau et al., 2005). Following trauma exposure animals show exaggerated fear to the single footshock context. This enhancement of fear learning is the defining feature of the SEFL model and appears to reflect a nonassociative sensitization of the

fear learning process. SEFL cannot be explained by generalization of fear from the stress to novel situations, as previous studies from our laboratory show that extinction of fear to the stress context does not mitigate SEFL (Long & Fanselow, 2012; Rau et al., 2005) and manipulations that produce amnesia of the trauma leave SEFL unaffected (Poulos et al., 2014; Rau et al., 2005).

While the SEFL model is characterized by enhanced fear learning to a mild stressor that is reminiscent of the original stressor, it also produces a number of other behavioral changes reflective of PTSD symptomology including extreme fear of stimuli associated with the traumatic event, increased anxiety, increased startle reactivity and altered glucocorticoid signaling (Pennington et al., 2017; Perusini et al., 2016; Poulos et al., 2015). Lastly, the SEFL model produces a long-lasting increase in alcohol drinking, reflecting the comorbidity between PTSD and substance abuse (Meyer et al., 2013). Importantly, we have demonstrated enhanced fear learning in the SEFL model in both male and female rodents (Poulos et al., 2015). The SEFL model is therefore a powerful tool for studying sex differences in stress reactivity across a variety of behavioral domains.

The need for studies of stress susceptibility in females

Rodent models of stress exposure that reproduce aspects of PTSD symptomology are a powerful tool for understanding the biological mechanisms of this disorder. However, despite the fact that PTSD is twice in common in women than in men, the overwhelming majority of research using rodent models of PTSD has been conducted exclusively in males (Beery & Zucker, 2011). There is also a need for rodent models that can capture the variability seen in PTSD (Shansky, 2015). To date there has been relatively little investigation of the individual factors that promote susceptibility versus resilience to the effects of stress. Many of the studies

that do investigate susceptibility versus resilience are again only conducted in males (Colucci et al., 2020; Covington et al., 2010; Jeong et al., 2020; Ritov et al., 2016). For example, the social defeat stress model is the most commonly used to probe the factors that promote stress susceptibility and resilience, but since female rodents typically do not show the same aggressive behaviors as males the use of this model is limited to males (Covington et al., 2010; Golden et al., 2011). As a result, there is a serious lack of research that investigates individual susceptibility factors in both males and females.

Stress susceptibility and resilience in the SEFL model

The standard 15-footshock stress used in the SEFL model produces a reliable, robust enhancement in fear learning in nearly all subjects without the need for separating subjects into susceptible or resilient groups (Rau et al., 2005; Rau & Fanselow, 2009). While this makes the SEFL model a powerful tool for probing the mechanisms of PTSD, it makes it difficult to probe the underlying factors that may promote stress susceptibility or resilience. However, closer inspection of previous studies reveals that a small proportion of stress-exposed subjects do fail to show a substantial enhancement in fear learning relative to unstressed controls, suggesting that individual differences in stress susceptibility may influence performance in the SEFL model. One advantage of the SEFL model is that its parameters are uniquely quantifiable and can be easily altered to allow such underlying differences to have a greater impact. Many other rodent models of stress exposure use unquantifiable stressors such as social defeat stress, predator odor, or combinations of multiple stressors (Cohen et al., 2006; Golden et al., 2011; Willner et al., 1987). In contrast, the intensity of the SEFL stressor can be readily manipulated by changing the number or amplitude of footshocks. By reducing the stressor severity through decreasing the

number of footshocks from 15 to 4, it may be possible to uncover distinct susceptible and resilient populations in both male and female subjects.

The fear learning network

It is likely that the changes that support SEFL and the elevated fear seen in PTSD involves changes in the neural network that underlies fear conditioning, of which the basolateral amygdala (BLA) is a key component (Fendt & Fanselow, 1999). During fear conditioning, projections conveying sensory information from the sensory cortex and thalamus converge onto pyramidal neurons of the BLA. When activated by a stimulus signaling danger, the BLA drives a fear response via its projections to the central amygdala (CeA), which in turn projects to targets in the brainstem, midbrain and hypothalamus that produce specific fear responses such as freezing.

While the inputs carrying information about the neutral conditional stimulus are initially too weak to activate the BLA, during fear conditioning these connections become strengthened such that presentations of the previously neutral stimulus acquire the ability to activate the BLA (Kim & Jung, 2006; Rogan et al., 1997). This synaptic strengthening occurs via long-term potentiation (LTP), a process typically triggered by Ca^{2+} influx through N-methyl-D-aspartate (NMDA) glutamate receptors (Bliss & Collingridge, 1993; Maren, 1999). Consequently, blockade of NDMA receptors (NMDARs) during conditioning prevents the acquisition of fear (Fanselow & Kim, 1994; Kim et al., 1992). Importantly, the ion channels of NDMARs are typically blocked by Mg^{2+} ions and only allow Ca^{2+} entry when the channel is bound by presynaptic glutamate release and the Mg^{2+} block is removed by sufficient postsynaptic

depolarization. Thus NMDARs act as a coincidence detector, only allowing LTP to occur when both of these conditions are met.

The potential role of Ca^{2+}-permeable AMPA receptors in SEFL

An important open question concerns the mechanisms through which stress potentiates future fear learning. While the formation of fear memories is typically thought to be contingent on activation of NMDARs, increasing evidence suggests that under certain circumstances the BLA connections that support fear learning can be strengthened though an alternative, NMDAR-independent process.

α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are ionotropic glutamate receptors that mediate most of the fast excitatory transmission within the central nervous system and are composed of four subunits (GluA1-GluA4) (Lee et al., 2003; Malinow, 2003). In the adult mammalian brain AMPA receptors (AMPARs) typically exist as heteromers of either GluA1 and GluA2 or GluA2 and GluA3, with the GluA2 subunit rendering the ion channel impermeable to Ca^{2+} (Isaac et al., 2007). However, increased GluA1 levels can result in the formation of GluA1-only receptors (Hollmann et al., 1991). Because these receptors lack the GluA2 subunit, they are permeable to Ca^{2+} entry and could potentially trigger LTP without the aid of NDMA receptors. These Ca^{2+}-permeable AMPARs (CP-AMPARs) could therefore enhance learning and memory formation by augmenting canonical synaptic plasticity. In support of this possibility, mice with genetic deletion of the GluA2 subunit in the CA1 region of the hippocampus display a novel, NDMAR-independent form of LTP and are impaired on NDMAR-dependent learning tasks while showing no impairment on NMDAR-independent learning tasks (Wiltgen et al., 2010).

Our laboratory has recently demonstrated increased expression of the GluA1 AMPAR subunit in the BLA following stress exposure in male rats (Perusini et al., 2016). This increase appears to have some functional significance, as inhibition of corticosterone synthesis prior to stress exposure has been shown to block both the increase in BLA GluA1 levels and prevent enhancements in fear learning following stress. Given the results of Wiltgen et al (2010), this leads to the intriguing possibility that stress exposure increases CP-AMPARs within the BLA, augmenting canonical plasticity and ultimately enhancing fear learning. However, it has not yet been demonstrated whether an increase in CP-AMPARs within the BLA is sufficient to support enhanced fear learning.

Dissertation objectives

The objectives of this dissertation were to answer three main questions. First, how do males and females differ in stress reactivity? Second, what are the factors that mediate stress susceptibility? Third, what are the biological mechanisms that support changes in fear learning following stress? **In Chapter 1, I investigated whether males and females show similar changes in PTSD-related behaviors and whether they show similar changes in BLA GluA1 levels following stress exposure.** I hypothesized that females would show greater changes in fear and anxiety following stress, capturing the greater prevalence of PTSD among women. In contrast, I hypothesized that males would show greater changes in alcohol drinking, capturing the greater prevalence of SUDs among men. I found that while males and females showed similar changes in fear learning following stress, differences were observed in anxiety-related behavior and alcohol drinking. I further found that stress altered BLA GluA1 levels in males but not females.

In Chapter 2, I investigated whether using a modified version of the SEFL procedure (4 footshocks instead of the standard 15) would enable the emergence of distinct susceptible and resilient populations. I hypothesized that susceptible subjects would show a constellation of behavioral changes relevant to PTSD symptomology, and that baseline levels of anxiety and alcohol drinking would predict subsequent stress susceptibility. I found that this modified procedure revealed distinct susceptible and resilient populations and while some aspects of stress susceptibility were shared across sexes, other aspects manifested differently in males and females.

In Chapter 3, I tested the role of Ca^{2+}-permeable AMPA receptors in supporting stress-enhanced fear learning in both males and females. I hypothesized that increasing the availability of GluA1 subunits relative to GluA2 subunits within the BLA, either through overexpression of GluA1 or suppression of GluA2, would be sufficient to enhance fear learning in the absence of stress. I found that increasing GluA1 availability alone was insufficient to enhance fear learning, but suppression of GluA2 combined with a history of mild stress exposure selectively enhanced susceptibility to a future stressor in males but not females.

References

Anagnostaras, S. G., Gale, G. D., & Fanselow, M. S. (2001). Hippocampus and contextual fear conditioning: recent controversies and advances. *Hippocampus*, *11*(1), 8-17. https://doi.org/10.1002/1098-1063(2001)11:1<8::AID-HIPO1015>3.0.CO;2-7

APA. (2013). *Diagnostic and statistical manual of mental disorders (DSM-5)*. American Psychiatric Pub.

Baxter, M. G., & Murray, E. A. (2002). The amygdala and reward. *Nat Rev Neurosci*, *3*(7), 563-573. https://doi.org/10.1038/nrn875

Beery, A. K., & Zucker, I. (2011). Sex bias in neuroscience and biomedical research. *Neurosci Biobehav Rev*, *35*(3), 565-572. https://doi.org/10.1016/j.neubiorev.2010.07.002

Bliss, T. V., & Collingridge, G. L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. *Nature*, *361*(6407), 31-39. https://doi.org/10.1038/361031a0

Bremner, J. D., Krystal, J. H., Southwick, S. M., & Charney, D. S. (1995). Functional neuroanatomical correlates of the effects of stress on memory. *J Trauma Stress*, *8*(4), 527-553.

Breslau, N., Davis, G. C., Andreski, P., Peterson, E. L., & Schultz, L. R. (1997). Sex differences in posttraumatic stress disorder. *Arch Gen Psychiatry*, *54*(11), 1044-1048. https://doi.org/10.1001/archpsyc.1997.01830230082012

Brick, J., & Pohorecky, L. A. (1983). Effect of ethanol on surface body temperature as measured by infrared radiation detection. *Psychopharmacology (Berl)*, *81*(3), 244-246. https://doi.org/10.1007/BF00427271

Broadbent, N. J., Squire, L. R., & Clark, R. E. (2004). Spatial memory, recognition memory, and the hippocampus. *Proc Natl Acad Sci U S A*, *101*(40), 14515-14520. https://doi.org/10.1073/pnas.0406344101

Brunello, N., Davidson, J. R., Deahl, M., Kessler, R. C., Mendlewicz, J., Racagni, G., Shalev, A. Y., & Zohar, J. (2001). Posttraumatic stress disorder: diagnosis and epidemiology, comorbidity and social consequences, biology and treatment. *Neuropsychobiology*, *43*(3), 150-162. https://doi.org/10.1159/000054884

Cohen, H., Zohar, J., Gidron, Y., Matar, M. A., Belkind, D., Loewenthal, U., Kozlovsky, N., & Kaplan, Z. (2006). Blunted HPA axis response to stress influences susceptibility to posttraumatic stress response in rats. *Biol Psychiatry*, *59*(12), 1208-1218. https://doi.org/10.1016/j.biopsych.2005.12.003

Colucci, P., Marchetta, E., Mancini, G. F., Alva, P., Chiarotti, F., Hasan, M. T., & Campolongo, P. (2020). Predicting susceptibility and resilience in an animal model of post-traumatic stress disorder (PTSD). *Transl Psychiatry*, *10*(1), 243. https://doi.org/10.1038/s41398-020-00929-9

Conboy, L., & Sandi, C. (2010). Stress at learning facilitates memory formation by regulating AMPA receptor trafficking through a glucocorticoid action. *Neuropsychopharmacology*, *35*(3), 674-685. https://doi.org/10.1038/npp.2009.172

Conrad, C. D., Galea, L. A., Kuroda, Y., & McEwen, B. S. (1996). Chronic stress impairs rat spatial memory on the Y maze, and this effect is blocked by tianeptine pretreatment. *Behav Neurosci*, *110*(6), 1321-1334.

Conrad, C. D., LeDoux, J. E., Magariños, A. M., & McEwen, B. S. (1999). Repeated restraint stress facilitates fear conditioning independently of causing hippocampal CA3 dendritic atrophy. *Behav Neurosci*, *113*(5), 902-913.

Conway, K. P., Compton, W., Stinson, F. S., & Grant, B. F. (2006). Lifetime comorbidity of DSM-IV mood and anxiety disorders and specific drug use disorders: results from the National Epidemiologic Survey on Alcohol and Related Conditions. J*ournal of Clinical Psychiatry*, *67*(2), 247-257.

Cordero, M. I., Venero, C., Kruyt, N. D., & Sandi, C. (2003). Prior exposure to a single stress session facilitates subsequent contextual fear conditioning in rats. Evidence for a role of corticosterone. *Horm Behav*, *44*(4), 338-345.

Covington, H. E., Lobo, M. K., Maze, I., Vialou, V., Hyman, J. M., Zaman, S., LaPlant, Q., Mouzon, E., Ghose, S., Tamminga, C. A., Neve, R. L., Deisseroth, K., & Nestler, E. J. (2010). Antidepressant effect of optogenetic stimulation of the medial prefrontal cortex. *J Neurosci*, *30*(48), 16082-16090. https://doi.org/10.1523/JNEUROSCI.1731-10.2010

Craske, M. G., Kircanski, K., Zelikowsky, M., Mystkowski, J., Chowdhury, N., & Baker, A. (2008). Optimizing inhibitory learning during exposure therapy. *Behav Res Ther*, *46*(1), 5-27. https://doi.org/10.1016/j.brat.2007.10.003

Dachir, S., Kadar, T., Robinzon, B., & Levy, A. (1993). Cognitive deficits induced in young rats by long-term corticosterone administration. *Behav Neural Biol*, *60*(2), 103-109.

Deal, A. L., Bass, C. E., Grinevich, V. P., Delbono, O., Bonin, K. D., Weiner, J. L., & Budygin, E. A. (2020). Bidirectional Control of Alcohol-drinking Behaviors Through Locus Coeruleus Optoactivation. *Neuroscience*, *443*, 84-92. https://doi.org/10.1016/j.neuroscience.2020.07.024

Dykman, R. A., Ackerman, P. T., & Newton, J. E. (1997). Posttraumatic stress disorder: a sensitization reaction. *Integr Physiol Behav Sci*, *32*(1), 9-18.

Eberly, R. E., & Engdahl, B. E. (1991). Prevalence of somatic and psychiatric disorders among former prisoners of war. *Hosp Community Psychiatry*, *42*(8), 807-813.

Falls, W. A., Miserendino, M. J., & Davis, M. (1992). Extinction of fear-potentiated startle: blockade by infusion of an NMDA antagonist into the amygdala. *J Neurosci*, *12*(3), 854-863.

Fani, N., Tone, E. B., Phifer, J., Norrholm, S. D., Bradley, B., Ressler, K. J., Kamkwalala, A., & Jovanovic, T. (2012). Attention bias toward threat is associated with exaggerated fear

expression and impaired extinction in PTSD. *Psychol Med*, *42*(3), 533-543. https://doi.org/10.1017/S0033291711001565

Fanselow, M. S., & Kim, J. J. (1994). Acquisition of contextual Pavlovian fear conditioning is blocked by application of an NMDA receptor antagonist D,L-2-amino-5-phosphonovaleric acid to the basolateral amygdala. *Behav Neurosci*, *108*(1), 210-212.

Fendt, A., & Fanselow, M. S. (1999). The neuroanatomical and neurochemical basis of conditioned fear. *Neuroscience & Biobehavioral Reviews*, *23*(5), 734-760.

Gamwell, K., Nylocks, M., Cross, D., Bradley, B., Norrholm, S. D., & Jovanovic, T. (2015). Fear conditioned responses and PTSD symptoms in children: Sex differences in fear-related symptoms. *Dev Psychobiol*, *57*(7), 799-808. https://doi.org/10.1002/dev.21313

Golden, S. A., Covington, H. E., Berton, O., & Russo, S. J. (2011). A standardized protocol for repeated social defeat stress in mice. *Nat Protoc*, *6*(8), 1183-1191. https://doi.org/10.1038/nprot.2011.361

Goldstein, R. B., Smith, S. M., Chou, S. P., Saha, T. D., Jung, J., Zhang, H., Pickering, R. P., Ruan, W. J., Huang, B., & Grant, B. F. (2016). The epidemiology of DSM-5 posttraumatic stress disorder in the United States: results from the National Epidemiologic Survey on Alcohol and Related Conditions-III. *Soc Psychiatry Psychiatr Epidemiol*, *51*(8), 1137-1148. https://doi.org/10.1007/s00127-016-1208-5

Hembree, E. A., Foa, E. B., Dorfan, N. M., Street, G. P., Kowalski, J., & Tu, X. (2003). Do patients drop out prematurely from exposure therapy for PTSD? *J Trauma Stress*, *16*(6), 555-562. https://doi.org/10.1023/B:JOTS.0000004078.93012.7d

Hollmann, M., Hartley, M., & Heinemann, S. (1991). Ca2+ permeability of KA-AMPA--gated glutamate receptor channels depends on subunit composition. *Science*, *252*(5007), 851-853.

Hopper, J. W., Frewen, P. A., van der Kolk, B. A., & Lanius, R. A. (2007). Neural correlates of reexperiencing, avoidance, and dissociation in PTSD: symptom dimensions and emotion dysregulation in responses to script-driven trauma imagery. *J Trauma Stress*, *20*(5), 713-725. https://doi.org/10.1002/jts.20284

Isaac, J. T., Ashby, M. C., & McBain, C. J. (2007). The role of the GluR2 subunit in AMPA receptor function and synaptic plasticity. *Neuron*, *54*(6), 859-871. https://doi.org/10.1016/j.neuron.2007.06.001

Jacobson, L., & Sapolsky, R. (1991). The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. *Endocr Rev*, *12*(2), 118-134. https://doi.org/10.1210/edrv-12-2-118

Jeong, M. J., Lee, C., Sung, K., Jung, J. H., Pyo, J. H., & Kim, J. H. (2020). Fear response-based prediction for stress susceptibility to PTSD-like phenotypes. *Mol Brain*, *13*(1), 134. https://doi.org/10.1186/s13041-020-00667-5

Kessler, R. C., Aguilar-Gaxiola, S., Alonso, J., Benjet, C., Bromet, E. J., Cardoso, G., Degenhardt, L., de Girolamo, G., Dinolova, R. V., Ferry, F., Florescu, S., Gureje, O.,

Haro, J. M., Huang, Y., Karam, E. G., Kawakami, N., Lee, S., Lepine, J. P., Levinson, D., Navarro-Mateu, F., Pennell, B. E., Piazza, M., Posada-Villa, J., Scott, K. M., Stein, D. J., Ten Have, M., Torres, Y., Viana, M. C., Petukhova, M. V., Sampson, N. A., Zaslavsky, A. M., & Koenen, K. C. (2017). Trauma and PTSD in the WHO World Mental Health Surveys. *Eur J Psychotraumatol*, *8*(sup5), 1353383. https://doi.org/10.1080/20008198.2017.1353383

Kessler, R. C., Berglund, P., Demler, O., Jin, R., Merikangas, K. R., & Walters, E. E. (2005). Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. *Arch Gen Psychiatry*, *62*(6), 593-602. https://doi.org/10.1001/archpsyc.62.6.593

Kessler, R. C., Crum, R. M., Warner, L. A., Nelson, C. B., Schulenberg, J., & Anthony, J. C. (1997). Lifetime co-occurrence of DSM-III-R alcohol abuse and dependence with other psychiatric disorders in the National Comorbidity Survey. *Arch Gen Psychiatry*, *54*(4), 313-321. https://doi.org/10.1001/archpsyc.1997.01830160031005

Kessler, R. C., Sonnega, A., Bromet, E., Hughes, M., & Nelson, C. B. (1995). Posttraumatic stress disorder in the National Comorbidity Survey. *Arch Gen Psychiatry*, *52*(12), 1048-1060.

Keyes, K. M., Grant, B. F., & Hasin, D. S. (2008). Evidence for a closing gender gap in alcohol use, abuse, and dependence in the United States population. *Drug Alcohol Depend*, *93*(1-2), 21-29. https://doi.org/10.1016/j.drugalcdep.2007.08.017

Kim, J. J., Fanselow, M. S., DeCola, J. P., & Landeira-Fernandez, J. (1992). Selective impairment of long-term but not short-term conditional fear by the N-methyl-D-aspartate antagonist APV. *Behavioral neuroscience*, *106*(4), 1.

Kim, J. J., & Jung, M. W. (2006). Neural circuits and mechanisms involved in Pavlovian fear conditioning: a critical review. *Neurosci Biobehav Rev*, *30*(2), 188-202. https://doi.org/10.1016/j.neubiorev.2005.06.005

Kim, J. J., Rison, R. A., & Fanselow, M. S. (1993). Effects of amygdala, hippocampus, and periaqueductal gray lesions on short- and long-term contextual fear. *Behav Neurosci*, *107*(6), 1093-1098.

Kosten, T. R., & Krystal, J. (1988). Biological mechanisms in posttraumatic stress disorder. Relevance for substance abuse. *Recent Dev Alcohol*, *6*, 49-68. https://doi.org/10.1007/978-1-4615-7718-8_3

Lakshminarasimhan, H., & Chattarji, S. (2012). Stress leads to contrasting effects on the levels of brain derived neurotrophic factor in the hippocampus and amygdala. *PLoS One*, *7*(1), e30481. https://doi.org/10.1371/journal.pone.0030481

Lanius, R. A., Bluhm, R., Lanius, U., & Pain, C. (2006). A review of neuroimaging studies in PTSD: heterogeneity of response to symptom provocation. *J Psychiatr Res*, *40*(8), 709-729. https://doi.org/10.1016/j.jpsychires.2005.07.007

Lanius, R. A., Williamson, P. C., Boksman, K., Densmore, M., Gupta, M., Neufeld, R. W., Gati, J. S., & Menon, R. S. (2002). Brain activation during script-driven imagery induced dissociative responses in PTSD: a functional magnetic resonance imaging investigation. *Biol Psychiatry*, *52*(4), 305-311.

Lanius, R. A., Williamson, P. C., Densmore, M., Boksman, K., Gupta, M. A., Neufeld, R. W., Gati, J. S., & Menon, R. S. (2001). Neural correlates of traumatic memories in posttraumatic stress disorder: a functional MRI investigation. *Am J Psychiatry*, *158*(11), 1920-1922. https://doi.org/10.1176/appi.ajp.158.11.1920

Lee, H. K., Takamiya, K., Han, J. S., Man, H., Kim, C. H., Rumbaugh, G., Yu, S., Ding, L., He, C., Petralia, R. S., Wenthold, R. J., Gallagher, M., & Huganir, R. L. (2003). Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory. *Cell*, *112*(5), 631-643. https://doi.org/10.1016/s0092-8674(03)00122-3

Leeies, M., Pagura, J., Sareen, J., & Bolton, J. M. (2010). The use of alcohol and drugs to self-medicate symptoms of posttraumatic stress disorder. *Depress Anxiety*, *27*(8), 731-736. https://doi.org/10.1002/da.20677

Long, V. A., & Fanselow, M. S. (2012). Stress-enhanced fear learning in rats is resistant to the effects of immediate massed extinction. *Stress*, *15*(6), 627-636. https://doi.org/10.3109/10253890.2011.650251

Lovibond, P. F. (1992). Tonic and phasic electrodermal measures of human aversive conditioning with long duration stimuli. *Psychophysiology*, *29*(6), 621-632. https://doi.org/10.1111/j.1469-8986.1992.tb02037.x

Luine, V., Martinez, C., Villegas, M., Magariños, A. M., & McEwen, B. S. (1996). Restraint stress reversibly enhances spatial memory performance. *Physiol Behav*, *59*(1), 27-32.

Luine, V., Villegas, M., Martinez, C., & McEwen, B. S. (1994). Repeated stress causes reversible impairments of spatial memory performance. *Brain Res*, *639*(1), 167-170.

Malinow, R. (2003). AMPA receptor trafficking and long-term potentiation. *Philos Trans R Soc Lond B Biol Sci*, *358*(1432), 707-714. https://doi.org/10.1098/rstb.2002.1233

Maren, S. (1999). Long-term potentiation in the amygdala: a mechanism for emotional learning and memory. *Trends Neurosci*, *22*(12), 561-567.

Maren, S. (2001). Neurobiology of Pavlovian fear conditioning. *Annu Rev Neurosci*, *24*, 897-931. https://doi.org/10.1146/annurev.neuro.24.1.897

Maren, S., De Oca, B., & Fanselow, M. S. (1994). Sex differences in hippocampal long-term potentiation (LTP) and Pavlovian fear conditioning in rats: positive correlation between LTP and contextual learning. *Brain Res*, *661*(1-2), 25-34. https://doi.org/10.1016/0006-8993(94)91176-2

McFarlane, A. C. (1998). Epidemiological evidence about the relationship between PTSD and alcohol abuse: the nature of the association. *Addict Behav*, *23*(6), 813-825. https://doi.org/10.1016/s0306-4603(98)00098-7

Meyer, E. M., Long, V., Fanselow, M. S., & Spigelman, I. (2013). Stress increases voluntary alcohol intake, but does not alter established drinking habits in a rat model of posttraumatic stress disorder. *Alcohol Clin Exp Res*, *37*(4), 566-574. https://doi.org/10.1111/acer.12012

Milad, M. R., Orr, S. P., Lasko, N. B., Chang, Y., Rauch, S. L., & Pitman, R. K. (2008). Presence and acquired origin of reduced recall for fear extinction in PTSD: results of a twin study. *J Psychiatr Res*, *42*(7), 515-520. https://doi.org/10.1016/j.jpsychires.2008.01.017

Mills, K. L., Teesson, M., Ross, J., & Peters, L. (2006). Trauma, PTSD, and substance use disorders: findings from the Australian National Survey of Mental Health and Well-Being. *Am J Psychiatry*, *163*(4), 652-658. https://doi.org/10.1176/appi.ajp.163.4.652

Morey, R. A., Dunsmoor, J. E., Haswell, C. C., Brown, V. M., Vora, A., Weiner, J., Stjepanovic, D., Wagner, H. R., LaBar, K. S., & Workgroup, V. M.-A. M. (2015). Fear learning circuitry is biased toward generalization of fear associations in posttraumatic stress disorder. *Transl Psychiatry*, *5*, e700. https://doi.org/10.1038/tp.2015.196

Myers, K. M., & Davis, M. (2007). Mechanisms of fear extinction. *Mol Psychiatry*, *12*(2), 120-150. https://doi.org/10.1038/sj.mp.4001939

Norrholm, S. D., Jovanovic, T., Olin, I. W., Sands, L. A., Karapanou, I., Bradley, B., & Ressler, K. J. (2011). Fear extinction in traumatized civilians with posttraumatic stress disorder: relation to symptom severity. *Biol Psychiatry*, *69*(6), 556-563. https://doi.org/10.1016/j.biopsych.2010.09.013

Orr, S. P., Metzger, L. J., Lasko, N. B., Macklin, M. L., Peri, T., & Pitman, R. K. (2000). De novo conditioning in trauma-exposed individuals with and without posttraumatic stress disorder. *J Abnorm Psychol*, *109*(2), 290-298.

Pavlides, C., Nivón, L. G., & McEwen, B. S. (2002). Effects of chronic stress on hippocampal long-term potentiation. *Hippocampus*, *12*(2), 245-257. https://doi.org/10.1002/hipo.1116

Pennington, Z. T., Anderson, A. S., & Fanselow, M. S. (2017). The ventromedial prefrontal cortex in a model of traumatic stress: fear inhibition or contextual processing? *Learn Mem*, *24*(9), 400-406. https://doi.org/10.1101/lm.046110.117

Peri, T., Ben-Shakhar, G., Orr, S. P., & Shalev, A. Y. (2000). Psychophysiologic assessment of aversive conditioning in posttraumatic stress disorder. *Biol Psychiatry*, *47*(6), 512-519. https://doi.org/10.1016/s0006-3223(99)00144-4

Perkonigg, A., Kessler, R. C., Storz, S., & Wittchen, H.-U. (2000). Traumatic events and post-traumatic stress disorder in the community: prevalence, risk factors and comorbidity. *Acta Psychiatr Scand*, *101*(1), 46-59.

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*(1), 45-57. https://doi.org/10.1038/npp.2015.224

Poulos, A. M., Reger, M., Mehta, N., Zhuravka, I., Sterlace, S. S., Gannam, C., Hovda, D. A., Giza, C. C., & Fanselow, M. S. (2014). Amnesia for early life stress does not preclude the adult development of posttraumatic stress disorder symptoms in rats. *Biol Psychiatry, 76*(4), 306-314. https://doi.org/10.1016/j.biopsych.2013.10.007

Poulos, A. M., Zhuravka, I., Long, V., Gannam, C., & Fanselow, M. (2015). Sensitization of fear learning to mild unconditional stimuli in male and female rats. *Behav Neurosci, 129*(1), 62-67. https://doi.org/10.1037/bne0000033

Rau, V., DeCola, J. P., & Fanselow, M. S. (2005). Stress-induced enhancement of fear learning: an animal model of posttraumatic stress disorder. *Neurosci Biobehav Rev, 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

Ritov, G., Boltyansky, B., & Richter-Levin, G. (2016). A novel approach to PTSD modeling in rats reveals alternating patterns of limbic activity in different types of stress reaction. *Mol Psychiatry, 21*(5), 630-641. https://doi.org/10.1038/mp.2015.169

Rogan, M. T., Stäubli, U. V., & LeDoux, J. E. (1997). Fear conditioning induces associative long-term potentiation in the amygdala. *Nature, 390*(6660), 604-607. https://doi.org/10.1038/37601

Sandi, C., Loscertales, M., & Guaza, C. (1997). Experience-dependent facilitating effect of corticosterone on spatial memory formation in the water maze. *Eur J Neurosci, 9*(4), 637-642.

Shansky, R. M. (2015). Sex differences in PTSD resilience and susceptibility: Challenges for animal models of fear learning. *Neurobiol Stress, 1*, 60-65. https://doi.org/10.1016/j.ynstr.2014.09.005

Smith, S. M., & Vale, W. W. (2006). The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. *Dialogues Clin Neurosci, 8*(4), 383-395.

Strahlendorf, H. K., & Strahlendorf, J. C. (1983). Ethanol suppression of locus coeruleus neurons: relevancy to the fetal alcohol syndrome. *Neurobehav Toxicol Teratol, 5*(2), 221-224.

Turner, S., Mota, N., Bolton, J., & Sareen, J. (2018). Self-medication with alcohol or drugs for mood and anxiety disorders: A narrative review of the epidemiological literature. *Depress Anxiety, 35*(9), 851-860. https://doi.org/10.1002/da.22771

Vervliet, B., Vansteenwegen, D., & Eelen, P. (2004). Generalization of extinguished skin conductance responding in human fear conditioning. *Learn Mem, 11*(5), 555-558. https://doi.org/10.1101/lm.77404

Vyas, A., Mitra, R., Shankaranarayana Rao, B. S., & Chattarji, S. (2002). Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. *J Neurosci, 22*(15), 6810-6818. https://doi.org/20026655

Wassum, K. M., & Izquierdo, A. (2015). The basolateral amygdala in reward learning and addiction. *Neurosci Biobehav Rev*, *57*, 271-283. https://doi.org/10.1016/j.neubiorev.2015.08.017

Willner, P., Towell, A., Sampson, D., Sophokleous, S., & Muscat, R. (1987). Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant. *Psychopharmacology (Berl)*, *93*(3), 358-364. https://doi.org/10.1007/BF00187257

Wiltgen, B. J., Royle, G. A., Gray, E. E., Abdipranoto, A., Thangthaeng, N., Jacobs, N., Saab, F., Tonegawa, S., Heinemann, S. F., O'Dell, T. J., Fanselow, M. S., & Vissel, B. (2010). A role for calcium-permeable AMPA receptors in synaptic plasticity and learning. *PLoS One*, *5*(9). https://doi.org/10.1371/journal.pone.0012818

CHAPTER 1

Sex differences in fear learning, anxiety and alcohol intake following stress

Abstract

Post-traumatic stress disorder (PTSD) develops after exposure to traumatic events and can severely impact quality of life. PTSD is frequently comorbid with substance use disorder (SUDs), with alcohol abuse being the most common. The exact nature of the relationship between these two conditions is unclear, although gender appears to be an important mediator as PTSD is twice as common in women while SUDs including alcohol abuse are more common in men. To investigate the role of sex in the development of PTSD and SUDs, we utilized a rodent model of stress exposure termed stress-enhanced fear learning (SEFL) that captures several aspects of PTSD including elevated alcohol intake. Male and female rats were exposed to a battery of assessments of behaviors relevant to PTSD symptomology prior to and following exposure to an intense acute stressor (15 unsignalled footshocks over 90 minutes). We found that stress exposure had similar impacts on fear learning, generalization and extinction across sexes. Females generally showed decreased anxiety compared to males both prior to and following stress, while some aspects of anxiety-related behavior were differentially impacted by stress. We found no evidence of stress-induced changes in voluntary alcohol intake in either males or females, suggesting that the ability of stress to alter alcohol drinking is sensitive to variations in testing procedure. Lastly, we found that despite males and females showing similar behavioral changes following stress, stress exposure was associated with altered GluA1 levels within the basolateral amygdala of male but not female rats. These findings indicate that while male and female rats show similar behavioral responses to stress exposure, the mechanisms that support these changes may be distinct.

Introduction

Post-traumatic stress disorder (PTSD) is a debilitating condition develops after exposure to traumatic events and severely impacts quality of life. PTSD is often co-morbid with substance use disorders (SUDs), with alcohol abuse being the most common (Kessler et al., 1995; Mills et al., 2006; Perkonigg et al., 2000). Despite the high burden of this disorder, its neural mechanisms are poorly understood and currently available treatments such as exposure therapy are not always effective (Craske et al., 2008; Hembree et al., 2003).

Rates of PTSD and comorbidity between PTSD and SUDs differ greatly between men and women. Despite the fact that men are more likely to experience trauma, rates of PTSD are higher in women even when controlling for trauma type (Breslau et al., 1997; Kessler et al., 2017; Kessler et al., 1995) as are rates of anxiety disorders (Kessler et al., 2005; Kessler et al., 1995). Marked gender differences also appear to exist in the relationship between PTSD and SUDs. Overall, men show higher rates of alcohol use disorder (AUD) compared to women and a higher proportion of men with PTSD also show comorbid AUD (Kessler et al., 2005). However, despite showing overall lower rates of SUDs, women who do experience AUD are more likely to also have comorbid PTSD compared to men with AUD (Kessler et al., 1997). These findings indicate significant gender differences in PTSD and SUD susceptibility, as well in as the relationships between the two conditions. However, the causes for such differences remain unclear.

Rodent models of stress exposure that reproduce aspects of PTSD symptomology offer a powerful tool for understanding the biological basis of this disorder. Such studies have provided considerable insight into how PTSD develops, as well as the factors that mediate the relationship between stress exposure and drug use (Holly & Miczek, 2015; Lisieski et al., 2018; Pennington et al., 2020). However, one major limitation is that, despite the substantial gender differences

observed in the human population, most rodent studies are conducted exclusively with males (Shansky, 2015). To understand how sex differences may influence stress responsivity, it is crucial to study these effects in both males and females. This study therefore investigated whether males and females differ in fear, anxiety and/or alcohol drinking following stress exposure. We also investigated whether the same neural mechanisms underlie the effects of stress in both males and females. Specifically, we examined whether both males and females showed elevations in the GluA1 subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor within the basolateral amygdala (BLA) following stress, which has been proposed to support changes in fear learning (Perusini et al., 2016).

One characteristic of PTSD is an exaggerated response to mild stressors that are reminiscent of the original trauma, leading to inappropriate fear responses (Bremner et al., 1995; Dykman et al., 1997). Our laboratory has developed a rodent model of stress exposure termed stress-enhanced fear learning (SEFL) that uses aspects of Pavlovian fear conditioning to capture this exaggerated fear response. In the SEFL model, animals are exposed to an intense acute stressor (typically 15 unsignaled footshocks) in a distinct context, followed by a single footshock in a different context (Rau et al., 2005). Following stress exposure animals show enhanced fear to the single footshock context, a result that appears to reflect a nonassociative sensitization of the fear learning process (Long & Fanselow, 2012; Poulos et al., 2014; Rau et al., 2005). While this enhancement of fear learning is the defining feature of the SEFL model, this procedure produces additional behavioral changes reflective of PTSD symptomology including extreme fear of stimuli associated with the traumatic event, increased anxiety, increased startle reactivity and altered glucocorticoid signaling (Meyer et al., 2013; Pennington et al., 2017; Perusini et al., 2016; Poulos et al., 2015). Lastly, the SEFL model produces a long-lasting increase in alcohol

consumption, reflecting the comorbidity between PTSD and substance abuse (Meyer et al., 2013).

Most of our work using the SEFL model has been conducted in males (Meyer et al., 2013; Perusini et al., 2016; Poulos et al., 2014; Rau et al., 2005; Rau & Fanselow, 2009). While we have demonstrated SEFL in female rats (Poulos et al., 2015), many other effects of stress exposure in males and females have not been directly compared. It is therefore unknown whether males and females will show same type or magnitude of behavioral changes following stress. Similarly, we have proposed that elevations in the GluA1 subunit AMPA receptor within the BLA following stress may underlie the effects of fear learning on stress (Perusini et al., 2016). However, because these elevations in GluA1 have only been demonstrated in males, it remains unknown whether females show a similar change following stress exposure or instead utilize a different neural mechanism.

In the present experiments we assessed changes in a variety of PTSD-relevant behaviors in males and females to determine how stress responsivity differs across sexes. In Experiment 1 subjects underwent assessments of baseline alcohol intake and anxiety prior to stress exposure, followed by a battery of assessments of fear, anxiety and alcohol-related behavior after which GluA1 levels within the BLA were analyzed. Given that PTSD and anxiety disorders are more common in women than in men, we hypothesized that females would show greater increases in fear and anxiety-related behaviors compared to males. Conversely, we anticipated that males would show greater changes in alcohol drinking following stress exposure.

Experiment 2 was designed to investigate several unexpected results emerging from Experiment 1. Specifically, Experiment 1 showed that stress exposure produced similar

increases in fear and anxiety-related behavior across sexes but failed to alter subsequent alcohol intake. In Experiment 2 we explored whether sex differences in alcohol intake prior to stress could have impacted the ability of stress to alter these behaviors.

Experiment 3 was designed to further investigate findings that emerged from Experiments 1 and 2. In particular, Experiments 1 and 2 resulted in no evidence of stress-induced changes in voluntary alcohol intake for either females or males despite previous reports showing increased alcohol drinking following stress exposure in males. In Experiment 3 we therefore tested the possibility that additional behavioral testing may have attenuated the ability of stress to impact alcohol drinking.

Experiment 1: Sex differences in fear learning, anxiety and alcohol intake following stress exposure

Experiments 1 assessed sex differences in fear, anxiety and alcohol drinking behaviors prior to and after stress as well as changes in BLA GluA1 levels. Given that PTSD and anxiety disorders are more common in women than in men, we hypothesized that females would show greater increases in fear and anxiety-related behaviors compared to males. Conversely, because SUDs are more common in men than in women, we anticipated that males would show greater changes in alcohol drinking following stress exposure.

Methods

Subjects

84 adult male and female Long-Evans rats (Envigo, Indianapolis, IN, USA) were used, approximately 10-11 weeks old at the start of the experiment. Animals were individually housed under a 12-hour light/dark cycle. Food and water were available *ad libitum* in the home cage and

during intermittent access 2-bottle choice environmental enrichment in the form of paper twists was provided. Animals were handled daily for 60 seconds each day for 1 week prior to the start of the experiment. The UCLA Institutional Animal Care and Use Committee approved all procedures involving animals.

Experimental timeline

Male and female subjects were equally divided into Stress and No Stress conditions. All subjects underwent a battery of fear learning, anxiety and alcohol intake assessments as outlined in Table 1-1.

Day	*Task*
1-14	Continuous access 2-bottle choice
15	Light-dark transition test
16	Stress
17	Generalization test
18	Single footshock
19	Stress-enhanced fear learning test
20	Open field test
21	Elevated plus maze
22	Light-dark transition test
23-27	Extinction
28	Aversive acoustic stimulus
29	Context test
30-37	Rest
38-96	Intermittent access 2-bottle choice
97-98	Tissue collection for Western blot

Table 1-1. Timeline for Experiments 1 and 2.

Apparatus and procedures

Stress-enhanced fear learning. All fear conditioning and extinction took place in four sets of four identical fear conditioning chambers housed in sound-attenuating shells (Med Associates Inc, St. Albans, VT, USA). Context A (stress context) contained flat grid floors, was lit by a white house light, and was scented with 50% Windex solution. Ventilation fans provided background noise. Context B (generalization context) was identical to Context A except the grid floors were made of alternating thick and thin bars and chambers were scented with 1:30 Simple Green solution. Context C (fear conditioning context) contained a black triangular insert and floors composed of staggered grid bars, and was scented with 1% acetic acid. Each set of grids was wired to a shock generator and scrambler. Stimulus delivery was controlled and freezing automatically scored using VideoFreeze software (Med Associates Inc).

Subjects were first transported to Context A in their homecages where they received a stressor consisting of either 15 1-sec, 1-mA unsignalled footshocks randomly distributed over 90 minutes (Stress condition) or received equivalent context exposure without footshock (No Stress condition). In Experiments 1 and 2, fear generalization was assessed one day later by exposing subjects to Context B which shared some features with the original stress context. Subjects were transported to Context B in clean cages and were exposed to the context for 8 minutes without footshock. The next day subjects were transported to Context C in a black plastic tub divided into 4 quadrants. Following a 3-minute baseline period, all subjects received a single 1-sec, 1-mA footshock and were removed 30 seconds later. Subjects were returned to Context C the following day for 8 minutes to assess fear to the fear conditioning context. Fear was measured via freezing.

Fear extinction. Fear extinction took place in the fear conditioning context (Context C). Subjects received 5 days of extinction training, in which they were exposed to Context C for 30 minutes per day without footshock delivery. Extinction across sessions was measured by freezing during the first 5 minutes of each session.

Aversive acoustic stimulus. Context D consisted of four identical fear conditioning chambers housed in sound-attenuating shells (Med Associates Inc). Each chamber contained a curved white plastic wall and white plastic floor inserts and was scented with 1:30 Simple Green solution. Acoustic stimuli were delivered using Goldwood GT-1005 wide dispersion piezo tweeters mounted to the wall of the chambers and connected to an amplifier. Following a three-minute baseline period, all subjects received a 100-msec, 110-dB burst of white noise and were removed 30 seconds later. The next day subjects were returned to Context D for 8 minutes to assess fear to the context.

Light-dark transition test. The light-dark transition test is a classic test of anxiety in rodents (Bourin & Hascoët, 2003; Crawley & Goodwin, 1980). The apparatus consisted of a Plexiglas arena (100 cm x 40 cm x 30 cm). The arena was divided into a light compartment with white walls and floors (75 cm x 40 cm) and a dark compartment with black walls and floors (25 cm x 40 cm). A 10 cm x 10 cm opening allowed movement between the two compartments. The room was dimly lit by a lamp in the corner of the room. Each session was recorded using an overhead camera and behavior was automatically tracked using EthoVision software (Noldus, Wageningen, Netherlands).

Subjects were individually placed in the dark compartment of the apparatus and allowed to freely move between the dark and light compartments for 10 minutes. Anxiety-related behavior was scored by the latency to enter the light compartment, the total time spent in the light compartment and the number of entries into the light compartment. Unlike most other tests of anxiety-related behavior, previous reports indicate that the light-dark transition test can be reliably conducted multiple times within the same subject (Ballaz et al., 2007; Banasikowski et al., 2015; Onaivi & Martin, 1989). In Experiments 1 and 2 the light-dark transition test was therefore run prior to and following stress (Table 1-1).

Open field test. This task utilized a modified version of the open field test (Godsil et al., 2005; Godsil & Fanselow, 2004). The apparatus consisted of a clear plastic container (78 cm x 39 cm x 30 cm) divided into 4 equal-sized zones (19.5 cm x 39 cm). Three LED lamps were located around each end of the apparatus, one lamp located against the center of each short wall and one lamp located against the ends of each long wall. Turning on the lamps on one end created a light gradient across the apparatus, with Zone 1 brightest (2160 Lux) and Zone 4 dimmest (260 Lux). The illuminated side of the apparatus was counterbalanced across subjects. The room was lit by a

red light throughout the session, and each session was recorded using an overhead camera and behavior automatically tracked using EthoVision software (Noldus).

Subjects were individually placed in the open field apparatus for 12 minutes and allowed to move freely. During the first 4 minutes, the apparatus was lit only by a red light. During minutes 5-8, the lamps around one side of the apparatus were illuminated creating a light gradient across the floor. During minutes 9-12, the lamps were turned off. Anxiety-related behavior was measured by general exploratory behavior and by the amount of time spent in Zone 4, indicating avoidance of the lights. Data was lost for one subject due to a computer error.

Elevated plus maze. The elevated plus maze is another classic rodent test of anxiety-related behavior (Rodgers & Dalvi, 1997). The apparatus consisted of a plus-shaped maze with each arm measuring 40 cm x 12.5 cm that was elevated 60 cm above the ground. The closed arms had black Plexiglas walls (17.5 cm), while the open arms did not. The room was lit by a red light throughout the session, and each session was recorded using an overhead camera and behavior automatically tracked using EthoVision software (Noldus). Subjects were placed in the center of the maze and allowed to freely explore for 6 minutes. Anxiety-related behavior was measured by the amount of time spent in the open arms of the maze, with less time indicating higher anxiety.

Continuous access two-bottle choice (2BC) drinking paradigm. All fluids were presented in 250-ml drinking bottles with low-leak drinking spouts accessible through the top of the home cage. Rats in Experiment 1 had access to one bottle containing 10% EtOH (w/v) and one bottle containing regular drinking water, while in Experiment 2 both bottles contained drinking water. Rats received continuous access to both bottles for two weeks prior to stress exposure (Table 1-1). Bottles were weighed daily except Sundays and locations were alternated daily to control for

side preferences. Rats were weighed every other day to calculate the grams of solution consumed per kilogram of body weight in each 24 hour session (g/kg/24 hrs).

Intermittent access two-bottle choice (2BC) drinking paradigm. Subjects received 59 days of intermittent access 2-bottle choice following stress exposure as described previously (Meyer et al., 2013). During this time subjects were given access to one bottle of 10% EtOH (w/v) and one bottle of regular drinking water on Mondays, Wednesdays and Fridays. Drinking bottles were weighed at the beginning and end of each 24-hour drinking session and rats were weighed at the end of each session. Bottle locations were alternated each session. At the end of each session the bottle containing alcohol was replaced with a bottle containing drinking water until the start of the next session.

Tissue and Western blot analysis

Western blot analysis of basolateral amygdala (BLA) samples was used to assess the relative abundance of the AMPA receptor GluA1 subunit. Rats were euthanized 24 to 48 hours after the last ethanol presentation. The brain was removed and 400-μm-thick coronal slices were made, from which the BLA was microdissected and frozen at -80°C. Tissue was then thawed and homogenized in ice cold buffer containing phosphatase inhibitor cocktail (1X) (Thermo Scientific, Waltham, MA USA), 0.3 mM PMSF protease inhibitor (Thermo Scientific), and RIPA buffer (Thermo Scientific). Protein concentrations were determined with BCA Protein Assay Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Quantification of GluA1 expression level and total protein concentration were performed using the Wes system according to the manufacturer's instructions (Protein Simple, San Jose, CA, USA).

Briefly, each well dedicated for the samples were loaded with equal amount of protein (0.75 μg/well). To quantify GluA1 expression levels, wells dedicated for the antibodies were loaded with equal amount of rabbit polyclonal anti-GluA1 (AB1504, Millipore Sigma, St Louis, MO, USA) diluted at a concentration of 1:25. Each plate was designed to allow for the comparison of GluA1 expression between stress versus unstressed controls for each sex. Chemiluminescence signal was processed and analyzed using Compass for Simple Western software 4.0 (Protein Simple). The peak of chemiluminescence signal for GluA1 was detected at 123 KDa. The area under the curve of the GluA1 chemiluminescence signal was calculated for each sample using a Gaussian fit. The area under the curve of the chemiluminescence signal corresponding to the total protein content detected at 123 KDa was calculated using a dropped down fit, and used to normalize the relative expression of GluA1 for each sample. Thus, the ratio GluA1:Total protein at 123 KDa for each sample was used to quantify and compare GluA1 expression levels between groups.

Data analysis

Data were analyzed using IBM SPSS Statistical Software. Results of tasks with a single dependent variable were analyzed using 2-way ANOVA with Stress as the first factor and Sex as the second factor. Tasks using repeated measures were analyzed using mixed-model ANOVA. Violations of sphericity corrected for using the Greenhouse-Geisser correction. Data were plotted using GraphPad Prism.

Results

Females show higher alcohol consumption and lower anxiety prior to stress

While levels of alcohol intake prior to stress exposure remained relatively low across days, females showed elevated alcohol intake compared to males. Females showed higher alcohol consumption ($F_{1,78}$=4.15, p=0.045; Fig 1-1A) and while alcohol preference was also elevated in females this effect was not significant ($F_{1,78}$=2.76, p=0.1; Fig 1-1B). To measure "binge-like" drinking, we counted the number of days that rats drank more than 5 g/kg. The mean number of binge-like days was 1.1 (SD=1.9) in females and 0.3 (SD=1.0) in males, a small but significant difference ($F_{1,78}$=6.47, p=0.01). Importantly, there were no pre-existing differences between Stress and No Stress groups in either alcohol consumption ($F_{1,78}$=0.004, p=0.95), preference ($F_{1,78}$=0.001, p=0.98) or number of binge-like days ($F_{1,78}$=0.02, p=0.9). Data from 2 subjects were excluded from these analyses due to bottle spillage.

Females also showed lower anxiety-like behavior prior to stress exposure as assessed by the light-dark transition test. Compared to males, females showed lower latency to enter the light compartment ($F_{1,80}$=11.00, p=0.001; Fig 1-1C), more time in the light compartment ($F_{1,80}$=16.00, p<0.001; Fig 1-1D) and more entries into the light compartment ($F_{1,80}$=6.66, p=0.012; Fig 1-1E). There were no baseline differences between Stress and No Stress groups on any of these measures (latency to enter light compartment: $F_{1,80}$=0.002, p=0.97; time in light compartment: $F_{1,80}$=0.53, p=0.47; entries into light compartment: $F_{1,80}$=0.09, p=0.77).

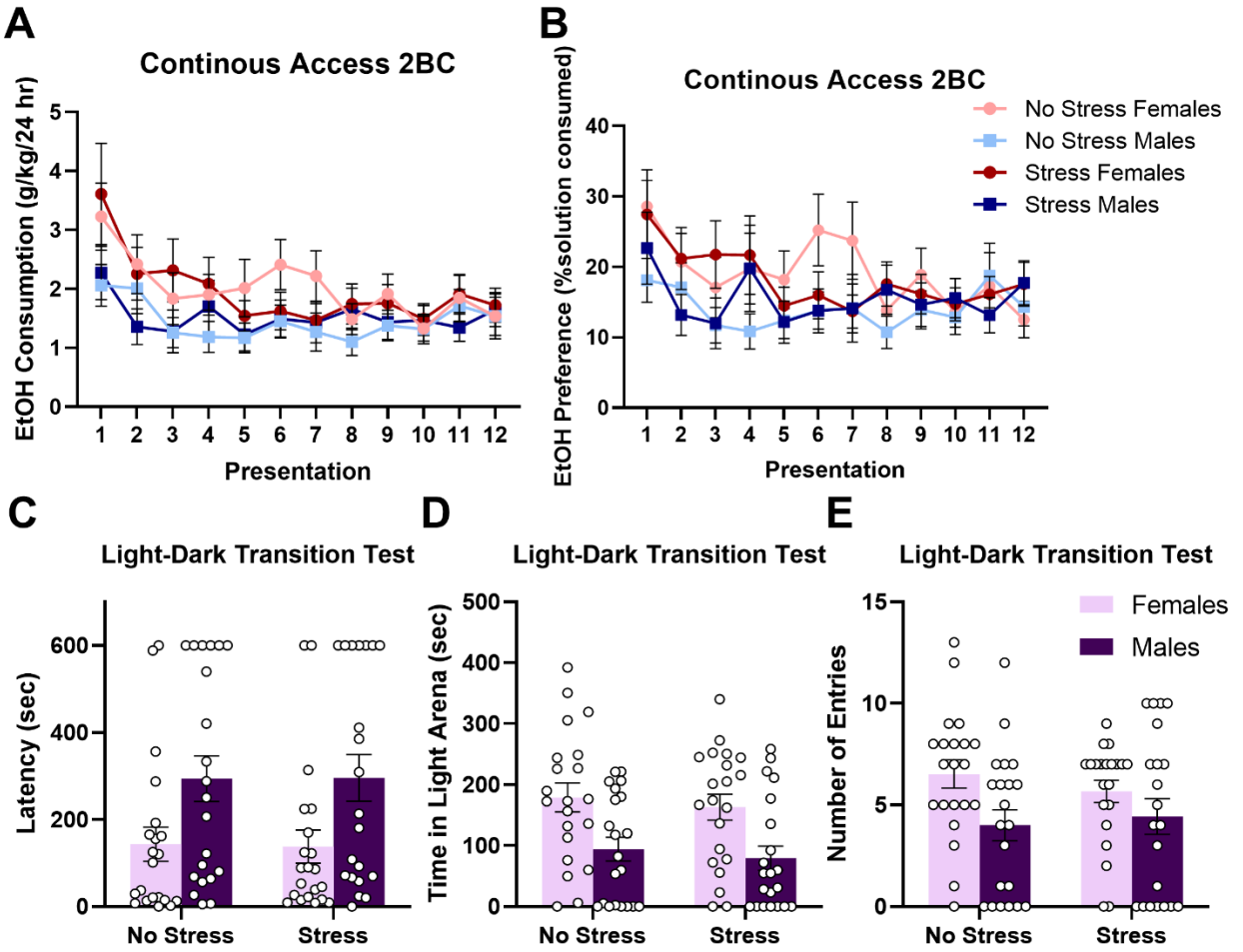


Figure 1-1. Experiment 1 baseline alcohol intake (10% EtOH w/v) and anxiety results. **A.** Females show more alcohol consumption compared to males during continuous access 2-bottle choice (Days 1-14). **B.** No differences in alcohol preference (Days 1-14). **C-E.** Results of light-dark transition test (Day 15). Females show reduced anxiety-related behavior as indicated by reduced latency to enter the light arena **(C)**, more time spent in the light arena **(D)** and more entries into the light arena **(E)**. Errors represent standard error of the mean.

Males and females do not differ during SEFL procedure

Following exposure to an acute stressor in Context A, subjects were placed in a novel context that shared some features with the original stress context (Context B). Stress subjects showed substantial fear generalization compared to unstressed controls ($F_{1,80}$=97.95, p<0.001; Fig 1-2A). However, fear generalization did not differ between males and females (Sex: $F_{1,80}$=2.06, p=0.16; Sex*Stress: $F_{1,80}$=1.83, p=0.18).

When exposed to a completely novel context (Context C), Stress subjects showed a small but significant amount of fear prior to receiving a single footshock ($F_{1,80}$=5.45, p=0.02; Fig 1-2B). While this effect appeared to be due to high levels of freezing in a small number of males, there was no significant effect of sex ($F_{1,80}$=3.37, p=0.07) or interaction ($F_{1,80}$=3.01, p=0.09). When returned to the fear conditioning context the following day, Stress subjects showed a dramatic elevation in fear ($F_{1,80}$=157, p<0.001; Fig 1-2C). However, there was no difference between males and females (Sex: $F_{1,80}$=1.33, p=0.25; Stress*Sex: $F_{1,80}$=0.11, p=0.75).

Males and females do not differ on additional aspects of fear learning

While there were no differences in fear to the SEFL context between males and females, we observed that Stress subjects showed near maximal levels of fear. This suggested that perhaps differences between males and females were present but obscured by a ceiling effect. To address this possibility and assess differences in fear extinction, extinction of fear to the SEFL context (Context C) was evaluated. While Stress subjects eventually extinguished to the levels of No Stress controls (Fig 1-2D), no differences between males and females were observed (Session*Sex: $F_{3.11,248.97}$=1.35, p=0.26; Session*Stress*Sex: $F_{3.11,248.97}$=1.25, p=0.29).

To examine changes in fear to a novel aversive stimulus, subjects were placed in a novel context (Context D) in which they received an aversive acoustic stimulus consisting of a 100-ms, 110-dB burst of white noise. Stress subjects showed more fear to Context D when returned the following day ($F_{1,80}$=46.16, p<0.001; Fig 1-2F), although no difference between males and females were observed ($F_{1,80}$=0.04, p=0.85). However, Stress subjects also showed elevated freezing prior to stimulus delivery ($F_{1,80}$=13.02, p=0.001; Fig 1-2E), suggesting some amount of fear generalization.

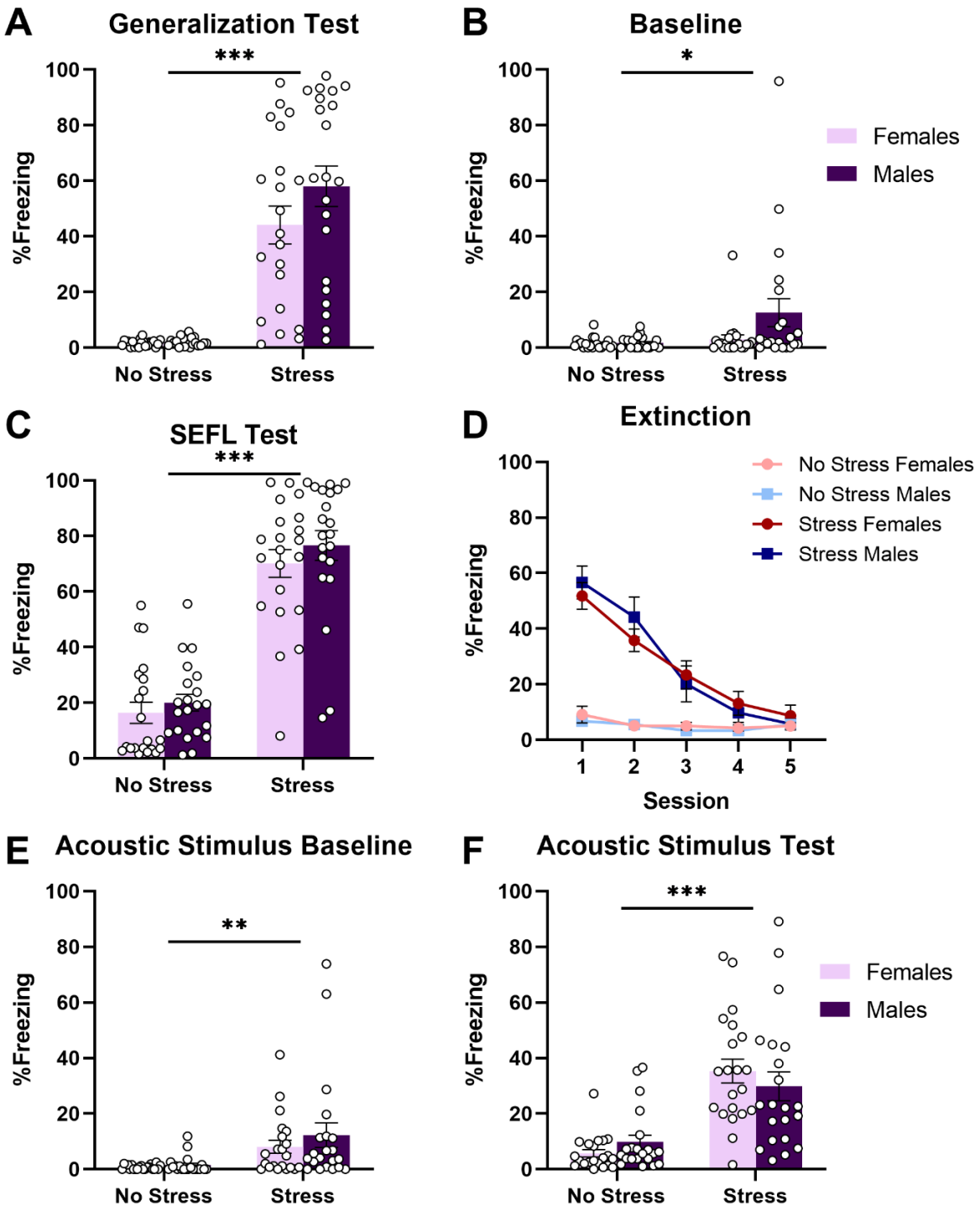


Figure 1-2. Experiment 1 fear learning results. **A.** Stress subjects show elevated fear generalization to a novel context that shared some features with the stress context. (Day 17). **B.** Stress subjects elevated fear to a novel context prior to footshock (Day 18). **C.** Stress subjects show enhanced fear to SEFL context (Day 19). **D.** No sex differences in fear extinction (Days 23-27). **E.** Stress subjects show elevated fear prior to acoustic stimulus delivery (Day 28). **F.** Stress subjects show elevated fear to acoustic stimulus context (Day 29). *p<0.05, **p<0.01, ***p<0.001. Errors represent standard error of the mean.

Males and females differ on some aspects of anxiety

Stress was found to significantly reduce exploratory behavior during the open field test (Fig 1-3A) as indicated by a significant Stress by Minute interaction ($F_{8.41,664.36}$=2, p=0.04; Fig 1-3B). Follow-up simple main effects indicated that Stress subjects showed reduced exploratory behavior at multiple timepoints throughout the session (ps<0.05-0.001). Females also showed greater locomotion compared to males ($F_{1,79}$=10.95, p=0.001) although no interaction was observed ($F_{1,79}$=0.05, p=0.83). Data from one subject was lost due to a computer error.

While stress similarly reduced exploratory behavior in both males and females, males and females differed in response to an anxiogenic stimulus. Stress differentially impacted avoidance of the light stimulus during the open field test indicated by a significant Stress*Sex*Minute interaction ($F_{6.09,480.82}$=3.74, p=0.001; Fig 1-3C). Follow-up mixed-model ANOVAs indicated that while females showed increased time spent in Zone 4 following light onset, there was no difference between Stress and No Stress females ($F_{1,39}$=0.04, p=0.85). In contrast, Stressed and No Stress males responded differently to light onset as indicated by a significant Minute*Condition interaction ($F_{5.55,222.01}$=4.03, p=0.001). Stress males displayed a blunted response to the light onset as indicated by decreased time in Zone 4 at multiple timepoints both during onset (ps<0.05-0.01) and following light termination (ps<0.05).

Observations indicated that this blunted response to light onset may have been due to some Stress males displaying a strong preference for Zone 1 that persisted even following light onset. To test this possibility, subjects were first classified as preferring either Zone 1 or Zone 4 based on which zone they spent more time in during the 4 minutes prior to light onset. Among the Zone 1 preferring-animals, a significant interaction between sex, stress exposure, and time was

observed ($F_{5.24,157.11}$=3.96, p=0.002; Fig 1-3D). Among males, a significant interaction between time and stress exposure was observed ($F_{3.79,53.04}$=3.09, p=0.03). Follow-up simple main effects revealed that while no differences between Stress and No Stress males were observed prior to light onset (ps>0.05), Stress males showed significantly less time in Zone 4 during and following light onset compared to No Stress males (ps<0.05). In contrast, no such interaction was observed either in Zone 1-preferring females ($F_{4.99,79.82}$=1.66, p=0.15) or in subjects that initially preferred Zone 4 ($F_{5.80,260.79}$=1.03, p=0.41; Fig 1-3E). These analyses suggest that Stress males had a uniquely reduced probability of leaving their preferred location.

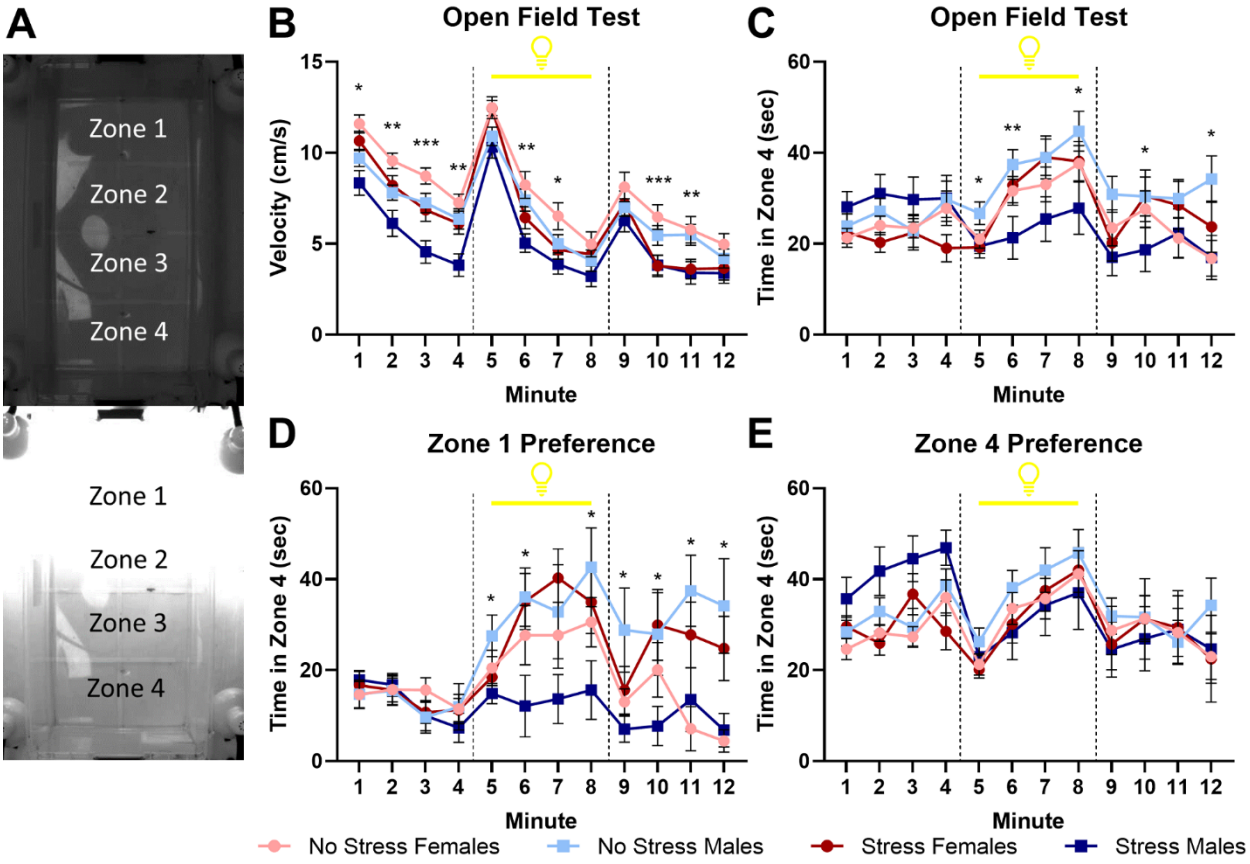


Figure 1-3. Experiment 1 open field test results (Day 20). **A.** Open field apparatus showing zone locations during lamps off (top) and lamps on (bottom). **B.** Stress reduces exploratory behavior at multiple timepoints. **C.** Following light onset, Stress males show reduced time in Zone 4 compared to No Stress males. **D.** Zone 1-preferring Stress males show reduced time in Zone 4 compared to No Stress males following light onset. **E.** No effects of stress in Zone 4-preferring subjects. *p<0.05, **p<0.01, ***p<0.001. Errors represent standard error of the mean.

Anxiety-related behavior was next evaluated using the elevated plus maze. However, no effects of stress exposure or sex were observed (Stress: $F_{1,66}$=2.13, p=0.15; Sex: $F_{1,66}$=0.21, p=0.65; Stress*Sex: $F_{1,66}$=2,87, p=0.1; Fig 1-4A). 14 subjects fell off the maze and were removed from this analysis. Anxiety-related behavior was last assessed using the light-dark transition test. However, no effects of stress or sex differences were observed on latency to enter the light compartment (Stress: $F_{1,80}$=0.05, p=0.83; Sex: $F_{1,80}$=2.1, p=0.15; Sex*Stress: $F_{1,80}$=0.41, p=0.52; Fig 1-4B), time in the light compartment (Stress: $F_{1,80}$=1.47, p=0.23; Sex: $F_{1,80}$=0.21, p=0.65; Sex*Stress: $F_{1,80}$=1.85, p=0.18; Fig 1-4C) or entries into the light compartment (Stress: $F_{1,80}$=3.49, p=0.07; Sex: $F_{1,80}$=0.09, p=0.77; Sex*Stress: $F_{1,80}$=0.7, p=0.4; Fig 1-4D).

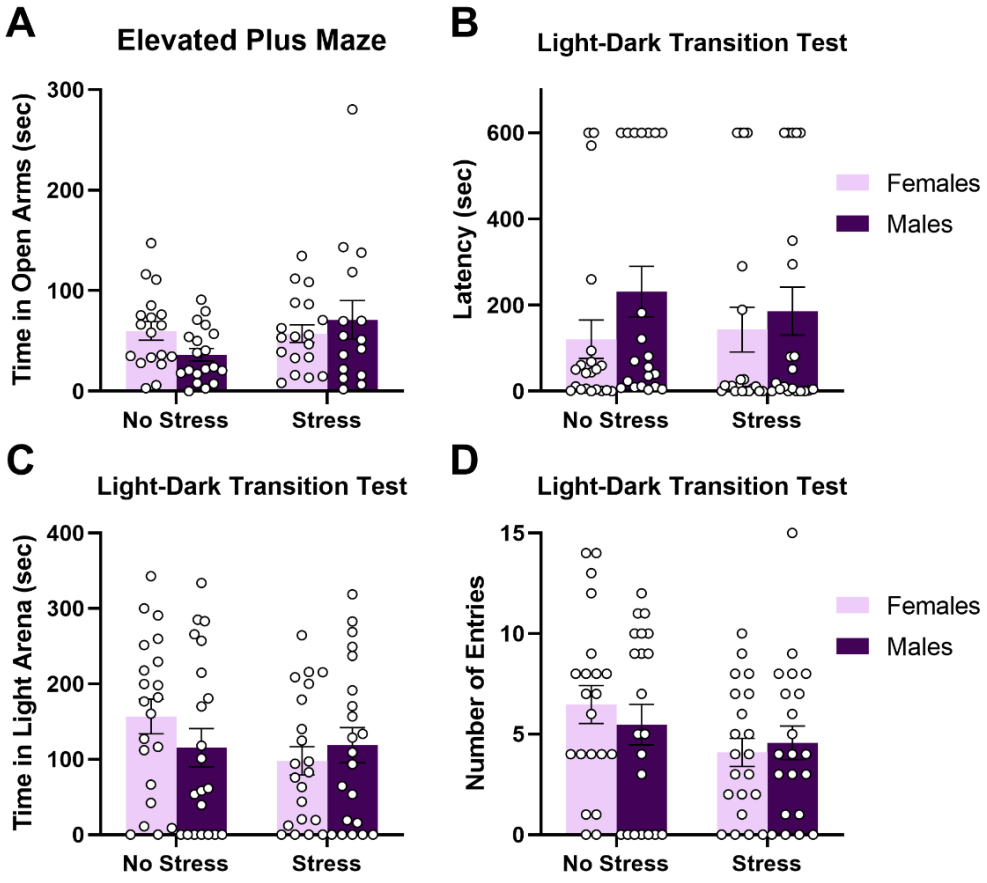


Figure 1-4. Experiment 1 elevated plus maze and light-dark transition test results. **A.** No differences in time spent in open arms of elevated plus maze (Day 21). **B-D.** Results of light-dark transition test (Day 22). No differences on latency to enter the light arena **(B)**, time spent in the light arena **(C)** or number of entries into the light arena **(D)**. Errors represent standard error of the mean.

No effects of stress on voluntary alcohol intake

Following completion of fear and anxiety testing we evaluated the effects of stress on voluntary alcohol intake. During intermittent access 2-bottle choice, females showed elevated alcohol intake as indicated by greater alcohol consumption compared to males ($F_{1,80}$=28.50, p<0.001; Fig 1-5A), although no differences in alcohol preference were observed ($F_{1,80}$=1.97, p=0.16; Fig 1-5B). The number of binge-like days was also greater in females (mean=12.2, SD=7) compared to males (mean=4.5, SD=6.2) ($F_{1,80}$=29.1, p<0.001). However, no effects of stress were observed on alcohol consumption (Stress: $F_{1,80}$=1.36, p=0.25; Stress*Sex: $F_{1,80}$=0.06, p=0.8), alcohol preference (Stress: $F_{1,80}$=0.73, p=0.4; Stress*Sex: $F_{1,80}$=1.09, p=0.3) or number of binge like-days (Stress: $F_{1,80}$=2.4, p=0.13; Stress*Sex: $F_{1,80}$=0.06, p=0.8).

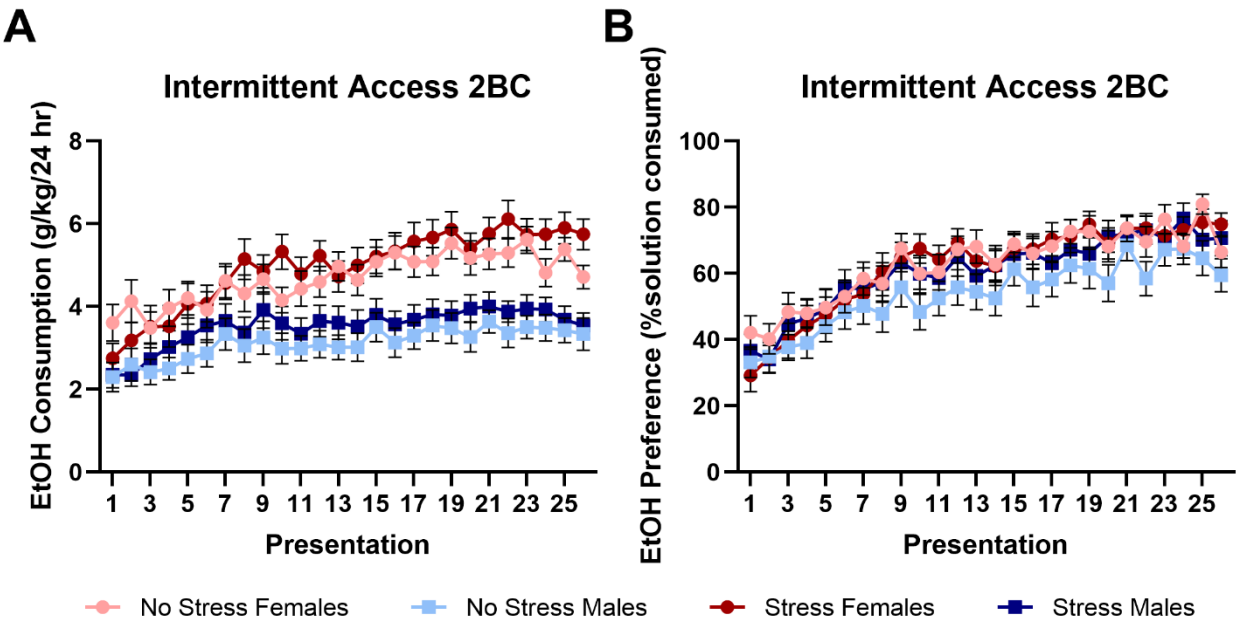


Figure 1-5. Experiment 1 intermittent access 2-bottle choice results (Days 38-96). **A.** Females show greater alcohol consumption (10% EtOH w/v) compared to males. **B.** No differences in alcohol preference. Error bars represent standard error of the mean.

Stress exposure is associated with decreased BLA GluA1 in males but not females

BLA were collected after the two-month long period of voluntary ethanol exposure and GluA1 levels relative to total protein levels at 123 KDa were measured. Owing to the large number of samples, Western blot plates were set up to individually compare the effects of stress within males and females. We found that Stress males showed decreased GluA1 relative to No Stress males (t_{41}=4.06, p<0.001; Fig 1-6B). In contrast, no effect of stress exposure was observed in females (t_{41}=0.38, p=0.71; Fig 1-6A).

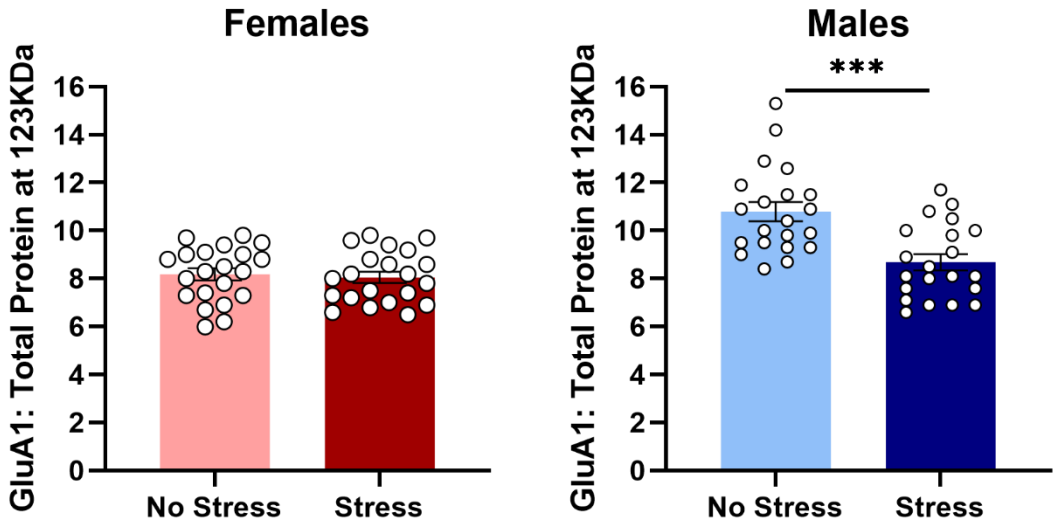


Figure 1-6. Experiment 1 Western blot results showing ratio of GluA1 to total protein at 123 KDa within the BLA. **A.** No differences in GluA1 between Stress and No Stress females. **B.** Stress males show reduced GluA1 compared to No Stress males. ***p<0.001. Error bars represent standard error of the mean.

Collectively, the results of Experiment 1 indicate that males and females show similar behavioral changes following stress exposure. Stress exposure produced comparable enhancements in fear learning to both footshock and an aversive acoustic stimulus, and no differences in fear generalization or extinction were observed between sexes. While females

showed less anxiety-related behavior and more alcohol intake both prior to and following stress, there were generally no interactions between stress exposure and sex, with the exception of the response to the light stimulus. An interaction between stress exposure and sex was observed in BLA GluA1 levels, as stress decreased GluA1 levels in males while having no effect in females.

Experiment 2: Effects of stress exposure in the absence of prior alcohol access

Experiment 2 investigated the unexpected results emerging from Experiment 1. Contrary to expectations, Experiment 1 showed that stress exposure produced similar increases in fear and anxiety-related behavior across sexes but did not alter alcohol intake following stress. Furthermore, stress impacted GluA1 levels within the BLA in males while producing no effect in females. Given that females consumed greater amounts of alcohol prior to stress exposure, in Experiment 2 we tested the possibility that alcohol access prior to stress could have impacted the ability of stress to alter fear, anxiety and alcohol drinking. We hypothesized that eliminating pre-stress alcohol exposure could allow sex differences in PTSD-related behaviors to emerge, including enhanced alcohol intake in stressed males.

Methods

Subjects.

42 adult male and female Long-Evans rats (Envigo) were used, approximately 10-11 weeks old at the start of the experiment. Housing and handling procedures were the same as in Experiment 1.

Experimental Timeline.

Male and female rats were equally divided into Stress and No Stress conditions. All subjects received exposure to the same battery of procedures as in Experiment 1 (Table 1-1), except subjects received no alcohol access prior to stress as described below.

Apparatus and Procedures

The apparatus and procedures were identical to those used in Experiment 1, except during continuous access two-bottle choice (Days 1-14) both bottles contained only regular drinking water. Bottle locations were swapped daily and subjected weighed every other day to ensure that all handling and experiences remained the same.

Tissue and Western blot analysis

Western blot analysis was performed as described in Experiment 1.

Data analysis

Data analysis was performed as described in Experiment 1.

Results

Baseline anxiety prior to stress

Baseline anxiety-related behavior was assessed using the light-dark transition test. While males showed numerically greater latency to enter the light compartment, less time spent in the light compartment and fewer entries into the light compartment as in Experiment 1, these differences were not significant (latency: $F_{1,38}$=0.24, p=0.63; light time: $F_{1,38}$=1.35, p=0.25; entries: $F_{1,38}$=0.46, p=0.5; Figs 1-7A-C). Importantly, no baseline differences in anxiety-related behavior were observed between Stress and No Stress conditions (latency: $F_{1,38}$=0.04, p=0.84; light time: $F_{1,38}$=0.62, p=0.44; entries: $F_{1,38}$=0.05, p=0.82).

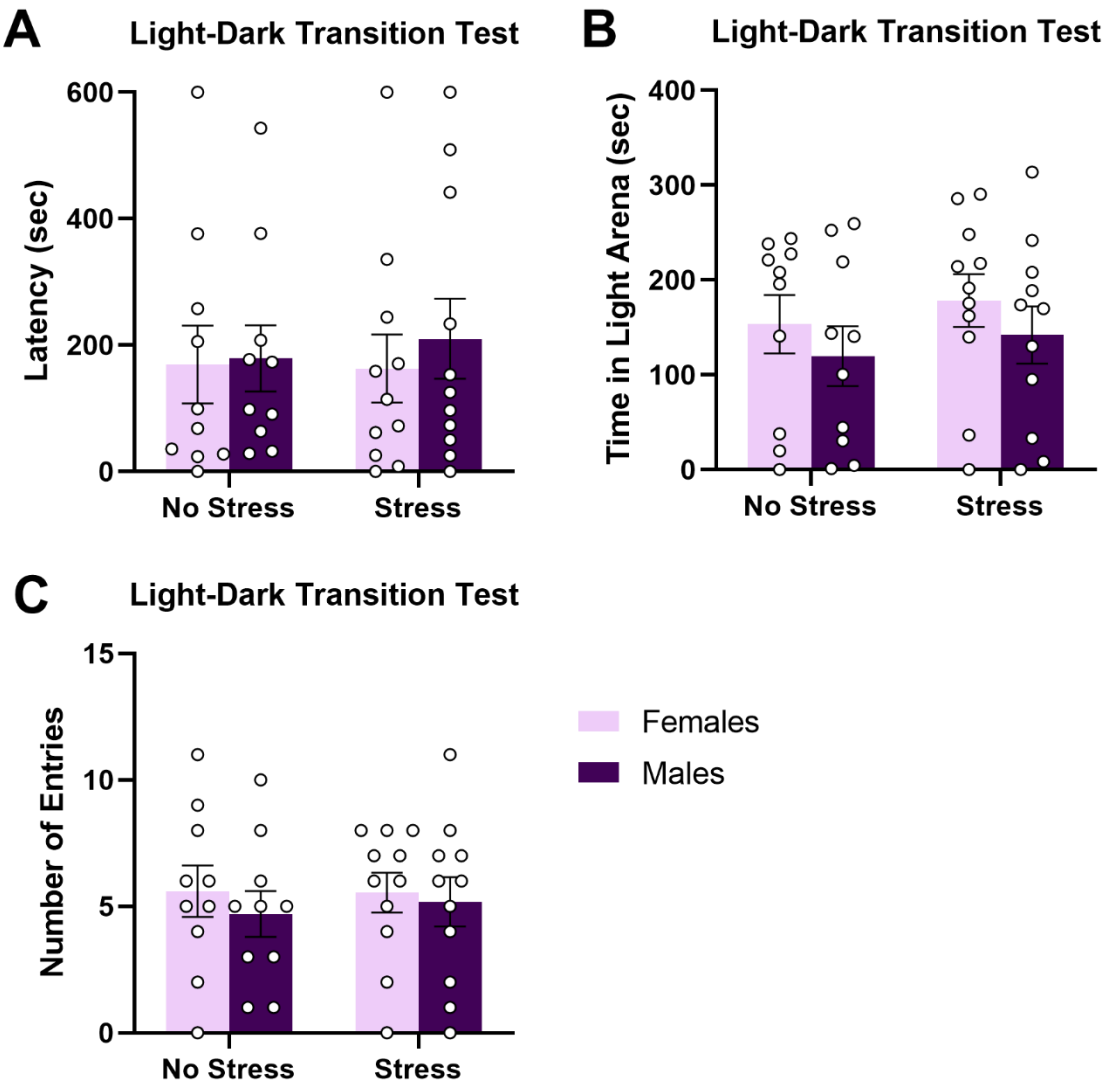


Figure 1-7. Experiment 2 pre-stress light-dark transition test results (Day 15). No significant differences were observed in latency to enter the light arena **(A)**, time in the light arena **(B)** or entries into the light arena **(C)**. Errors represent standard error of the mean.

Effects of stress on fear learning are not dependent on prior alcohol exposure

Similar patterns of changes in fear learning were observed as in Experiment 1, indicating that these effects were not influenced by alcohol exposure prior to stress. During the generalization test (Context B) Stress subjects showed enhanced fear generalization compared to

No Stress controls ($F_{1,38}$=51.84, p<0.001; Fig 1-8A), but no sex differences were observed (Sex: $F_{1,38}$=0.11, p=0.74; Sex*Condition: $F_{1,38}$=0.12, p=0.73).

When exposed to a completely novel context (Context C), no differences in baseline fear prior to the single footshock were observed (Stress: $F_{1,38}$=3.34, p=0.08; Sex: $F_{1,38}$=0.39, p=0.54; Sex*Stress: $F_{1,38}$=0.04, p=0.84; Fig 1-8B). When returned to the fear conditioning context the following day, Stress subjects showed enhanced fear ($F_{1,38}$=82.83, p<0.001; Fig 1-8C) but there was no difference between males and females (Sex: $F_{1,38}$=0.04, p=0.85; Stress*Sex: $F_{1,38}$=0.46, p=0.5).

During fear extinction to Context C, Stress subjects again extinguished to the levels of No Stress controls, but no differences between males and females were observed (Session*Sex: $F_{3,102}$=0.54, p=0.64; Session*Stress*Sex: $F_{3,102}$ =1.06, p=0.0.37; Fig 1-8D). Stress subjects showed greater fear to Context D prior to white noise delivery ($F_{1,38}$=13.7, p=0.001; Fig 1-8E) and when returned the following day ($F_{1,38}$=14.33, p=0.001; Fig 1-8F). However, no differences between males and females were observed either prior to white noise exposure (Sex: $F_{1,38}$=1.39, p=0.25; Sex*Condition: $F_{1,38}$=0.77, p=0.39) or during the context test the following day (Sex: $F_{1,38}$=1.97, p=0.17; Sex*Condition: $F_{1,38}$=1.07, p=0.31).

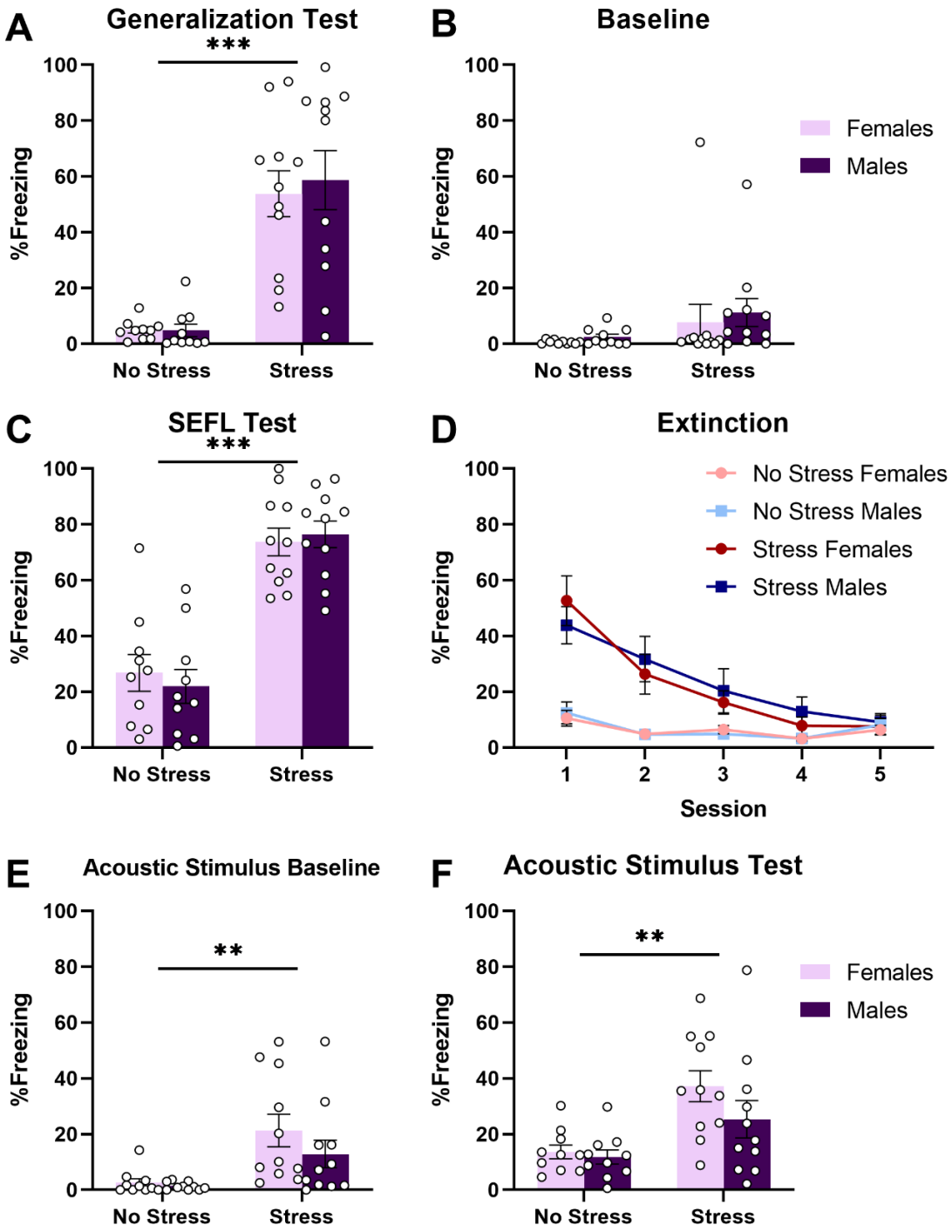


Figure 1-8. Experiment 2 fear learning results. **A.** Stress subjects show greater fear generalization to a context that shared some features with the stress context (Day 17). **B.** No difference in baseline fear to novel context prior to single footshock (Day 18). **C.** Stress subjects show elevated fear to SEFL context (Day 19). **D.** No differences between males and females were observed in extinction of fear to SEFL context (Days 23-27). **E.** Stress subjects show some fear generalization to novel context prior to acoustic stimulus delivery (Day 28). **F.** Stress subjects show elevated fear to context paired with acoustic stimulus (Day 29). **p<0.01, ***p<0.001. Error bars represent standard error of the mean.

Stress increases anxiety-related behavior in the absence of alcohol exposure

Stress produced similar changes in anxiety-related behavior as observed in Experiment 1. Stress decreased exploratory behavior during the Open Field Test as indicated by a significant Minute*Condition interaction ($F_{11,418}$=1.91, p=0.04; Fig 1-9A), with follow-up simple main effects revealing reduced locomotion in Stress subjects at multiple timepoints (ps<0.05). While females showed more exploratory behavior compared to males ($F_{1,38}$=6.48, p=0.02), no interaction between stress exposure and sex was observed ($F_{1,38}$=1.78, p=0.19). No differences in time spent in Zone 4 were observed (Minute*Condition*Sex: $F_{8,289}$=1.49, p=0.17; Fig 1-9B).

Anxiety was also assessed using the elevated plus maze and light-dark transition test. However, no differences were observed in time spent in the open arms of the elevated plus maze (Stress: $F_{1,34}$=2.79, p=0.1; Sex: $F_{1,34}$=0.15, p=0.7; Stress*Sex: $F_{1,34}$=0.01, p=0.99; Fig 1-9C). 4 subjects fell off the maze and were excluded from the analysis of this task. Similarly, no differences were observed in the latency to enter the light compartment of the light-dark transitions test (Stress: $F_{1,38}$=2.72, p=0.11; Sex: $F_{1,38}$=0.48, p=0.49; Stress*Sex: $F_{1,38}$=0.01, p=0.99; Fig 1-9D), time spent in the light compartment (Stress: $F_{1,38}$=3.14, p=0.09; Sex: $F_{1,38}$=0.06, p=0.81; Stress*Sex: $F_{1,38}$=0.34, p=0.56; Fig 1-9E) or entries into the light compartment (Stress: $F_{1,38}$=0.78, p=0.38; Sex: $F_{1,38}$=1.33, p=0.26; Stress*Sex: $F_{1,38}$=56, p=0.46; Fig 1-9F).

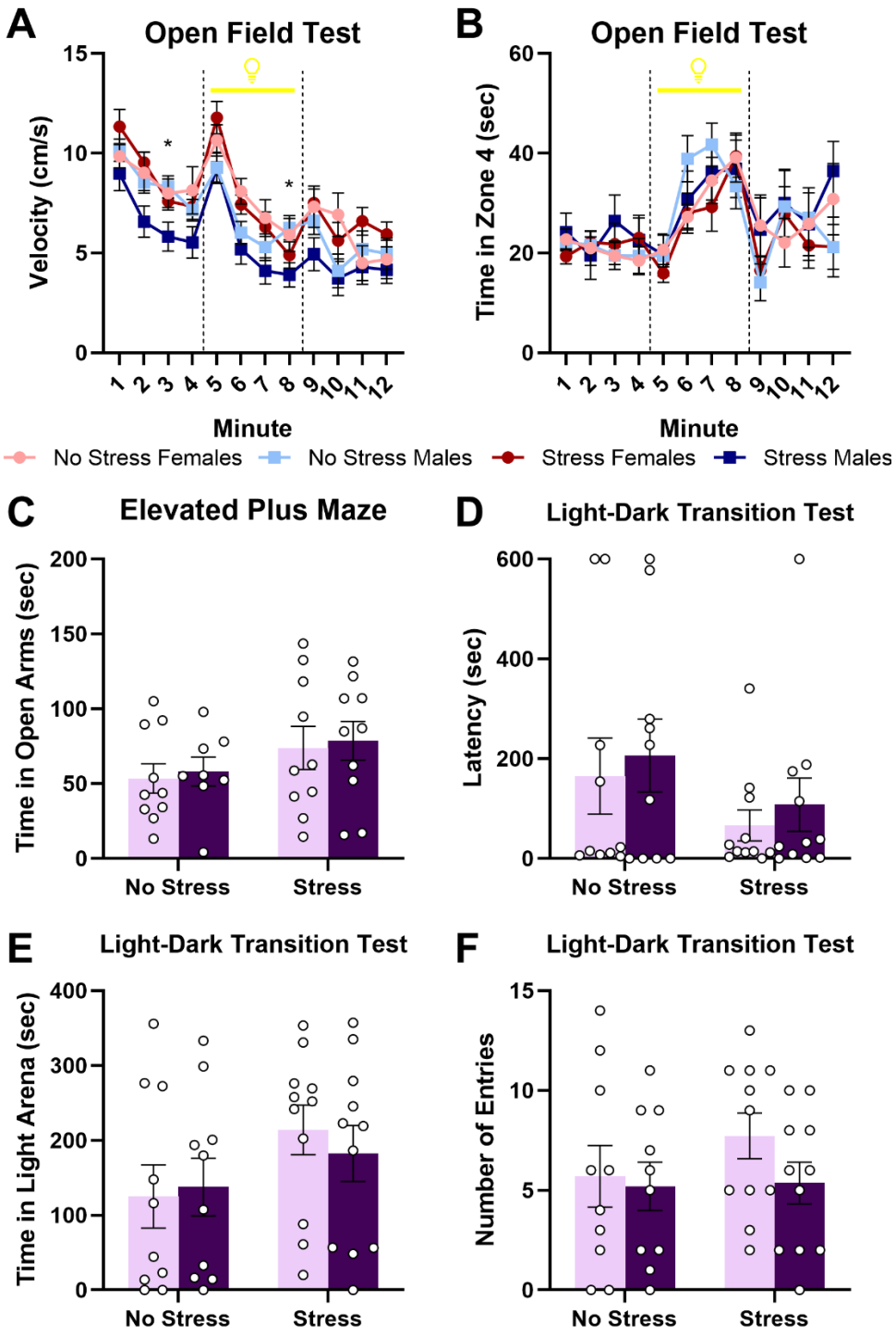


Figure 1-9. Experiment 2 anxiety test results. **A.** During open field test (Day 20) Stress subjects show reduced locomotion compared to No Stress subjects at minutes 3 and 8. **B.** No differences in time spent in Zone 4 of open field. **C.** No differences in time spent in open arms of elevated plus maze (Day 21). **D-F.** Results of light-dark transition test following stress (Day 22). No differences in latency to enter the light arena **(D)**, time in the light arena **(E)** or number of entries into the light arena **(F)**. *p<0.05. Error bars represent standard error of the mean.

Alcohol access prior to stress does not prevent stress-induced changes in alcohol intake

The absence of stress-induced increases in alcohol intake in Experiment 1 were unexpected given that we have previously shown increased alcohol intake in male Long-Evans rats following the same acute stressor (Meyer et al, 2013). One potential explanation is that alcohol access prior to stress had prevented any stress-induced enhancement, as establishment of drinking habits prior to stress exposure can prevent changes in alcohol intake following stress (Meyer et al, 2013). If this was the case, then we would expect to see increased alcohol intake in Stress subjects in Experiment 2. However, following stress exposure no differences were observed on either alcohol consumption (Stress: $F_{1,38}$=1.08, p=0.31; Stress*Sex: $F_{1,38}$=1.45, p=0.24; Fig 1-10A), alcohol preference (Stress: $F_{1,38}$=1.22, p=0.28; Stress*Sex: $F_{1,38}$=0.78, p=0.38; Fig 1-10B), or number of binge-like days (Stress: $F_{1,38}$=0.4, p=0.53; Stress*Sex: $F_{1,38}$=3, p=0.09). This indicates that the lack of a stress-induced increase in alcohol intake in Experiment 1 was not due to alcohol exposure prior to stress.

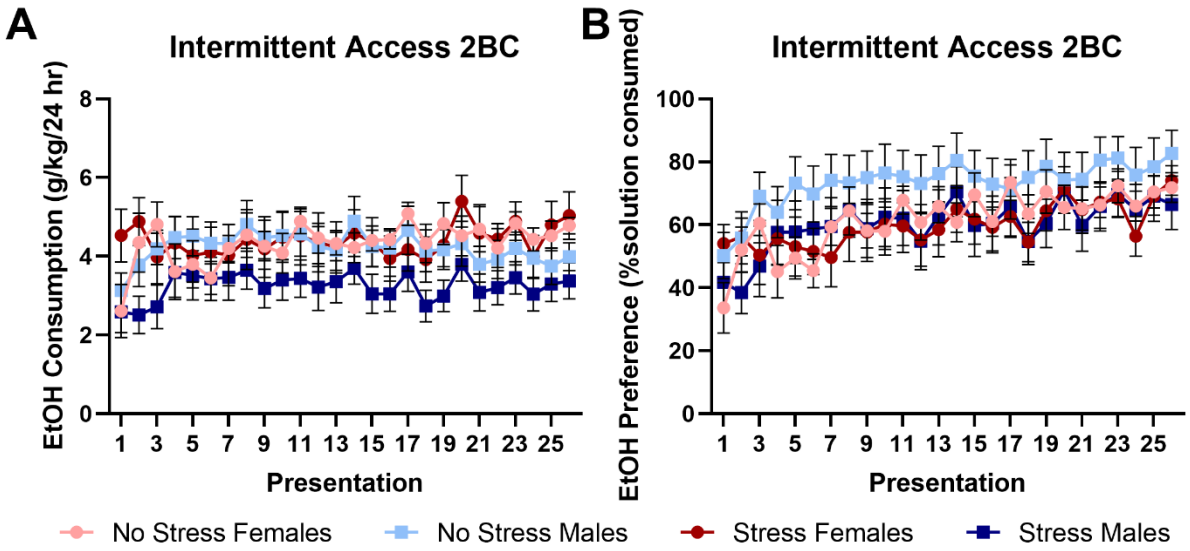


Figure 1-10. Experiment 2 intermittent access 2-bottle choice results (Days 38-96). **A.** No effects of stress exposure or sex in alcohol consumption (10% EtOH w/v) in subjects not exposed to alcohol prior to stress. **B.** No effects of stress exposure or sex in alcohol preference in subjects not exposed to alcohol prior to stress. Error bars represent standard error of the mean.

Changes in GluA1 in males following stress exposure

GluA1 levels within the BLA were measured after 2 months of intermittent access 2-bottle choice, with Western blot plates set up to compare the effects of stress within males and females. We again saw an effect of stress exposure in males, with Stress males showing marginally elevated GluA1 compared to No Stress males (t_{20}=2.03, p=0.06; Fig 1-11B). In contrast, no effects of stress exposure were observed in females (t_{20}=0.11, p=0.92; Fig 1-11A).

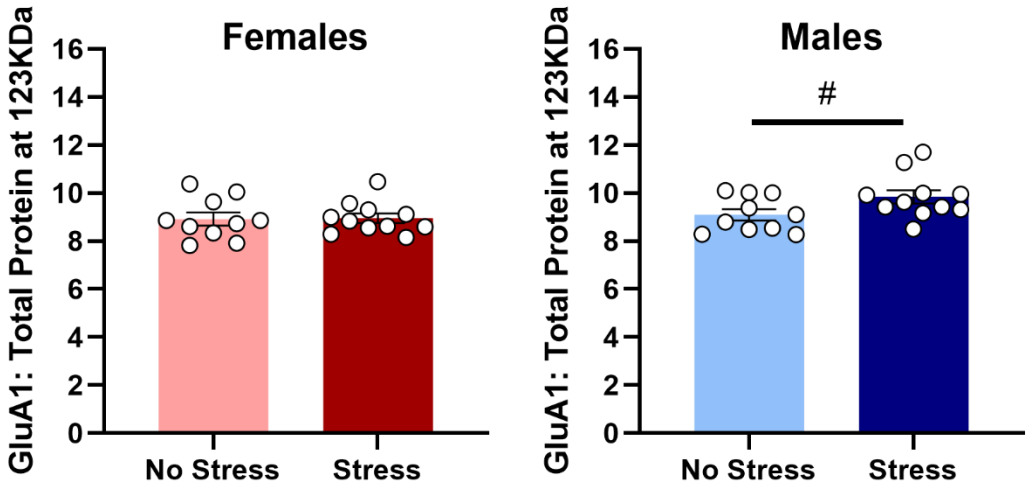


Figure 1-11. Experiment 2 Western blot results showing ratio of GluA1 to total protein at 123 KDa within the BLA. **A.** No differences in GluA1 between Stress and No Stress females. **B.** Stress males show marginally elevated GluA1 compared to No Stress males. #p<0.10. Error bars represent standard error of the mean.

Collectively, Experiment 2 indicates that the unexpected results of Experiment 1 were not influenced by pre-stress alcohol access. We generally observed similar effects of stress exposure in alcohol-naïve subjects as were observed in Experiment 1, with no interaction between stress and sex on most measures of fear or anxiety-related behavior. While we hypothesized that a lack of pre-stress alcohol access would enable stress to alter subsequent alcohol intake, no such

effects were observed. We also replicated the finding that stress exposure altered BLA GluA1 levels in males but not females, although the direction of this change was reversed.

Experiment 3: Effects of environmental enrichment and stress on alcohol intake

Experiment 3 further investigated findings that emerged from Experiments 1 and 2. In particular, Experiments 1 and 2 produced no evidence of stress-induced changes in voluntary alcohol intake for either females or males despite previous reports showing increased alcohol drinking following stress exposure in males. In Experiment 3 we therefore tested the possibility that the additional behavioral testing between stress exposure and alcohol access may have attenuated the ability of stress to impact alcohol drinking.

Methods

Subjects

Experiment 3 used 34 male Long-Evans rats (Envigo) approximately 10-11 weeks old at the start of the experiment. Housing and handling procedures were the same as described in Experiments 1 and 2.

Experimental Timeline

Subjects were divided into Stress and No Stress conditions. Both groups were further divided into Enrichment and No Enrichment conditions. Subjects in the Enrichment condition received a battery of fear learning and anxiety assessments following stress exposure as outlined in Table 1-2, while subjects in the No Enrichment remained in their home cages between the SEFL test and alcohol access.

Day	*Enrichment*	*No Enrichment*
1	Stress	Stress
2	Single footshock	Single footshock
3	Stress-enhanced fear learning test	Stress-enhanced fear learning test
4	Open field test	Rest
5	Elevated plus maze	↓
6	Light-dark transition test	
7	Aversive acoustic stimulus	
8	Context test	
9-15	Rest	
16-74	Intermittent access two-bottle choice	Intermittent access two-bottle choice

Table 1-2. Timeline for Experiment 3.

Apparatus and Procedures.

Stress-enhanced fear learning. All fear conditioning and extinction took place in three sets of four identical fear conditioning chambers housed in sound-attenuating shells (Med Associates Inc). Context A (stress context) contained flat grid floors, was lit by a white house light, and was scented with 50% Windex solution. Ventilation fans provided background noise. Context B (fear conditioning context) contained a black triangular insert and floors composed of staggered grid bars, and was scented with 1% acetic acid. Each set of grids was wired to a shock generator and scrambler. Stimulus delivery was controlled and freezing automatically scored using VideoFreeze software (Med Associates Inc).

All subjects were first transported to Context A in their homecages where they received a stressor consisting of either 15 1-sec, 1-mA unsignalled footshocks randomly distributed over 90 minutes (Stress condition) or received equivalent context exposure without footshock (No Stress condition). The next day subjects were transported to Context B in a black plastic tub divided into 4 quadrants. Following a 3-minute baseline period, all subjects received a single 1-sec, 1-mA footshock and were removed 30 seconds later. Subjects were returned to Context B the

following day for 8 minutes to assess fear to the fear conditioning context. Fear was measured via freezing.

Battery of additional tests. Subjects in the Enrichment condition underwent the open field test, the elevated plus maze and the light-dark transition test as described in Experiment 1. Following, Enrichment subjects underwent fear conditioning to a novel aversive acoustic stimulus as described in Experiment 1. Each day subjects in the No Enrichment condition were briefly handled and otherwise remained in their homecage.

Intermittent-access two-bottle choice. All subjects underwent 59 days of voluntary alcohol access as described in Experiment 1.

Data analysis

Data were analyzed using IBM SPSS Statistical Software. Results of tasks with a single dependent variable were analyzed using either 1-way ANOVA (comparing effect of Stress within the Enrichment condition) or 2-way ANOVA (Stress as the first factor and Enrichment as the second factor). Tasks using repeated measures were analyzed using mixed-model ANOVA. Violations of sphericity corrected for using the Greenhouse-Geisser correction. Data were plotted using GraphPad Prism.

Results

Results of SEFL procedure

While Meyer et al (2013) demonstrated increased alcohol intake following stress, in Experiments 1 and 2 we failed to replicate this finding. We therefore investigated other potential differences in experimental procedures that could account for this discrepancy. One notable

difference is that in Experiments 1 and 2 subjects received an extensive battery of behavioral tests in between stress exposure and alcohol access, while Meyer et al 2013 did not include any additional testing between the SEFL Test and alcohol access. This additional testing could act as form of environmental enrichment, which has been shown to attenuate the effects of stress (Francis et al, 2002). Experiment 3 tested whether a history of behavioral testing between the SEFL procedure and alcohol access could alter the ability of stress to influence alcohol intake.

We first ensured that there were no pre-existing differences in SEFL between the Enrichment and No Enrichment groups. While Stress subjects showed some generalization from the stress context to the fear conditioning context ($F_{1,30}$=43.12, p<0.001; Fig 1-12A), there were no differences between Enrichment and No Enrichment groups (Enrichment: $F_{1,30}$=2.04, p=0.16; Stress*Enrichment: $F_{1,30}$=1.74, p=0.2). Stress subjects showed a dramatic enhancement in freezing to the fear conditioning context ($F_{1,30}$=494.62, p<0.001; Fig 1-12B) while no differences were observed between Enrichment and No Enrichment groups (Enrichment: $F_{1,30}$=0.56, p=0.46; Stress*Enrichment: $F_{1,30}$=0.16, p=0.70).

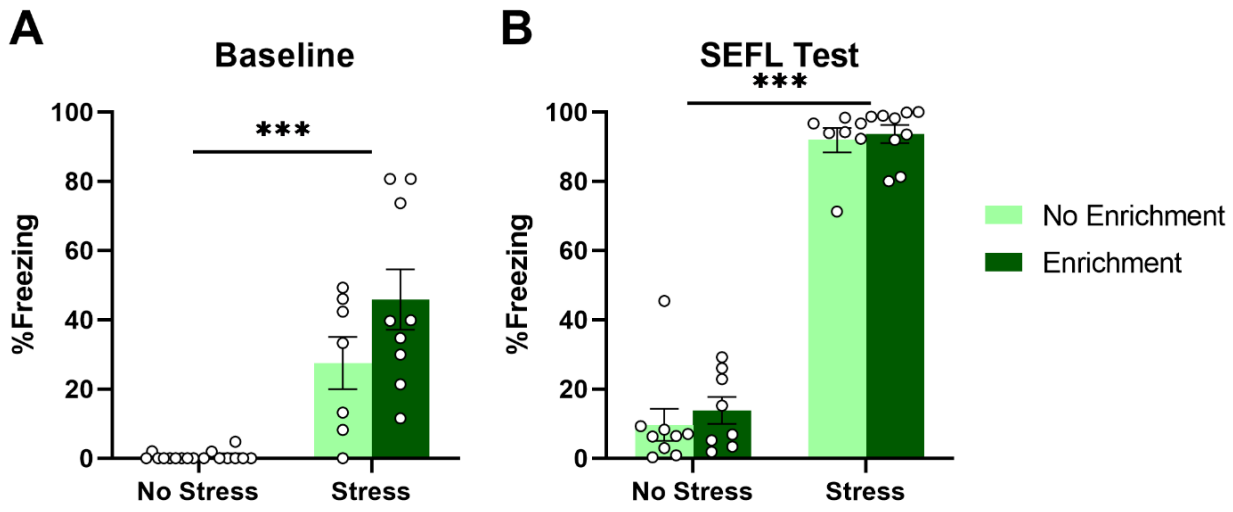


Figure 1-12. Experiment 3 SEFL Results. **A.** Stress subjects showed elevated fear to a novel context prior to footshock exposure (Day 2). **B.** Stress subjects show elevated fear to SEFL context (Day 3). ***p<0.001. Error bars represent standard error of the mean.

Stress increases anxiety on the open field test

Following the SEFL procedure, subjects in the Enrichment group received an additional battery of fear learning and anxiety assessments. As seen in Experiments 1 and 2, stress exposure reduced exploratory behavior during the open field test as indicated by a Minute*Stress interaction ($F_{11,165}$=3.76, p<0.001; Fig 1-13A). Follow-up simple main effects revealed that Stress subjects showed reduced exploratory behavior at multiple timepoints throughout the 12-minute task (ps<0.05-0.001). No significant differences between groups were seen in time spent in Zone 4 (Stress: $F_{1,15}$=0.29, p=0.6; Stress*Minute: $F_{11,165}$=0.92, p=0.52; Fig 1-13B). However, stress exposure did appear to cause an exaggerated location preference as was observed in Experiment 1. Among subjects that initially preferred Zone 1, Stress subjects showed less time in Zone 4 compared to No Stress subjects at multiple timepoints ($F_{11,66}$=1.99, p=0.04; Fig 1-13C). In contrast, no significant effect of stress exposure was observed in Zone 4-preferring animals ($F_{1,7}$=3.87, p=0.09; Fig 1-13D).

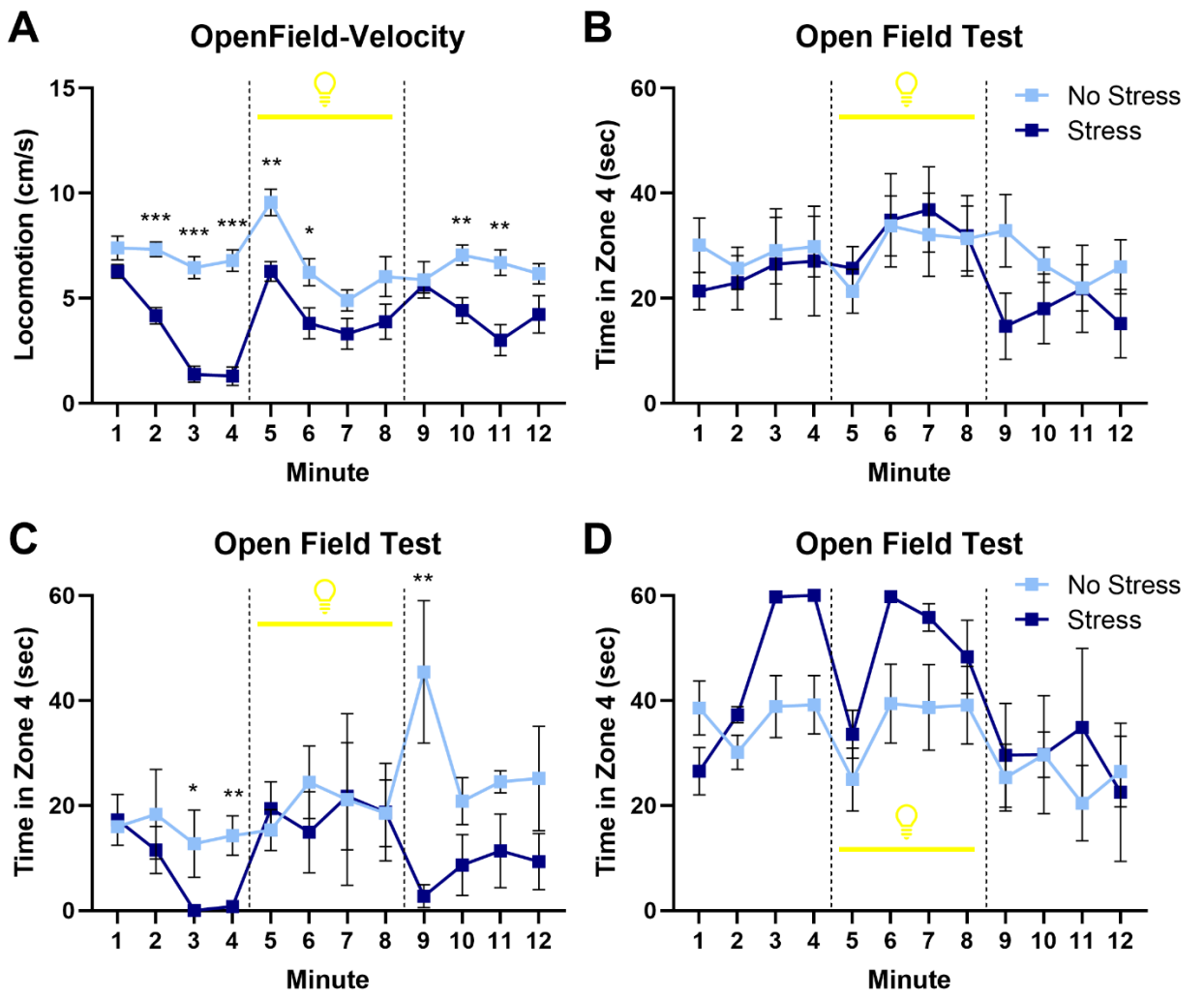


Figure 1-13. Experiment 3 open field test results from subjects in the Enrichment condition (Day 4). **A.** Stress subjects show reduced locomotion at multiple timepoints. **B.** No differences between Stress and No Stress subjects in time spent in Zone 4 of the open field test. **C.** Among subjects that initially preferred Zone 1, Stress subjects show reduced time in Zone 4 prior to light onset (minutes 3 and 4) and following light termination (minute 9). **D.** Among subjects that initially preferred Zone 4, no differences in time spent in Zone 4 were observed. *p<0.05, **p<0.01, ***p<0.001. Error bars represent standard error of the mean.

Anxiety was also assessed by the elevated plus maze and light-dark transition test. However, as with Experiments 1 and 2 no effects of Stress exposure were observed on time spent on the open arms of the elevated plus maze ($F_{1,15}$=0.01, p=0.92; Fig 1-14A). Similarly, on the

light-dark transition test no differences were observed in latency to enter the light compartment ($F_{1,15}$=0.06, p=0.81; Fig 1-14B), time in the light compartment ($F_{1,15}$=0.12, p=0.74; Fig 1-14C) or entries into the light compartment ($F_{1,15}$=0.04, p=0.84; Fig 1-14D).

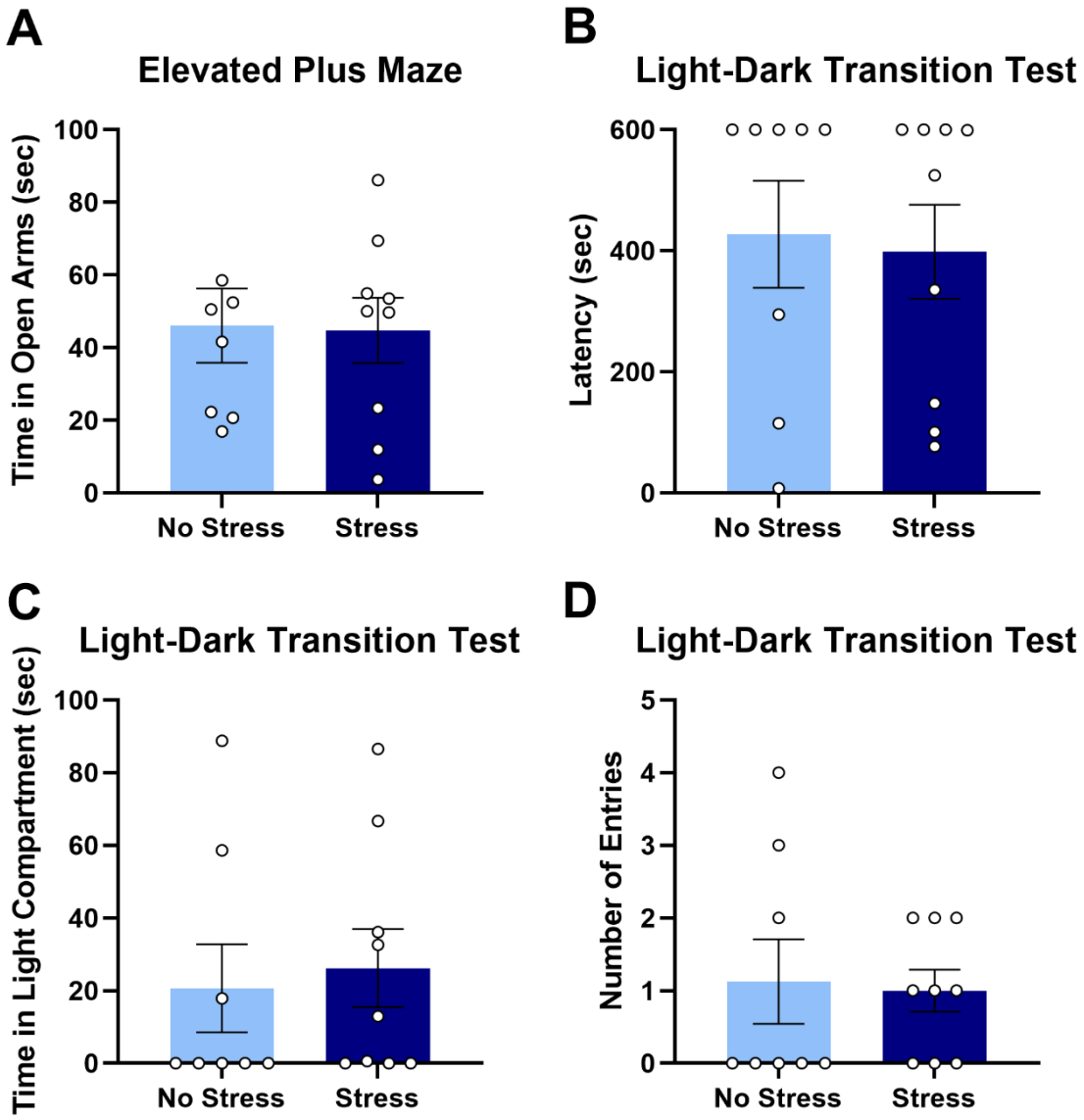


Figure 1-14. Experiment 3 results of elevated plus maze and light-dark transition test. **A.** No effects of stress exposure on time spent in the open arms of the elevated plus maze (Day 5). **B-D.** Results of light-dark transition test (Day 6). No effects of stress exposure on latency to enter the light arena **(B)**, time spent in the light arena **(C)** or number of entries into the light arena **(D)**. Error bars represent standard error of the mean.

Stress increases fear to an aversive acoustic stimulus

Following tests of anxiety-like behavior, the Enrichment group also underwent fear conditioning to an aversive acoustic stimulus in a novel context. Replicating the effects seen in Experiments 1 and 2, Stress subjects showed elevated fear to the novel context both prior to ($F_{1,15}$=19.98, p<0.001; Fig 1-15A) and following stimulus exposure ($F_{1,15}$=50.48, p<0.001; Fig 1-15B).

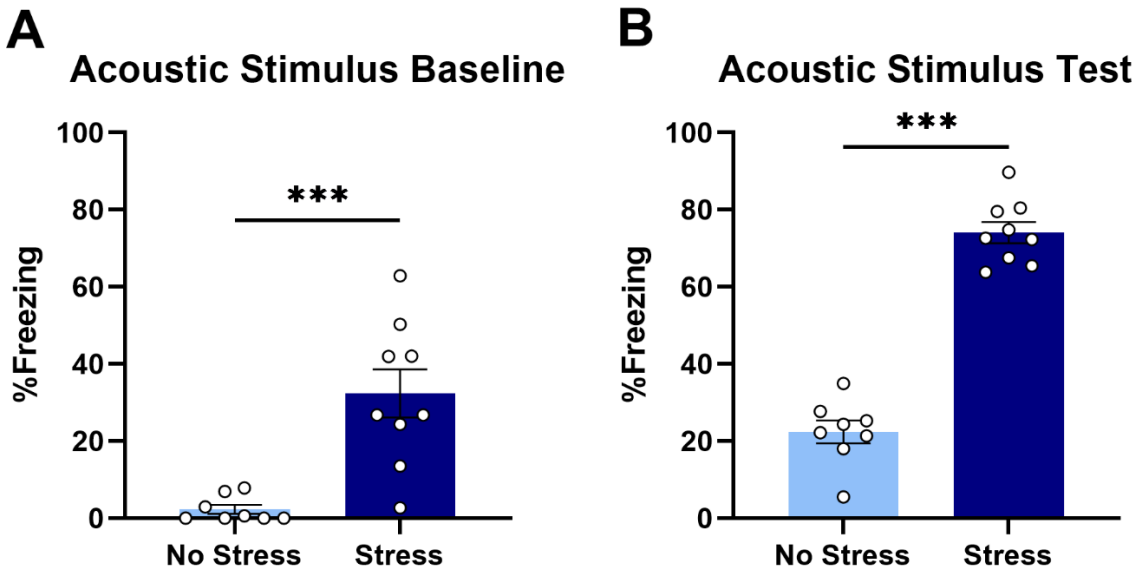


Figure 1-15. Experiment 3 acoustic stimulus results. **A.** Stress subjects showed elevated fear to the novel context prior to acoustic stimulus delivery (Day 7). B**.** Stress subjects show elevated fear to the acoustic stimulus context the following day (Day 8). ***p<0.001. Error bars represent standard error of the mean.

Stress and behavioral testing may interact to alter alcohol intake

Following completion of fear and anxiety testing, all subjects received two months of intermittent access 2-bottle choice. No significant effects of either stress exposure or enrichment were observed in either alcohol consumption (Stress: $F_{1,30}$=1.7, p=0.20; Enrichment: $F_{1,30}$=2.18,

p=0.15; Fig 1-16A), alcohol preference (Stress: $F_{1,30}$=0.26, p=0.62; Enrichment: $F_{1,30}$=0.43, p=0.52; Fig 1-16B) or number of binge-like days (Stress: $F_{1,30}$=2.47, p=0.13; Enrichment: $F_{1,30}$=3.79, p=0.06). While we hypothesized that enrichment could influence the ability of stress to alter alcohol intake, no significant interaction was observed on either alcohol consumption ($F_{1,30}$=0.83, p=0.37), alcohol preference ($F_{1,30}$=1.16, p=0.29) or number of binge-like days ($F_{1,30}$=1.88, p=0.18). However, inspection of the data revealed that there was a general trend towards elevated alcohol intake in the subjects that had undergone both stress and enrichment relative to the other three groups.

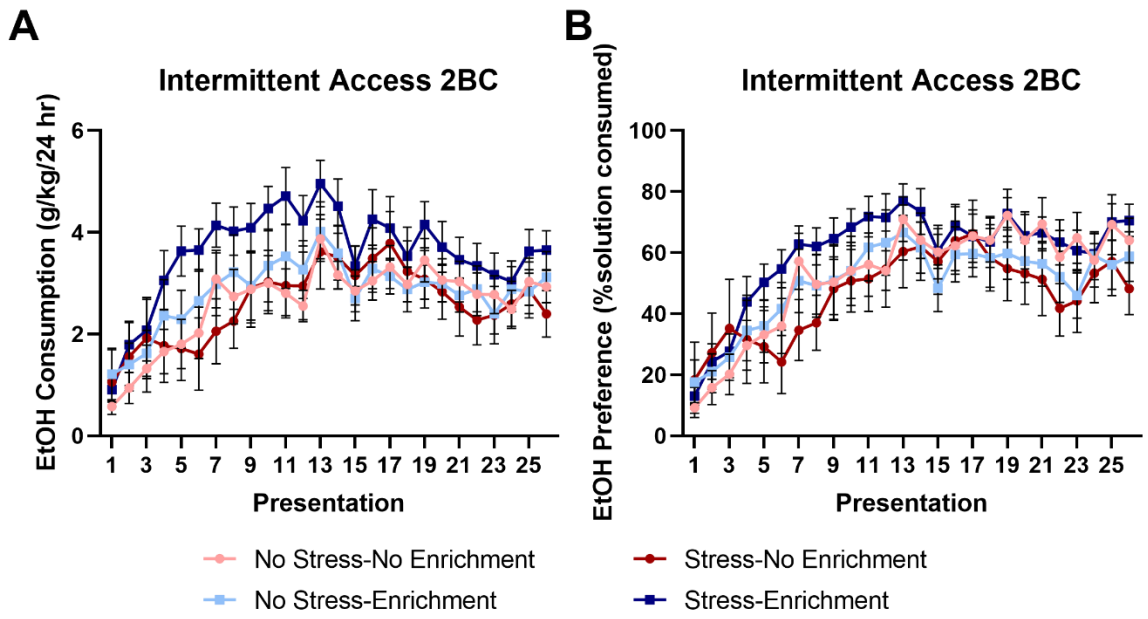


Figure 1-16. Experiment 3 intermittent access 2-bottle choice results (Days 16-74). **A.** Stress-Enrichment subjects show numerically elevated alcohol consumption (10% EtOH w/v) compared to other groups, but no significant effects of stress or enrichment were observed. **B.** Stress-Enrichment subjects show numerically elevated alcohol preference compared to other groups, but no significant effects of stress or enrichment were observed. Error bars represent standard error of the mean.

Experiments 1 and 2 showed no evidence of stress-induced changes in alcohol drinking despite previous reports of increased alcohol drinking following stress exposure in males (Meyer et al., 2013). As environmental enrichment has been shown to attenuate the effects of stress

exposure, Experiment 3 investigated whether behavioral testing functioned as a form of enrichment to prevent the effects of stress on alcohol intake. These results indicate that stress exposure and additional behavioral testing may interact to alter alcohol intake. Specifically, subjects that received both stress and additional testing showed numerically elevated alcohol intake, suggesting that additional testing instead functioned as an additional source of stress.

Discussion

Rodent models of stress exposure that recapitulate features of PTSD are a valuable tool for understanding the biological mechanisms of this disorder, yet much of the research in this field has been conducted exclusively in males despite the fact that women are twice as likely to develop PTSD compared to men (Kessler et al., 2017; Kessler et al., 1995). In these experiments we investigated potential sex differences in the responses of male and female rats to the SEFL procedure, looking both at fear learning and a constellation of other behavioral changes relevant to PTSD symptomology. We further examined sex differences in the effects of stress on GluA1 levels within the BLA.

Stress produces similar changes in fear learning, generalization and extinction across sexes

Women show higher rates of PTSD compared to men even when controlling for trauma type (Breslau et al., 1997; Kessler et al., 2017; Kessler et al., 1995). Furthermore, women with PTSD show stronger fear conditioning and fear generalization compared to men (Gamwell et al., 2015; Inslicht et al., 2013). We therefore hypothesized that female rats would show more fear during the SEFL test, as well as elevated fear generalization and impaired fear extinction. However, in Experiments 1 and 2 we found that while stress exposure produced a robust enhancement in fear learning to a novel context there were no differences between males and

females, nor did differences in emerge during fear extinction to the SEFL context. Similarly, no sex differences were observed in fear generalization or in fear to a context associated with a novel aversive stimulus. While it has been suggested that female rats express fear in a manner other than freezing such as darting and rearing (Choy et al., 2012; Gruene et al., 2015), we found no evidence of such behaviors in either males or females.

Alcohol exposure during early life and adolescence has been reported to disrupt fear learning in rats (Bergstrom et al., 2006; Hamilton et al., 2011; Weitemier & Ryabinin, 2003). Given that females showed elevated baseline alcohol intake in Experiment 1, one possibility was that the lack of a sex difference in fear learning was due to females receiving more alcohol exposure. We tested this possibility in Experiment 2, exposing male and female subjects to the same battery of assessments as used in Experiment 1 with the exception that no alcohol was provided prior to stress. However, we again observed no differences in fear learning, generalization or extinction between males and females. These results suggest that the lack of sex differences in fear learning were not influenced by alcohol exposure.

An additional explanation for the lack of sex differences in fear-related behavior is the intensity of the stressor used. The 15-footshock stress used in SEFL experiments produces exceptionally robust, reliable enhancements in fear learning that can last for at least 90 days (Rau & Fanselow, 2009). Some rodent models of stress exposure have been reported to produce both "susceptible" groups that show behavioral changes following stress and "resilient" groups that do not (Covington et al., 2010; Jeong et al., 2020; Ritov et al., 2016). However, the fear-enhancing ability of the SEFL model is such that we have never had to apply such separation criterion to subjects exposed to the 15-footshock stress (Perusini et al., 2016; Rau et al., 2005; Rau & Fanselow, 2009). It is therefore plausible sex differences in stress susceptibility exist but were

obscured by the nature of the stressor used. Utilizing the uniquely quantifiable nature of the SEFL procedure by testing the effects of a less intense stressor (e.g. 4 footshocks instead of the standard 15) may be more effective for revealing differences in stress susceptibility.

Males and females differ in anxiety-related behavior

While no sex differences in fear-related behavior were found, we did observe differences in anxiety-related behavior between males and females. Previous studies have found evidence of more locomotion and less anxiety-like behavior in female rats (Johnston & File, 1991; Scholl et al., 2019; Zimmerberg & Farley, 1993). Similarly, in Experiments 1 and 2 we demonstrated that females show less anxiety-related behavior both prior to and following stress, although stress exposure produced similar reductions in general exploratory behavior across both sexes during the open field test.

We did observe that stress exposure differentially impacted the response of males and females to an anxiogenic light stimulus. In Experiment 1, both Stress and No Stress females showed similar avoidance of the brightest zone following light onset, which is in agreement with a previous report from our laboratory showing that footshock exposure does not impact light avoidance on this task in females (Godsil et al., 2005). However, Experiments 1 and 3 indicated that stress-exposed males possess exaggerated preferences for specific locations within the arena and are unwilling to move from those locations even when they become aversive. This may reflect reduced behavioral flexibility in males, as stress exposure has previously been shown to impair performance on assessment of flexibility such as reversal learning and set-shifting as well as fear extinction (Holmes & Wellman, 2009; Izquierdo et al., 2006). Interestingly, acute stress has been shown to disrupt cognitive flexibility in men but not women (Sheilds et al., 2016).

Stress exposure did not influence voluntary alcohol intake

A significant challenge in treating PTSD is the high levels of comorbidity between PTSD and alcohol abuse (Kessler et al., 1995). Because alcohol abuse occurs more frequently in men than in women, we anticipated that males would show a greater increase in alcohol intake following stress compared to females. However, in Experiments 1 and 2 we did not observe any effects of stress on alcohol intake in either males or females. This was surprising given previous evidence of increased alcohol intake following SEFL (Meyer et al., 2013). We hypothesized that the results of Experiment 1 could be explained by the fact that these subjects received alcohol exposure prior to stress, as establishment of drinking habits prior to stress have been shown to prevent stress-induced increases in alcohol intake (Meyer et al., 2013). However, in Experiment 2 we still failed to see an effect of stress despite these subjects being alcohol-naïve prior to stress. In Experiment 3, we further explored aspects of our experimental design that may have masked the effects of stress on alcohol drinking.

Another potential explanation for the discrepancy between the results of Experiments 1 and 2 and that of Meyer et al (2013) was the extensive battery of behavioral testing subjects received in between stress and intermittent access 2-bottle choice. It is conceivable that the additional testing functioned as a form of environmental enrichment, which has been shown to attenuate the effects of stress (Francis et al., 2002; Solinas et al., 2008). However, the results of Experiment 3 indicated that this was not the case. Instead, we saw a trend towards increased alcohol intake in subjects that had received both stress and additional testing. It is possible that rather than acting as enrichment, the additional testing instead served as an additional form of stress that allowed the emergence of a stress-induced increase in alcohol intake. Since all subjects in Experiments 1

and 2 received this additional testing, it is possible that the lack of a difference was not due to no effects of stress but rather all subjects showing stress-induced elevations in drinking.

It is worth noting that despite alcohol abuse occurring more frequently in men compared to women, rodent studies frequently report more alcohol drinking in females compared to males (Lancaster et al., 1996; Priddy et al., 2017). Accordingly, in Experiment 1 we observed greater alcohol drinking in females both before and after stress. This discrepancy between human epidemiology findings and the rodent literature may be a reminder that alcohol use is influenced by environmental as well as biological factors.

Stress influences BLA GluA1 levels in males but not females

While males and females generally showed similar changes in fear and anxiety following stress, one question that remains is whether the biological changes that underlie such behaviors are the same. It is likely that the fear sensitization demonstrated in both SEFL and PTSD patients is due to changes in the neural network that support fear conditioning, of which the basolateral amygdala (BLA) is a key component (Fendt & Fanselow, 1999). Fear memories are thought to be formed by the strengthening of synaptic inputs conveying sensory information to the BLA (Kim & Jung, 2006; Rogan et al., 1997). This occurs via long-term potentiation (LTP), which is typically triggered by Ca^{2+} influx through N-Methyl-D-aspartic acid (NMDA) glutamate receptors (Maren, 1999). However, we hypothesize that the availability of an alternative mechanism of synaptic strengthening may underlie the elevated fear learning observed in SEFL. We have previously observed increased protein levels of the AMPAR GluA1 subunit within the BLA of male rats following stress (Perusini et al., 2016). While AMPA receptors typically exist as heteromers of GluA1 and GluA2, with the GluA2 subunit rendering the receptor impermeable

to Ca^{2+} entry, increased GluA1 levels can result in the formation of GluA1-only receptors that allow Ca^{2+} entry and could thus enable an alternative form of LTP (Hollmann et al., 1991; Wright & Vissel, 2012) This leads to the possibility that stress exposure increases Ca^{2+}-permeable AMPA receptor levels, which could augment canonical BLA plasticity.

In Experiments 1 and 2 we observed altered GluA1 levels within the BLA of stress-exposed males. Curiously, while GluA1 levels were elevated as expected in Experiment 2 they were decreased in Experiment 1. One potential explanation is that GluA1 levels were altered by the extensive behavioral testing in between stress exposure and tissue collection. Many types of learning experiences have been shown to produce transient increases in GluA1-containing AMPA receptors at the synapse, although these increases are typically followed by the insertion of GluA2-containing AMPA receptors (Matsuo et al., 2008; Wright & Vissel, 2012). Alcohol access has also been shown to increase GluA1 phosphorylation within the amygdala (Agoglia et al., 2015; McGinn et al., 2016), a process required for the trafficking of AMPA receptors to the synapse (Lee et al., 2010). This suggest that perhaps the two months of voluntary alcohol access prior to tissue collection further impacted BLA GluA1 levels.

In contrast to the effects on males, among females we observed no effects of stress exposure on BLA GluA1 levels in either Experiment 1 or Experiment 2. One possibility is that the aforementioned complications obscured any effects of stress, although why this would occur differently in males compared to females is unclear. Alternatively, it is possible that females do not in fact show altered GluA1 levels following stress and instead rely on an entirely different mechanism. There is evidence of differences in GluA1 and GluA2 expression between males and females. Females have been reported to show increased GluA1 in the nucleus accumbens (NAc)

and hippocampus and increased GluA2 within the ventral tegmental area (VTA) (Castro-Zavala et al., 2020; Katsouli et al., 2014).

There is also evidence that stress differentially impacts GluA1 in males compared to females. Many of the studies that have reported changes in BLA GluA1 expression, trafficking and phosphorylation following stress exposure were conducted in males only (Caudal et al., 2010; Hubert et al., 2014; Perusini et al., 2016). Among studies that do include both males and females, early life stress combined with cocaine exposure has been shown to increase the ratio of GluA1 to GluA2 in the NAc in males but not in females (Castro-Zavala et al., 2020), while early life stress decreases BLA GluA2 levels in males but not females (Guadagno et al., 2020). While it is unclear whether these changes in protein are supported by corresponding changes in transcription of *gria1* and *gria2*, which encode GluA1 and GluA2 respectively, social stress has been reported to alter BLA *gria2* levels in male but not female hamsters (McCann et al., 2018). Lastly, contextual fear conditioning is impaired in GluA1-knockout male mice, but not in GluA1-knockout female mice (Dachtler et al., 2011). Collectively, these results indicate that while males and females show comparable levels of fear enhancement following stress, the mechanisms through which fear memories are formed and impacted by stress may be very different.

Comparison of results with PTSD epidemiology

Given the higher prevalence of PTSD among women, we hypothesized that the SEFL procedure would produce greater changes in fear and anxiety behavior among. Contrary to these expectations, we found no sex differences on either fear learning, fear generalization or fear extinction. While this may be surprising from a human epidemiology viewpoint, these results are

in fact in agreement with rodent studies that have investigated sex differences in fear learning (Dalla & Shors, 2009). Several studies investigating fear learning to discrete tone CSs have reported that fear levels in females are comparable (Graham et al., 2009; Markus & Zecevic, 1997) or reduced (Pryce et al., 1999) compared to that of males. Studies of contextual fear conditioning have typically reported less context fear in females compared to males, which may reflect differences in hippocampal processing of contextual stimuli in females (Daviu et al., 2014; Maren et al., 1994; Markus & Zecevic, 1997; Poulos et al., 2015). Although we did not find significant sex differences on any measures of fear behavior, inspection of the data does indicate that females overall showed slightly lower levels of freezing compared to males.

Similarly, the small number of studies that do evaluate sex differences in fear learning following stress exposure rarely find more pronounced effects in females compared to males (Shansky, 2015). Previous studies have reported either no sex differences in fear learning (Poulos et al., 2015) or found that stress exposure enhances fear learning and impairs fear extinction in males but not females (Baran et al., 2009; Keller et al., 2015; Wood & Shors, 1998). Collectively, the results of our current experiments corroborate previous reports showing comparable or reduced freezing in females compared to males across fear acquisition and extinction procedures.

While males and females showed similar effects of stress on fear and anxiety in the present experiments, this does not mean that the biological mechanisms that support these behaviors are the same. As mentioned above, stress exposure was associated with altered GluA1 in males but not females suggesting that stress-enhanced fear learning may be supported different mechanisms. Sex differences have also been reported in the hypothalamus-pituitary-adrenal (HPA) axis, which governs the stress response. The HPA axis involves the cascading

release of endocrine signals that ultimately results in the release of corticosterone, the primary stress hormone in rodents (Smith & Vale, 2006). Females show a stronger HPA axis response to stress compared to males as indicated by greater production and release corticosterone and other stress hormones (Heck & Handa, 2019). HPA axis activity is terminated by the binding of corticosterone onto glucocorticoid receptors (GRs) in the hippocampus and hypothalamus. Evidence suggests that this negative feedback is weaker in females compared to males, as females require longer for corticosterone levels to return to baseline following stress termination and loss of GRs within forebrain regions including the hippocampus causes HPA axis dysregulation in males but not females (Heck & Handa, 2019; Solomon et al., 2012). These differences are thought to be due in part to the activity of gonadal hormones, with HPA axis activity varying along with the estrous cycle of female rodents (Viau & Meaney, 1991).

Collectively, these findings indicate that while the behavioral results of the SEFL procedure do not precisely reflect the epidemiology of PTSD observed in humans, this procedure will still be valuable for probing the biological mechanisms that support these behaviors. In particular, it is likely that interactions between gonadal hormones and HPA axis activity may mediate the differences in stress susceptibility between men and women.

Limitations

One limitation of this study was that for each experiment all testing was done within the same subjects and counterbalancing the order of each test was not feasible owing to the large battery of tests used. While efforts were made to conduct the task in increasing order of stress severity (apart from stress exposure itself), it is possible that performance was influenced by the animals' history of testing. For example, anxiety tests are particularly sensitive to novelty and

there is evidence that exposure on one anxiety test can influence performance on others (McIlwain et al., 2001; Rodgers & Shepherd, 1993). In future studies it will be informative to replicate and explore specific results in the absence of additional testing, such as the differential effects of stress on responses to an anxiogenic light stimulus during the open field test. Similarly, given the intriguing sex-specific effects of stress exposure on GluA1 levels within the BLA, follow-up studies will be necessary to investigate whether these effects reflect a genuine sex difference or are an artifact of the testing procedures. Lastly, while the results of Experiment 3 suggest that stress and a history of behavioral testing may interact to influence alcohol intake, the relatively small number of subjects meant that our ability to find significant effects was underpowered. In future studies it will be worth replicating this effect with a larger sample size.

Conclusion

In these experiments we show a detailed characterization of sex differences in fear, anxiety and alcohol intake following an intense acute stressor. We found that despite the higher rates of PTSD among women compared to men, male and female rats showed similar changes in fear learning following stress exposure. Sex differences were observed in anxiety-related behavior, with females showing more exploratory behavior and stress-exposed males showing a blunted response to an anxiogenic stimulus. We further found evidence that behavioral testing and stress exposure may interact to enhance alcohol intake. Lastly, we found that stress altered BLA GluA1 levels in males but not females despite producing similar behavioral changes, suggesting that these animals may show similar behaviors via different mechanisms.

References

Agoglia, A. E., Holstein, S. E., Reid, G., & Hodge, C. W. (2015). CaMKIIα-GluA1 Activity Underlies Vulnerability to Adolescent Binge Alcohol Drinking. *Alcohol Clin Exp Res*, *39*(9), 1680-1690. https://doi.org/10.1111/acer.12819

Ballaz, S. J., Akil, H., & Watson, S. J. (2007). Previous experience affects subsequent anxiety-like responses in rats bred for novelty seeking. *Behav Neurosci*, *121*(5), 1113-1118. https://doi.org/10.1037/0735-7044.121.5.1113

Banasikowski, T. J., Cloutier, C. J., Ossenkopp, K. P., & Kavaliers, M. (2015). Repeated exposure of male mice to low doses of lipopolysaccharide: dose and time dependent development of behavioral sensitization and tolerance in an automated light-dark anxiety test. *Behav Brain Res*, *286*, 241-248. https://doi.org/10.1016/j.bbr.2015.03.004

Baran, S. E., Armstrong, C. E., Niren, D. C., Hanna, J. J., & Conrad, C. D. (2009). Chronic stress and sex differences on the recall of fear conditioning and extinction. *Neurobiol Learn Mem*, *91*(3), 323-332. https://doi.org/10.1016/j.nlm.2008.11.005

Bergstrom, H. C., McDonald, C. G., & Smith, R. F. (2006). Alcohol exposure during adolescence impairs auditory fear conditioning in adult Long-Evans rats. *Physiol Behav*, *88*(4-5), 466-472. https://doi.org/10.1016/j.physbeh.2006.04.021

Bremner, J. D., Krystal, J. H., Southwick, S. M., & Charney, D. S. (1995). Functional neuroanatomical correlates of the effects of stress on memory. *J Trauma Stress*, *8*(4), 527-553.

Breslau, N., Davis, G. C., Andreski, P., Peterson, E. L., & Schultz, L. R. (1997). Sex differences in posttraumatic stress disorder. *Arch Gen Psychiatry*, *54*(11), 1044-1048. https://doi.org/10.1001/archpsyc.1997.01830230082012

Castro-Zavala, A., Martín-Sánchez, A., & Valverde, O. (2020). Sex differences in the vulnerability to cocaine's addictive effects after early-life stress in mice. *Eur Neuropsychopharmacol*, *32*, 12-24. https://doi.org/10.1016/j.euroneuro.2019.12.112

Caudal, D., Godsil, B. P., Mailliet, F., Bergerot, D., & Jay, T. M. (2010). Acute stress induces contrasting changes in AMPA receptor subunit phosphorylation within the prefrontal cortex, amygdala and hippocampus. *PLoS One*, *5*(12), e15282. https://doi.org/10.1371/journal.pone.0015282

Choi, J. S., & Kim, J. J. (2010). Amygdala regulates risk of predation in rats foraging in a dynamic fear environment. *Proc Natl Acad Sci U S A*, *107*(50), 21773-21777. https://doi.org/10.1073/pnas.1010079108

Choy, K. H., Yu, J., Hawkes, D., & Mayorov, D. N. (2012). Analysis of vigilant scanning behavior in mice using two-point digital video tracking. *Psychopharmacology (Berl)*, *221*(4), 649-657. https://doi.org/10.1007/s00213-011-2609-5

Covington, H. E., Lobo, M. K., Maze, I., Vialou, V., Hyman, J. M., Zaman, S., LaPlant, Q., Mouzon, E., Ghose, S., Tamminga, C. A., Neve, R. L., Deisseroth, K., & Nestler, E. J.

(2010). Antidepressant effect of optogenetic stimulation of the medial prefrontal cortex. *J Neurosci*, *30*(48), 16082-16090. https://doi.org/10.1523/JNEUROSCI.1731-10.2010

Craske, M. G., Kircanski, K., Zelikowsky, M., Mystkowski, J., Chowdhury, N., & Baker, A. (2008). Optimizing inhibitory learning during exposure therapy. *Behav Res Ther*, *46*(1), 5-27. https://doi.org/10.1016/j.brat.2007.10.003

Dachtler, J., Fox, K. D., & Good, M. A. (2011). Gender specific requirement of GluR1 receptors in contextual conditioning but not spatial learning. *Neurobiol Learn Mem*, *96*(3), 461-467. https://doi.org/10.1016/j.nlm.2011.07.001

Dalla, C., & Shors, T. J. (2009). Sex differences in learning processes of classical and operant conditioning. *Physiol Behav*, *97*(2), 229-238. https://doi.org/10.1016/j.physbeh.2009.02.035

Daviu, N., Andero, R., Armario, A., & Nadal, R. (2014). Sex differences in the behavioural and hypothalamic-pituitary-adrenal response to contextual fear conditioning in rats. *Horm Behav*, *66*(5), 713-723. https://doi.org/10.1016/j.yhbeh.2014.09.015

Dykman, R. A., Ackerman, P. T., & Newton, J. E. (1997). Posttraumatic stress disorder: a sensitization reaction. *Integr Physiol Behav Sci*, *32*(1), 9-18.

Fendt, A., & Fanselow, M. S. (1999). The neuroanatomical and neurochemical basis of conditioned fear. *Neuroscience & Biobehavioral Reviews*, *23*(5), 734-760.

Francis, D. D., Diorio, J., Plotsky, P. M., & Meaney, M. J. (2002). Environmental enrichment reverses the effects of maternal separation on stress reactivity. *J Neurosci*, *22*(18), 7840-7843.

Gamwell, K., Nylocks, M., Cross, D., Bradley, B., Norrholm, S. D., & Jovanovic, T. (2015). Fear conditioned responses and PTSD symptoms in children: Sex differences in fear-related symptoms. *Dev Psychobiol*, *57*(7), 799-808. https://doi.org/10.1002/dev.21313

Godsil, B. P., Blackmore, M. A., & Fanselow, M. S. (2005). Modulation of an activity response with associative and nonassociative fear in the rat: a lighting differential influences the form of defensive behavior evoked after fear conditioning. *Learn Behav*, *33*(4), 454-463.

Godsil, B. P., & Fanselow, M. S. (2004). Light stimulus change evokes an activity response in the rat. *Learn Behav*, *32*(3), 299-310.

Graham, L. K., Yoon, T., Lee, H. J., & Kim, J. J. (2009). Strain and sex differences in fear conditioning: 22 kHz

ultrasonic vocalizations and freezing in rats. *Psychology & Neuroscience*, *2*(2), 219-225.

Gruene, T. M., Flick, K., Stefano, A., Shea, S. D., & Shansky, R. M. (2015). Sexually divergent expression of active and passive conditioned fear responses in rats. *Elife*, *4*. https://doi.org/10.7554/eLife.11352

Guadagno, A., Verlezza, S., Long, H., Wong, T. P., & Walker, C. D. (2020). It Is All in the Right Amygdala: Increased Synaptic Plasticity and Perineuronal Nets in Male, But Not Female,

Juvenile Rat Pups after Exposure to Early-Life Stress. *J Neurosci, 40*(43), 8276-8291. https://doi.org/10.1523/JNEUROSCI.1029-20.2020

Hamilton, G. F., Murawski, N. J., St Cyr, S. A., Jablonski, S. A., Schiffino, F. L., Stanton, M. E., & Klintsova, A. Y. (2011). Neonatal alcohol exposure disrupts hippocampal neurogenesis and contextual fear conditioning in adult rats. *Brain Res, 1412*, 88-101. https://doi.org/10.1016/j.brainres.2011.07.027

Heck, A. L., & Handa, R. J. (2019). Sex differences in the hypothalamic-pituitary-adrenal axis' response to stress: an important role for gonadal hormones. *Neuropsychopharmacology, 44*(1), 45-58. https://doi.org/10.1038/s41386-018-0167-9

Hembree, E. A., Foa, E. B., Dorfan, N. M., Street, G. P., Kowalski, J., & Tu, X. (2003). Do patients drop out prematurely from exposure therapy for PTSD? *J Trauma Stress, 16*(6), 555-562. https://doi.org/10.1023/B:JOTS.0000004078.93012.7d

Hollmann, M., Hartley, M., & Heinemann, S. (1991). Ca2+ permeability of KA-AMPA--gated glutamate receptor channels depends on subunit composition. *Science, 252*(5007), 851-853.

Holly, E. N., & Miczek, K. A. (2015). Capturing Individual Differences: Challenges in Animal Models of Posttraumatic Stress Disorder and Drug Abuse. *Biol Psychiatry, 78*(12), 816-818. https://doi.org/10.1016/j.biopsych.2015.09.015

Holmes, A., & Wellman, C. L. (2009). Stress-induced prefrontal reorganization and executive dysfunction in rodents. *Neurosci Biobehav Rev, 33*(6), 773-783. https://doi.org/10.1016/j.neubiorev.2008.11.005

Hubert, G. W., Li, C., Rainnie, D. G., & Muly, E. C. (2014). Effects of stress on AMPA receptor distribution and function in the basolateral amygdala. *Brain Struct Funct, 219*(4), 1169-1179. https://doi.org/10.1007/s00429-013-0557-z

Inslicht, S. S., Metzler, T. J., Garcia, N. M., Pineles, S. L., Milad, M. R., Orr, S. P., Marmar, C. R., & Neylan, T. C. (2013). Sex differences in fear conditioning in posttraumatic stress disorder. *J Psychiatr Res, 47*(1), 64-71. https://doi.org/10.1016/j.jpsychires.2012.08.027

Izquierdo, A., Wellman, C. L., & Holmes, A. (2006). Brief uncontrollable stress causes dendritic retraction in infralimbic cortex and resistance to fear extinction in mice. *Journal of Neuroscience, 26*(21), 5733-5738. https://doi.org/10.1523/JNEUROSCI.0474-06.2006

Jeong, M. J., Lee, C., Sung, K., Jung, J. H., Pyo, J. H., & Kim, J. H. (2020). Fear response-based prediction for stress susceptibility to PTSD-like phenotypes. *Mol Brain, 13*(1), 134. https://doi.org/10.1186/s13041-020-00667-5

Johnston, A. L., & File, S. E. (1991). Sex differences in animal tests of anxiety. *Physiol Behav, 49*(2), 245-250. https://doi.org/10.1016/0031-9384(91)90039-q

Katsouli, S., Stamatakis, A., Giompres, P., Kouvelas, E. D., Stylianopoulou, F., & Mitsacos, A. (2014). Sexually dimorphic long-term effects of an early life experience on AMPA receptor subunit expression in rat brain. *Neuroscience, 257*, 49-64. https://doi.org/10.1016/j.neuroscience.2013.10.073

Keller, S. M., Schreiber, W. B., Staib, J. M., & Knox, D. (2015). Sex differences in the single prolonged stress model. *Behav Brain Res*, *286*, 29-32. https://doi.org/10.1016/j.bbr.2015.02.034

Kessler, R. C., Aguilar-Gaxiola, S., Alonso, J., Benjet, C., Bromet, E. J., Cardoso, G., Degenhardt, L., de Girolamo, G., Dinolova, R. V., Ferry, F., Florescu, S., Gureje, O., Haro, J. M., Huang, Y., Karam, E. G., Kawakami, N., Lee, S., Lepine, J. P., Levinson, D., Navarro-Mateu, F., Pennell, B. E., Piazza, M., Posada-Villa, J., Scott, K. M., Stein, D. J., Ten Have, M., Torres, Y., Viana, M. C., Petukhova, M. V., Sampson, N. A., Zaslavsky, A. M., & Koenen, K. C. (2017). Trauma and PTSD in the WHO World Mental Health Surveys. *Eur J Psychotraumatol*, *8*(sup5), 1353383. https://doi.org/10.1080/20008198.2017.1353383

Kessler, R. C., Berglund, P., Demler, O., Jin, R., Merikangas, K. R., & Walters, E. E. (2005). Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. *Arch Gen Psychiatry*, *62*(6), 593-602. https://doi.org/10.1001/archpsyc.62.6.593

Kessler, R. C., Crum, R. M., Warner, L. A., Nelson, C. B., Schulenberg, J., & Anthony, J. C. (1997). Lifetime co-occurrence of DSM-III-R alcohol abuse and dependence with other psychiatric disorders in the National Comorbidity Survey. *Arch Gen Psychiatry*, *54*(4), 313-321. https://doi.org/10.1001/archpsyc.1997.01830160031005

Kessler, R. C., Sonnega, A., Bromet, E., Hughes, M., & Nelson, C. B. (1995). Posttraumatic stress disorder in the National Comorbidity Survey. *Arch Gen Psychiatry*, *52*(12), 1048-1060.

Kim, J. J., & Jung, M. W. (2006). Neural circuits and mechanisms involved in Pavlovian fear conditioning: a critical review. *Neurosci Biobehav Rev*, *30*(2), 188-202. https://doi.org/10.1016/j.neubiorev.2005.06.005

Lancaster, F. E., Brown, T. D., Coker, K. L., Elliott, J. A., & Wren, S. B. (1996). Sex differences in alcohol preference and drinking patterns emerge during the early postpubertal period. *Alcohol Clin Exp Res*, *20*(6), 1043-1049. https://doi.org/10.1111/j.1530-0277.1996.tb01945.x

Lee, H. K., Takamiya, K., He, K., Song, L., & Huganir, R. L. (2010). Specific roles of AMPA receptor subunit GluR1 (GluA1) phosphorylation sites in regulating synaptic plasticity in the CA1 region of hippocampus. *J Neurophysiol*, *103*(1), 479-489. https://doi.org/10.1152/jn.00835.2009

Lisieski, M. J., Eagle, A. L., Conti, A. C., Liberzon, I., & Perrine, S. A. (2018). Single-Prolonged Stress: A Review of Two Decades of Progress in a Rodent Model of Post-traumatic Stress Disorder. *Front Psychiatry*, *9*, 196. https://doi.org/10.3389/fpsyt.2018.00196

Long, V. A., & Fanselow, M. S. (2012). Stress-enhanced fear learning in rats is resistant to the effects of immediate massed extinction. *Stress*, *15*(6), 627-636. https://doi.org/10.3109/10253890.2011.650251

Maren, S. (1999). Long-term potentiation in the amygdala: a mechanism for emotional learning and memory. *Trends Neurosci*, *22*(12), 561-567.

Maren, S., De Oca, B., & Fanselow, M. S. (1994). Sex differences in hippocampal long-term potentiation (LTP) and Pavlovian fear conditioning in rats: positive correlation between LTP and contextual learning. *Brain Res*, *661*(1-2), 25-34. https://doi.org/10.1016/0006-8993(94)91176-2

Markus, E. J., & Zecevic, M. (1997). Sex differences and estrous cycle changes in hippocampus-dependent fear conditioning. *PSychobiology*, *25*(3), 246-252.

Matsuo, N., Reijmers, L., & Mayford, M. (2008). Spine-type-specific recruitment of newly synthesized AMPA receptors with learning. *Science*, *319*(5866), 1104-1107. https://doi.org/10.1126/science.1149967

McCann, K. E., Sinkiewicz, D. M., Rosenhauer, A. M., Beach, L. Q., & Huhman, K. L. (2019). Transcriptomic analysis reveals sex-dependent expression patterns in the basolateral amygdala of dominant and subordinate animals after acute social conflict. *Molecular neurobiology*, *56*(5), 3768-3779.

McGinn, M. A., Paulsen, R. I., Itoga, C. A., Farooq, M. A., Reppel, J. E., Edwards, K. N., Whitaker, A. M., Gilpin, N. W., & Edwards, S. (2016). Withdrawal from Chronic Nicotine Exposure Produces Region-Specific Tolerance to Alcohol-Stimulated GluA1 Phosphorylation. *Alcohol Clin Exp Res*, *40*(12), 2537-2547. https://doi.org/10.1111/acer.13258

McIlwain, K. L., Merriweather, M. Y., Yuva-Paylor, L. A., & Paylor, R. (2001). The use of behavioral test batteries: effects of training history. *Physiol Behav*, *73*(5), 705-717. https://doi.org/10.1016/s0031-9384(01)00528-5

Meyer, E. M., Long, V., Fanselow, M. S., & Spigelman, I. (2013). Stress increases voluntary alcohol intake, but does not alter established drinking habits in a rat model of posttraumatic stress disorder. *Alcohol Clin Exp Res*, *37*(4), 566-574. https://doi.org/10.1111/acer.12012

Mills, K. L., Teesson, M., Ross, J., & Peters, L. (2006). Trauma, PTSD, and substance use disorders: findings from the Australian National Survey of Mental Health and Well-Being. *Am J Psychiatry*, *163*(4), 652-658. https://doi.org/10.1176/appi.ajp.163.4.652

Onaivi, E. S., & Martin, B. R. (1989). Neuropharmacological and physiological validation of a computer-controlled two-compartment black and white box for the assessment of anxiety. *Prog Neuropsychopharmacol Biol Psychiatry*, *13*(6), 963-976. https://doi.org/10.1016/0278-5846(89)90047-x

Pennington, Z. T., Anderson, A. S., & Fanselow, M. S. (2017). The ventromedial prefrontal cortex in a model of traumatic stress: fear inhibition or contextual processing? *Learn Mem*, *24*(9), 400-406. https://doi.org/10.1101/lm.046110.117

Pennington, Z. T., Trott, J. M., Rajbhandari, A. K., Li, K., Walwyn, W. M., Evans, C. J., & Fanselow, M. S. (2020). Chronic opioid pretreatment potentiates the sensitization of fear

learning by trauma. *Neuropsychopharmacology*, *45*(3), 482-490. https://doi.org/10.1038/s41386-019-0559-5

Perkonigg, A., Kessler, R. C., Storz, S., & Wittchen, H.-U. (2000). Traumatic events and post-traumatic stress disorder in the community: prevalence, risk factors and comorbidity. *Acta Psychiatr Scand*, *101*(1), 46-59.

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*(1), 45-57. https://doi.org/10.1038/npp.2015.224

Poulos, A. M., Reger, M., Mehta, N., Zhuravka, I., Sterlace, S. S., Gannam, C., Hovda, D. A., Giza, C. C., & Fanselow, M. S. (2014). Amnesia for early life stress does not preclude the adult development of posttraumatic stress disorder symptoms in rats. *Biol Psychiatry*, *76*(4), 306-314. https://doi.org/10.1016/j.biopsych.2013.10.007

Poulos, A. M., Zhuravka, I., Long, V., Gannam, C., & Fanselow, M. (2015). Sensitization of fear learning to mild unconditional stimuli in male and female rats. *Behav Neurosci*, *129*(1), 62-67. https://doi.org/10.1037/bne0000033

Priddy, B. M., Carmack, S. A., Thomas, L. C., Vendruscolo, J. C., Koob, G. F., & Vendruscolo, L. F. (2017). Sex, strain, and estrous cycle influences on alcohol drinking in rats. *Pharmacol Biochem Behav*, *152*, 61-67. https://doi.org/10.1016/j.pbb.2016.08.001

Pryce, C. R., Lehmann, J., & Feldon, J. (1999). Effect of sex on fear conditioning is similar for context and discrete CS in Wistar, Lewis and Fischer rat strains. *Pharmacol Biochem Behav*, *64*(4), 753-759. https://doi.org/10.1016/s0091-3057(99)00147-1

Rau, V., DeCola, J. P., & Fanselow, M. S. (2005). Stress-induced enhancement of fear learning: an animal model of posttraumatic stress disorder. *Neurosci Biobehav Rev*, *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

Ritov, G., Boltyansky, B., & Richter-Levin, G. (2016). A novel approach to PTSD modeling in rats reveals alternating patterns of limbic activity in different types of stress reaction. *Mol Psychiatry*, *21*(5), 630-641. https://doi.org/10.1038/mp.2015.169

Rodgers, R. J., & Dalvi, A. (1997). Anxiety, defence and the elevated plus-maze. *Neurosci Biobehav Rev*, *21*(6), 801-810. https://doi.org/10.1016/s0149-7634(96)00058-9

Rodgers, R. J., & Shepherd, J. K. (1993). Influence of prior maze experience on behaviour and response to diazepam in the elevated plus-maze and light/dark tests of anxiety in mice. *Psychopharmacology (Berl)*, *113*(2), 237-242. https://doi.org/10.1007/BF02245704

Rogan, M. T., Stäubli, U. V., & LeDoux, J. E. (1997). Fear conditioning induces associative long-term potentiation in the amygdala. *Nature*, *390*(6660), 604-607. https://doi.org/10.1038/37601

Scholl, J. L., Afzal, A., Fox, L. C., Watt, M. J., & Forster, G. L. (2019). Sex differences in anxiety-like behaviors in rats. *Physiol Behav*, *211*, 112670. https://doi.org/10.1016/j.physbeh.2019.112670

Shansky, R. M. (2015). Sex differences in PTSD resilience and susceptibility: Challenges for animal models of fear learning. *Neurobiol Stress*, *1*, 60-65. https://doi.org/10.1016/j.ynstr.2014.09.005

Smith, S. M., & Vale, W. W. (2006). The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. *Dialogues Clin Neurosci*, *8*(4), 383-395.

Solinas, M., Chauvet, C., Thiriet, N., El Rawas, R., & Jaber, M. (2008). Reversal of cocaine addiction by environmental enrichment. *Proc Natl Acad Sci U S A*, *105*(44), 17145-17150. https://doi.org/10.1073/pnas.0806889105

Solomon, M. B., Furay, A. R., Jones, K., Packard, A. E., Packard, B. A., Wulsin, A. C., & Herman, J. P. (2012). Deletion of forebrain glucocorticoid receptors impairs neuroendocrine stress responses and induces depression-like behavior in males but not females. *Neuroscience*, *203*, 135-143. https://doi.org/10.1016/j.neuroscience.2011.12.014

Viau, V., & Meaney, M. J. (1991). Variations in the hypothalamic-pituitary-adrenal response to stress during the estrous cycle in the rat. *Endocrinology*, *129*(5), 2503-2511. https://doi.org/10.1210/endo-129-5-2503

Weitemier, A. Z., & Ryabinin, A. E. (2003). Alcohol-induced memory impairment in trace fear conditioning: a hippocampus-specific effect. *Hippocampus*, *13*(3), 305-315. https://doi.org/10.1002/hipo.10063

Wood, G. E., & Shors, T. J. (1998). Stress facilitates classical conditioning in males, but impairs classical conditioning in females through activational effects of ovarian hormones. *Proc Natl Acad Sci U S A*, *95*(7), 4066-4071. https://doi.org/10.1073/pnas.95.7.4066

Wright, A., & Vissel, B. (2012). The essential role of AMPA receptor GluR2 subunit RNA editing in the normal and diseased brain. *Front Mol Neurosci*, *5*, 34. https://doi.org/10.3389/fnmol.2012.00034

Zimmerberg, B., & Farley, M. J. (1993). Sex differences in anxiety behavior in rats: role of gonadal hormones. *Physiol Behav*, *54*(6), 1119-1124. https://doi.org/10.1016/0031-9384(93)90335-d

CHAPTER 2

Impacts of stress resilience and susceptibility on fear learning, anxiety, and alcohol intake

Abstract

Post-traumatic stress disorder (PTSD) can develop after exposure to traumatic events and severely impacts the quality of life. PTSD is frequently comorbid with substance use disorders, with alcoholism being particularly common. However, not everyone who experiences trauma develops PTSD and the factors that render individuals susceptible or resilient to the effects of stress are unknown although gender appears to play an important role. Rodent models of stress exposure such as stress-enhanced fear learning (SEFL) recapitulate some aspects of PTSD symptomology, making them an invaluable tool for studying this disorder. This study examined whether exposure to a modified version of the SEFL procedure (4 footshocks instead of the standard 15 over 90 minutes) would reveal both susceptible and resilient subjects. Following stress exposure, distinct susceptible and resilient groups emerged that differed in fear learning and anxiety-related behavior as well as voluntary alcohol intake. Some aspects of stress susceptibility manifested differently in males compared to females, with susceptibility associated with increased alcohol intake in males and increased baseline anxiety in females.

Introduction

Post-traumatic stress disorder (PTSD) may develop after exposure to traumatic events and severely impacts the quality of life. PTSD patients experience a number of debilitating symptoms including hypervigilance and avoidance of stimuli reminiscent of the trauma (APA, 2013). PTSD is also often co-morbid with drug abuse, with alcohol abuse being the most common (Kessler et al., 1995; Mills et al., 2006; Perkonigg et al., 2000). Despite the high burden of this disorder, its mechanisms are not fully understood and currently available treatments such as exposure therapy are not always effective (Craske et al., 2008; Hembree et al., 2003; Milad et al., 2009)

A critical question is why some individuals develop the disorder following trauma while others do not. It has been estimated that while approximately one third of the population will experience a trauma during their lifetime, only 10-20% of these individuals will develop PTSD with women twice as likely to develop the disorder compared to men (Brunello et al., 2001; Kessler et al., 1995; Kessler et al, 2017). Furthermore, not all PTSD patients display the same types or intensity of symptoms, and neuroimaging studies suggest that different categories of symptoms may be supported by different neural mechanisms (Lanius et al., 2006; Lanius et al., 2002; Lanius et al., 2001). Determining the factors that promote susceptibility or resilience to developing the disorder is therefore crucial for understanding the disorder and developing more effective, targeted treatments.

Animal models that capture some of the behavioral symptoms of PTSD are a powerful tool to study the mechanisms of this disorder. One characteristic of PTSD is an exaggerated response to mild stressors that are reminiscent of the original trauma, leading to inappropriate fear responses (Bremner et al., 1995; Dykman et al., 1997). Our laboratory has developed a rodent model of stress exposure termed stress-enhanced fear learning (SEFL) that uses aspects of

Pavlovian fear conditioning to capture this exaggerated fear response. In standard Pavlovian fear conditioning, a neutral conditional stimulus (CS), such as a discrete cue or a context, is paired with an aversive unconditional stimulus (US), such as a footshock. Following the formation of a CS-US association, the CS will elicit a fear response. In the SEFL model, animals are exposed to an intense acute stressor (typically 15 unsignaled footshocks) in a distinct context, followed by a mild fear conditioning event (a single footshock) in a different conditioning context (Rau et al., 2005). Following stress exposure animals show exaggerated fear to the fear conditioning context. This enhancement of fear learning is the defining feature of the SEFL model and reflects a nonassociative sensitization of the fear learning process as it is not prevented by amnesia for, or extinction of, fear to the stress context (Long & Fanselow, 2012; Poulos et al., 2014; Rau et al., 2005). Additionally, the acute stress will also enhance future fear learning to an auditory stimulus paired with shock even though there are no auditory stimuli present during stress (Pennington et al., 2017).

While enhanced fear learning is the key feature of the SEFL model, this procedure produces a number of other behavioral changes reflective of PTSD symptoms including extreme fear of stimuli associated with the original stressor, increased anxiety-related behavior, increased startle reactivity, and altered glucocorticoid signaling (Meyer et al., 2013; Pennington et al., 2017; Perusini et al., 2016; Poulos et al., 2015). Importantly, the SEFL model produces a long-lasting increase in voluntary alcohol intake, reflecting the comorbidity between PTSD and substance abuse (Meyer et al., 2013).

While a number of stress exposure models have been used to study PTSD, these models often consist of unquantifiable stressors whose parameters cannot be quantitatively manipulated such as social defeat stress, predator odor, or combinations of multiple stressors (Cohen et al.,

2006; Golden et al., 2011; Willner et al., 1987). In addition, few studies have examined the responses of males and females to the same stressor despite the fact that women are twice as likely to develop PTSD compared to men after adjusting for stress severity (Goldstein et al., 2016; Kessler et al., 1995). The stressor typically used in our experiments (15 unsignaled footshocks over 90 minutes) produces a robust enhancement in fear learning in both males and females that is advantageous for studying the mechanisms of this enhancement but may obscure individual differences that promote susceptibility versus resilience to the effects of stress. However, this stressor is uniquely quantifiable such that stress severity can be readily manipulated by changing the number or amplitude of footshocks. By reducing the stressor severity to 4 footshocks, we sought to uncover both susceptible and resilient groups and determine whether females show increased stress sensitivity.

Many studies investigating stress susceptibility rely on post-hoc methods such as quantifying the behavioral outcome of interest in the data set under study (e.g. increased freezing or reduced social interaction) and defining the 20-30% of subjects at one extreme as susceptible and the 20-30% at the other extreme as resilient (Colucci et al., 2020; Covington et al., 2010; Jeong et al., 2020; Ritov et al., 2016). These methods of classification are limited as they are based just on the current sample and similar procedures are rarely applied to unstressed controls. We aimed to improve upon existing methods of classification by establishing an *a priori* criterion based on prior sampling of a large population of subjects showing a normal fear response.

In the following experiments we tested the hypothesis that using a novel *a priori* classification criterion would reveal both susceptible and resilient populations following a reduced 4-footshock stressor. We further hypothesized that susceptible subjects would

demonstrate a constellation of behavioral differences relevant to PTSD symptomology. We found that using a reduced-severity stressor produced distinct susceptible and resilient groups that differed on several measures of fear, anxiety, and alcohol-related behaviors. We further found that while males and females did not differ in rates of stress susceptibility, susceptible males and females showed different patterns of alcohol consumption and anxiety-related behavior.

Experiment 1: Characterization of distinct susceptible and resilient populations following stress exposure

In Experiment 1 we evaluated performance on a battery of fear, anxiety and alcohol assessments prior to and following stress. We hypothesized that distinct susceptible and resilient populations would emerge that differ on multiple aspects of PTSD-related behavior, and that males and females might show different clusters of behavioral changes.

Methods

Animals

66 adult male and female Long-Evans rats (Envigo, Indianapolis, IN, USA) were used, approximately 10-11 weeks old at the start of the experiment. Animals were individually housed under a 12-hour light/dark cycle. Food and water were available *ad libitum* in the home cage, and during intermittent access two-bottle choice enrichment in the form of paper twists was provided. Animals were handled daily for 60 seconds each day for 1 week prior to the start of the experiment. The UCLA Institutional Animal Care and Use Committee approved all procedures involving animals.

Establishment of "Susceptible" versus "Resilient" classification criterion

In the standard SEFL procedure, rats are first exposed to a stressor consisting of 15 unsignaled 1-sec, 1-mA footshocks or receive equivalent context exposure without footshock. Subjects then receive a mild fear conditioning event consisting of a single footshock in a novel context. During the critical test of the SEFL procedure, termed the SEFL test, subjects are returned to the fear conditioning context for 8 minutes during which fear is assessed via freezing. The key feature of SEFL is that stress-exposed subjects show enhanced fear to the fear conditioning context relative to unstressed controls.

To determine an *a priori* criterion for classifying whether performance on the SEFL test deviated from that of unstressed controls, we compiled SEFL test scores from a large number of unstressed control subjects (n=182), drawing from published and unpublished SEFL experiments previously run in our laboratory. These data were pooled over several years, but all experiments used male and/or female young adult Long-Evans rats (approximately 9-12 weeks old) and the same training parameters including number and intensity of footshock.

Subjects were classified as "Susceptible" if they showed performance on the SEFL test that was at least two standard deviations above the means of unstressed controls, which based on this dataset, was 58% freezing (Mean=18.8%, SD=19.6%). Subjects that showed less than 58% freezing during the SEFL test were classified as "Resilient". Nearly all unstressed control subjects (94.0%) met the Resilient criterion (distribution of SEFL Test scores shown in Fig 2-1A). Conversely, the same analysis performed on subjects exposed to the standard 15-shock stress (n=130) revealed that the majority of stressed subjects (79.2%) met the Susceptible criterion (Mean=74.5%, SD=22%, distribution of SEFL Test scores shown in Fig 2-1B). This classification criterion was therefore used to determine whether the 4-footshock stress would produce both Susceptible and Resilient populations (experimental timeline shown in Table 2-1).

Given that PTSD is more common in women than in men, we evaluated whether the susceptibility cutoff would differ if calculated separately for males and females (Supplementary Methods). We found no differences in the distribution of SEFL Test scores between unstressed males and females (Fig 2-7A-B), or in males and females exposed to the standard 15-footshock stress (Fig 2-7C-D). Importantly, we found that the susceptibility criterion remained the same when calculated separately for males and females (Supplementary Methods). It has been reported that female rats express fear via darting behavior, but to our knowledge this has only been reported during auditory fear conditioning (Gruene et al, 2015). Videos were screened for darting, but we did not observe any darting behavior in either males or females during the SEFL Test.

Day	*Task*
1-14	Continuous access 2-bottle choice
15	Light-dark transition test
16	Stress
17	Generalization test
18	Single footshock
19	Stress-enhanced fear learning test
20	Open field test
21	Elevated plus maze
22	Light-dark transition test
23-27	Extinction
28	Aversive acoustic stimulus
29	Context test
30-37	Rest
38-87	Intermittent access 2-bottle choice

Table 2-1. Timeline for Experiment 1.

Our approach focused on determining a criterion for susceptibility, i.e. identifying the population of subjects that show unusually high levels of fear. An alternative approach would be to determine the criterion for resilience, i.e. identifying stress-exposed subjects that show unusually low levels of fear. We performed a similar analysis to consider this alternative that was based on the 15 footshock-exposed subjects, but determined that this approach did not perform as well (Supplementary Materials).

Assessments of baseline alcohol consumption and anxiety-related behavior

Continuous access two-bottle choice (2BC) drinking procedure. All fluids were presented in 250-ml drinking bottles with low-leak drinking spouts (Ancare) accessible through the top of the home cage. Rats had access to one bottle containing 10% EtOH (w/v) and one bottle containing regular drinking water. Rats received continuous access to both bottles for two weeks, which in contrast to intermittent access produces relatively low levels of alcohol consumption (Brancato et al., 2016; Wise, 1973). Bottles were weighed daily except Sundays and locations were alternated daily to control for side preferences. Rats were weighed every other day to calculate the grams of solution consumed per kilogram of body weight in each 24-hour session (g/kg/24 hrs).

Light-dark transition test. The light-dark transition test is a classic test of anxiety-related behavior in rodents (Bourin & Hascoët, 2003; Crawley & Goodwin, 1980). The apparatus consisted of a Plexiglas arena (100 cm x 40 cm x 30 cm). The arena was divided into a light compartment with white walls and floors (75 cm x 40 cm x 30 cm) and a dark compartment with black walls and floors (25 cm x 40 cm x 30 cm), with a 10 cm x 10 cm opening allowing movement between the two compartments. The room was dimly lit by a lamp in the corner of the room. Each session was recorded using an overhead camera and behavior automatically tracked using EthoVision software (Noldus, Wageningen, Netherlands). Subjects were individually placed in the dark compartment of the light-dark transition test apparatus and allowed to freely move between the dark and light compartments for 10 minutes. Anxiety-related behavior was scored by the latency to enter the light compartment, the total time spent in the light compartment and the number of entries into the light compartment.

Unlike many rodent tests of anxiety-related behavior, previous reports indicate that the light-dark transition test can be reliably conducted multiple times within the same subject (Ballaz et al., 2007; Banasikowski et al., 2015; Onaivi & Martin, 1989). The light-dark transition test

was therefore run both prior to and following stress exposure while all other assessments of anxiety-related behavior were run only following stress exposure.

Fear conditioning

Stress-enhanced fear learning. All fear conditioning took place in four sets of four identical fear conditioning chambers housed in sound-attenuating shells (Med Associates Inc, St. Albans, VT, USA). Context A (stress context) contained flat grid floors, was lit by a white house light and scented with 50% Windex solution. Ventilation fans provided background noise. Context B (generalization context) was identical to Context A except the grid floors were made of alternating thick and thin bars and chambers were scented with 1:30 Simple Green solution. Context C (mild fear conditioning context) contained a black triangular insert and floors composed of grid bars at alternating vertical heights and was scented with 1% acetic acid. Each set of grids was wired to a shock generator and scrambler. Sessions were recorded by near-infrared cameras and freezing was automatically scored using VideoFreeze software (Med Associates Inc).

Subjects were first transported to Context A in their homecages where they received an acute stressor consisting of 4 1-sec, 1-mA unsignalled footshocks randomly distributed over 90 min. One day later, fear generalization was assessed by exposing subjects to a novel context that shared some features with the original stress context (Context B) for 8 minutes without footshock. The next day subjects were transported to Context C in a black plastic tub divided into 4 quadrants. Following a 3-minute baseline period, all subjects received a single 1-sec, 1-mA footshock and were removed 30 seconds later. All subjects were returned to Context C the

following day for 8 minutes to assess fear to the mild fear conditioning context. Freezing served as our index of fear conditioning.

Fear extinction. To assess fear extinction to the mild fear conditioning context (Context C), subjects received 5 days of extinction training, in which they were exposed to Context C for 30 minutes per day without footshock delivery. Extinction across sessions was measured by freezing during the first 5 minutes of each session. To account for differences in initial fear levels, extinction was also measured by the number of sessions required for each subject to reach 50% of their freezing on the first day of extinction. Subjects that did not reach this criterion were given a score of 5.

Aversive acoustic stimulus. Context D (white noise context) contained a curved white plastic wall and white plastic floor inserts and was scented with 1:30 Simple Green solution. Acoustic stimuli were delivered using Goldwood GT-1005 wide dispersion piezo tweeters mounted to the wall of the chambers and connected to an amplifier. Following a three-minute baseline period, all subjects received a 100-msec, 110-dB burst of white noise and were removed 30 seconds later. The next day subjects were returned to Context D for 8 minutes to assess fear to the context.

Assessments of anxiety-related behavior following stress

Open field test. This task utilized a modified version of the open field test, a classic test of anxiety-related behavior in rodents (Godsil et al., 2005; Godsil & Fanselow, 2004). The apparatus consisted of a clear plastic container (78 cm x 39 cm x 30 cm) divided into 4 equal-sized zones (19.5 cm x 39 cm). Three LED lamps were located around each end of the apparatus, one lamp located against the center of each short wall and one lamp located against the ends of each long wall. Turning on the lamps on one end created a light gradient across the apparatus,

with Zone 1 the brightest (2160 Lux) and Zone 4 the dimmest (260 Lux). The illuminated side of the apparatus was counterbalanced across subjects. The room was lit by a red light throughout the session, and each session was recorded using an overhead camera and behavior automatically tracked using EthoVision software (Noldus).

Subjects were individually placed in the open field apparatus for 12 minutes and allowed to roam freely. During the first 4 minutes, the apparatus was lit only by a red light. During minutes 5-8, the lamps around one side of the apparatus were illuminated creating a light gradient across the floor. During minutes 9-12, the lamps were turned off. To assess anxiety-related behavior, general exploratory behavior was measured throughout the session and by the amount of time spent in Zone 4, indicating avoidance of the lights.

Elevated plus maze. The elevated plus maze is another classic rodent test of anxiety-related behavior (Rodgers & Dalvi, 1997). The apparatus consisted of a plus-shaped maze with each arm measuring 40 cm x 12.5 cm that was elevated 60 cm above the ground. The closed arms had black Plexiglas walls (17.5 cm), while the open arms did not. The room was lit by a red light throughout the session, and each session was recorded using an overhead camera and behavior automatically tracked using EthoVision software (Noldus).

Subjects were placed in the center of the maze and allowed to freely explore for 6 minutes. Anxiety-related behavior was measured by the amount of time spent in the open arms of the maze, with decreased time indicating increased anxiety. 14 subjects (6 females and 8 males) fell off of the maze and were excluded from analysis of this task.

Intermittent access two-bottle choice (2BC) drinking paradigm

Subjects received 59 days of intermittent access 2-bottle choice as described previously (Meyer et al., 2013), which has been shown to produce escalating levels of alcohol consumption (Simms et al., 2008; Wise, 1973). During this time subjects were given access to one bottle of 10% ethanol (EtOH, w/v) and one bottle of regular drinking water on Mondays, Wednesdays and Fridays. Drinking bottles were weighed at the beginning and end of each 24-hour drinking session and rats were weighed at the end of each session. Bottle locations were alternated each session. At the end of each session the bottle containing alcohol was replaced with a bottle containing drinking water until the start of the next session.

Data analysis

To evaluate whether Susceptible and Resilient subjects differed on fear, anxiety and alcohol consumption-related behavior, performance on each individual measure was analyzed using IBM SPSS Statistics Software. Tasks with a single dependent variable were analyzed with 2-way ANOVA with Resilience as the first factor and Sex as the second factor. Tasks with repeated measures were analyzed using mixed-model ANOVA. Significant interactions were interpreted using simple main effects. Violations of sphericity in mixed-model ANOVA were addressed by adjusting the degrees of freedom (dfs) using the Greenhouse-Geisser correction. Data were plotted using GraphPad Prism

Analysis of whether baseline anxiety levels predicted post-stress task performance was performed using R 3.6.3 (R Core Team, 2020), the mosaic package (Pruim et al., 2017) and the psych package (Revelle, 2019). Principal component analysis was first performed to compute a single measure of baseline anxiety from the 3 measures of anxiety-related behavior from the light-dark transition test (entries, light time, latency). Backwards regression was then performed

to predict each post-stress measure from the principal component analysis variable, sex and the interaction term resampling 1000 times for each test. If the interaction term did not explain a significant proportion of variance it was removed from the analysis. Principal component analysis was also performed to compute a single measure of anxiety from the second light-dark transition test conducted following stress.

Results

4-shock stress produces distinct Susceptible and Resilient populations

Applying our *a priori* classification criterion to the results from previous studies indicated that unstressed control subjects are predominantly classified as Resilient, while subjects exposed to the standard 15-footshock stress are mainly classified as Susceptible (Figs 2-1A-B). In contrast, the presently used 4-shock stress produced a pronounced bimodal distribution, with 56.8% of subjects classified as Resilient and 43.2% classified as Susceptible (Fig 2-1C).

To verify that the differences between these groups during the SEFL test reflected differences in fear learning, rather than generalization of fear from the stress context, we examined freezing in the fear conditioning context prior to footshock delivery. No differences in baseline fear were observed between Susceptible and Resilient subjects (Resilience: $F_{1,40}$=3.18, p=0.08; Sex: $F_{1,40}$=1.75, p=0.19; Resilience*Sex: $F_{1,40}$=1.69, p=0.20; Fig 2-1D).

We next examined whether females were more likely to be classified as Susceptible compared to males. SEFL test performance did not differ between males and females (Sex: $F_{1,40}$=0.17, p=0.69; Sex*Resilience: $F_{1,40}$=1.24, p=0.27; Fig 2-1E; Fig 2-7E-F). While slightly fewer females were classified as Susceptible compared to males (Females: 36%; Males: 50%; Fig 2-1F), a chi-square test revealed no sex differences in the number of subjects classified as

Susceptible ($X^2(1,N=44)=0.83$, p=0.36). These results indicate that the 4-footshock stress can be used to reveal distinct Susceptible and Resilient populations, although males and females do not appear to differ in stress susceptibility. Performance of Susceptible and Resilient subjects on the battery of tasks used in this experiment compared to unstressed controls is shown in Table 2-3 (Supplementary Materials).

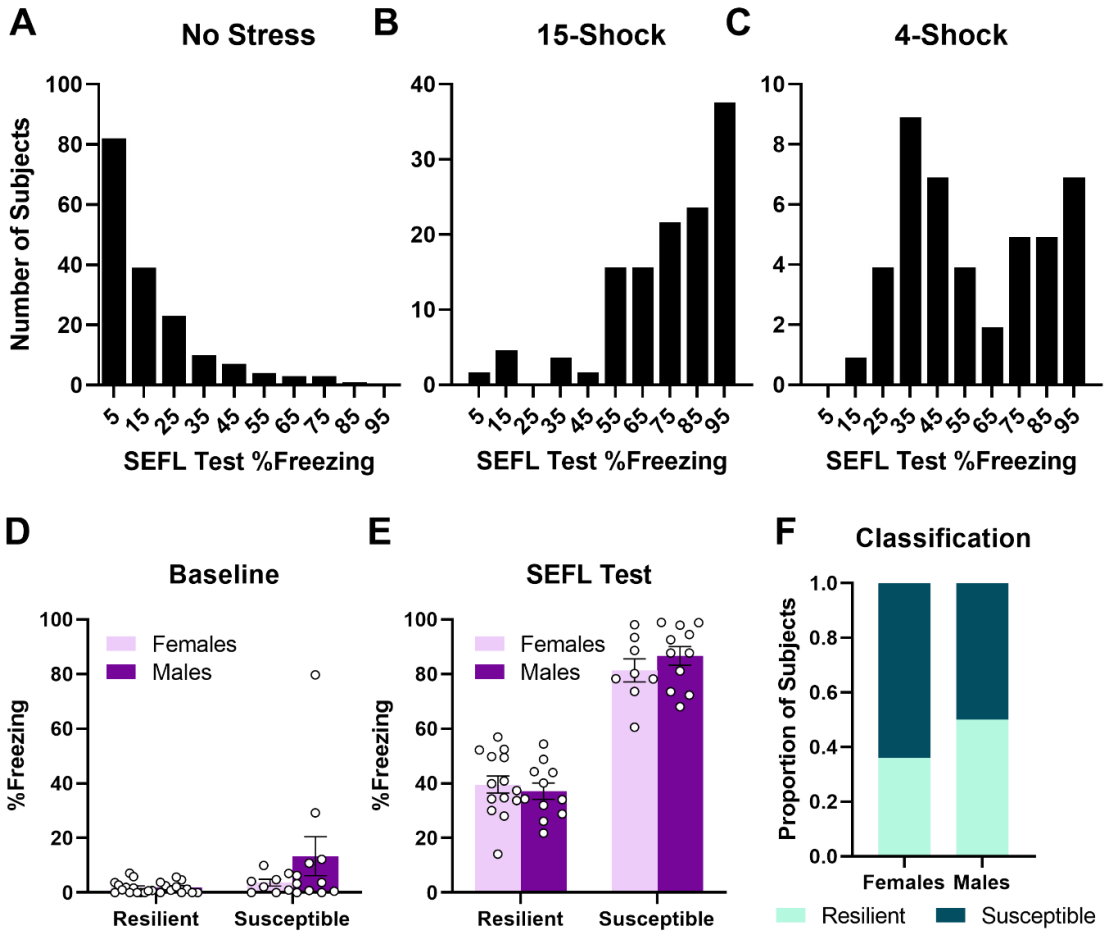


Figure 2-1. Stress-enhanced fear learning following different levels of stress. **A.** Distribution of SEFL test scores in unstressed controls (n=182 drawn from previous published and unpublished experiments). Tick marks indicate center of 10% bins. **B.** Distribution of SEFL test scores in subjects exposed to standard 15-footshock stress (n=130) drawn from previous published and unpublished experiments). **C.** Distribution of SEFL test scores in subjects exposed to 4-footshock stress in the present experiment (n=44). **D.** Susceptible and Resilient subjects do not differ in baseline fear during the three minutes prior to footshock delivery (Day 18). **E.** No differences between males and females were observed during the SEFL test (Day 19). **F.** Proportion of subjects classified as Susceptible or Resilient. Error bars represent standard error of the mean.

Susceptible subjects show increased fear generalization following stress exposure

To fully characterize the differences between Susceptible and Resilient subjects we first assessed whether these subjects differed on other aspects of fear learning that are relevant to PTSD symptomology. Following stress exposure but prior to receiving the single footshock, fear generalization was assessed by exposing subjects to a novel context that shared some features with the original stress context. Susceptible subjects showed increased fear generalization compared to Resilient subjects, ($F_{1,40}$=15.74, p<0.001; Fig 2-2A), though there were no differences between males and females (Sex: $F_{1,40}$=1.60, p=0.21; Sex*Resilience: $F_{1,40}$=2.96, p=0.09).

During fear extinction to the fear conditioning context, significant differences between Susceptible and Resilient subjects were observed (Session*Resilience: $F_{2.06,82.41}$=4.64, p=0.012, dfs adjusted using Greenhouse-Geisser correction; Fig 2-2B), with Susceptible subjects showing elevated freezing during all sessions (ps<0.01-0.001). To compare extinction rates between groups in a manner to compensate for the initial differences in freezing levels, we measured the number of extinction sessions required to reach 50% of initial freezing levels displayed on the first day of extinction. Results indicated that Susceptible subjects may show impaired fear extinction as they required a greater number of sessions to reach this criterion ($F_{1,40}$=11.82, p=0.001; Fig 2-2C). Males and females did not differ during extinction (Sex: $F_{1,40}$=0.02, p=0.90; Resilience*Sex: $F_{1,40}$=1.13, p=0.29) or in the number of sessions required to reach criterion (Sex: $F_{1,40}$=0.12, p=0.73; Sex*Resilience: $F_{1,40}$=0.001, p=0.99).

Susceptible subjects show increased fear to an aversive acoustic stimulus

While Susceptible subjects showed increased fear to a stimulus reminiscent of the original stress, it was unknown whether these subjects would show similarly enhanced fear to a novel aversive stimulus. Subjects were placed in a novel context (Context D) in which they received an aversive acoustic stimulus consisting of a 110-dB, 100-ms burst of white noise. Susceptible subjects showed greater fear to the white noise context ($F_{1,40}$=27.77, p<0.001; Fig 2-2E), although no differences between males and females were observed (Sex: $F_{1,40}$=0.41, p=0.53; Sex*Resilience: $F_{1,40}$=0.48, p=0.49). However, Susceptible subjects also showed increased freezing prior to stimulus delivery ($F_{1,40}$=14.79, p<0.001; Fig 2-2D), suggesting that the elevated fear seen during test could potentially be due to fear generalization.

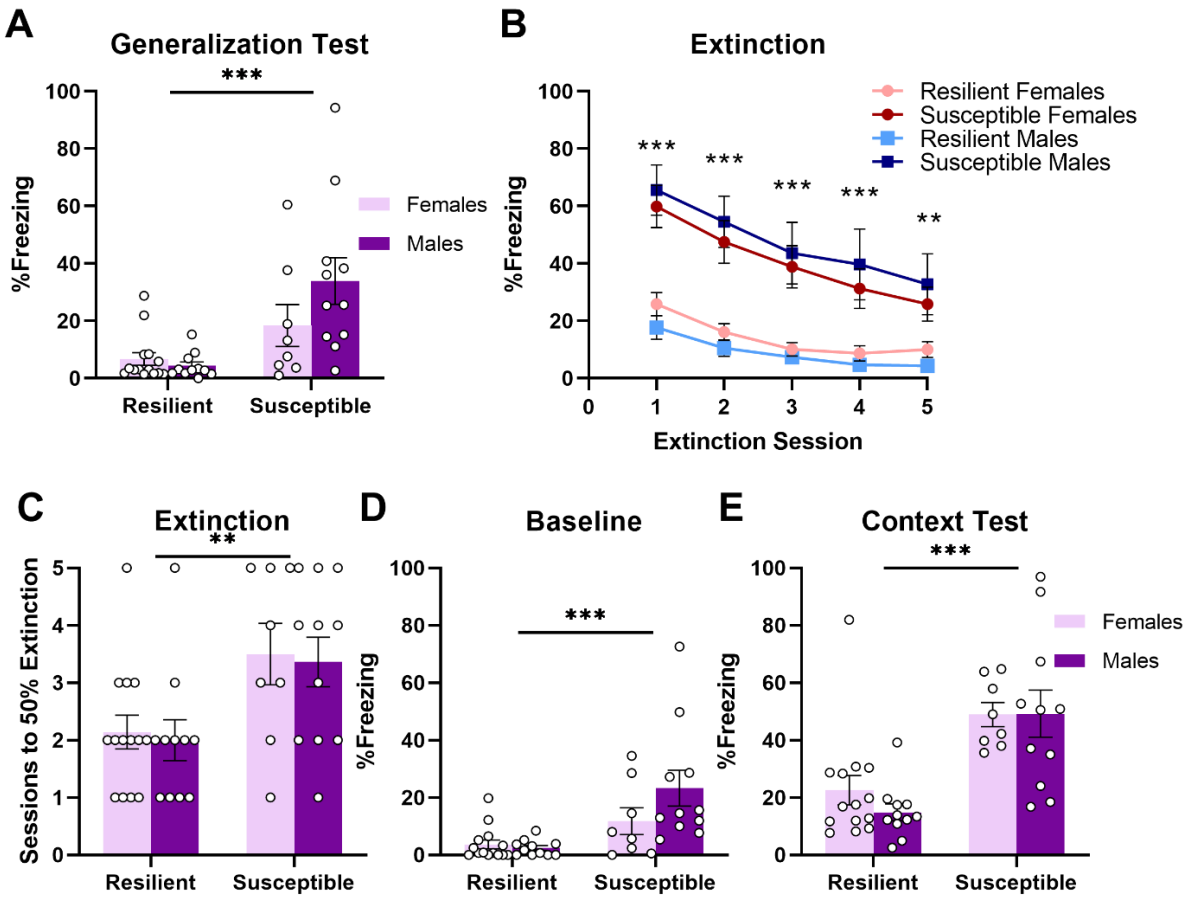


Figure 2-2. Susceptible and Resilient subjects differ on multiple aspects of fear learning. **A.** Susceptible subjects show increased fear generalization during the generalization test (Day 17). **B.** Susceptible subjects show increased fear throughout fear extinction (Days 23-27). Data points show freezing during the first 5 minutes of each session. **C.** Susceptible subjects require more sessions to reach 50% of the initial freezing levels on the first day of extinction. **D.** Susceptible subjects show increased baseline fear during the three minutes prior to acoustic stimulus delivery (Day 28). **E.** Susceptible subjects show increased fear to acoustic stimulus context (Day 29). **p<0.01, ***p<0.001. Error bars represent standard error of the mean.

Susceptible subjects show increased anxiety-related behavior following stress

We have previously shown that stress exposure increases anxiety-related behavior (Perusini et al., 2016). We therefore examined whether Susceptible subjects showed heightened anxiety using a battery of behavioral tasks following stress exposure. Susceptible subjects showed increased anxiety-related behavior as indicated by reduced exploratory behavior during the open field test ($F_{1,40}$=12.43, p=0.001; Fig 2-3A) and decreased entries into the light compartment during the light-dark transition test ($F_{1,40}$=10.86, p=0.002; Fig 2-3D). Numerical differences were also observed in time spent in the light compartment ($F_{1,40}$=2.94, p=0.09; Fig 3E), and latency to enter the light compartment ($F_{1,40}$=3.16, p=0.08; Fig 2-3F), but these effects fell short of statistical significance.

Analysis of time spent in Zone 4 of the open field test revealed a strong trend towards an interaction between time, sex, and stress susceptibility ($F_{5.88,235.15}$=1.97, p=0.07, dfs adjusted using Greenhouse-Geisser correction; Fig 2-3B). Female subjects showed light avoidance with no observed effects of Resilience ($F_{1,20}$=0.27, p=0.61) or Resilience by Minute interaction ($F_{4.79,95.78}$=0.83, p=0.53, dfs adjusted using Greenhouse-Geisser correction). In contrast, Susceptible males showed reduced time spent in Zone 4 during and after light onset as indicated by a significant Resilience by Minute interaction ($F_{5.02,100.38}$=2.43, p=0.04, dfs adjusted using

Greenhouse-Geisser correction). These results suggest that stress susceptibility is uniquely associated with a blunted response to light onset in males.

Time spent in the open arms of the elevated plus maze was also analyzed as a measure of anxiety-related behavior. However, no effects of sex or resilience were observed (Resilience: $F_{1,35}$=2.71, p=0.11; Sex: $F_{1,35}$=2.66, p=0.11; Sex*Resilience: $F_{1,35}$=1.57, p=0.22; Fig 2-3C). Lastly, we took advantage of the fact that the light-dark transition test had been used prior to and following stress to test the possibility that stress exposure differentially altered performance in Susceptible versus Resilient subjects. However, no interactions between stress exposure and resilience were observed on number of entries into the light arena ($F_{1,40}$=1.69, p=0.2; Fig 2-8A), time in the light arena ($F_{1,40}$=0.07, p=0.8; Fig 2-8B) or latency to enter the light arena ($F_{1,40}$=1.58, p=0.22; Fig 2-8C).

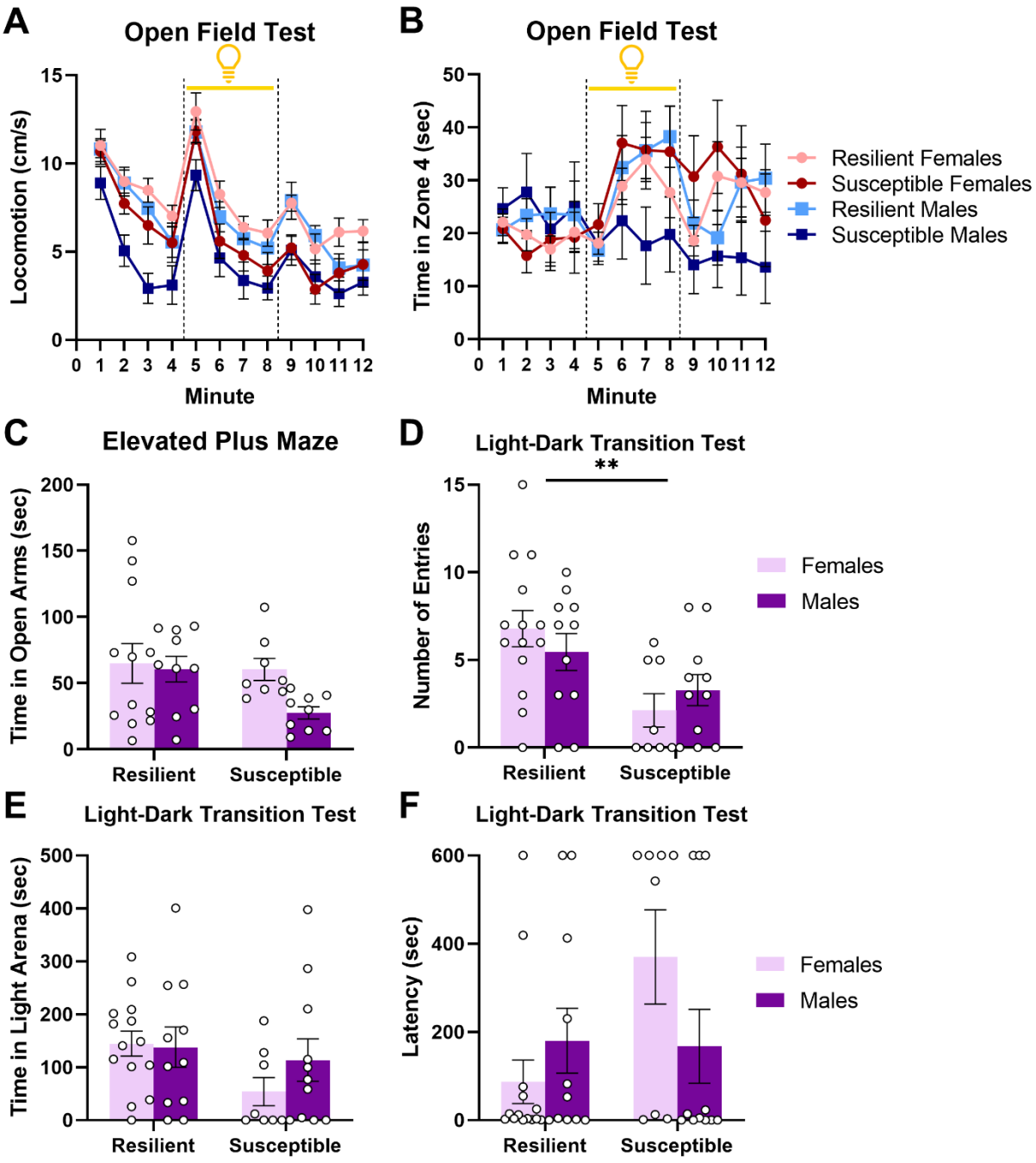


Figure 2-3. Susceptible subjects show increased anxiety-like behavior following stress exposure. **A.** Susceptible subjects show decreased locomotion during the open field test (Day 20). Dotted lines indicate transitions from lights off to on (first line) and lights on to off (second line). **B.** Susceptible males show a blunted response to light onset in comparison to Resilient males. **C.** No differences in time spent in the open arms of the elevated plus maze (Day 21). **D-F.** Results of the light-dark transition test (Day 22). Susceptible subjects show reduced entries into the light compartment (**D**), marginally decreased time in the light compartment (**E**) and marginally increased latency to enter the light compartment (**F**). **p<0.01. Error bars represent standard error of the mean.

Susceptible subjects show increased alcohol consumption in the absence of pre-stress alcohol exposure

Susceptibility did not appear to alter alcohol consumption following stress in subjects that had also been exposed to alcohol prior to stress. Differences between males and females emerged across presentations in alcohol consumption, with females showing increased consumption ($F_{7.68,307.19}$=3.76, p<0.001, dfs adjusted using Greenhouse-Geisser correction; Fig 2-4B). However, no difference in alcohol preference was observed ($F_{7.61,304.57}$=1.43, p=0.19, dfs adjusted using Greenhouse-Geisser correction; Fig 2-4A). There were no differences between Susceptible and Resilient subjects in either alcohol consumption (Resilience: $F_{1,40}$=0.12, p=0.73; Sex*Resilience: $F_{1,40}$=1.88, p=0.18) or alcohol preference (Resilience: $F_{1,40}$=0.17, p=0.69; Sex*Resilience: $F_{1,40}$=1.44, p=0.24).

Previous work indicates that while stress increases alcohol consumption, this effect is blocked if rats had established drinking habits via intermittent EtOH exposure prior to stress (Meyer et al., 2013). This suggests that alcohol access prior to stress exposure may have blunted potential differences between Susceptible and Resilient subjects. To address this possibility, a separate group of subjects received identical treatment except both bottles during continuous alcohol access prior to stress contained only drinking water.

A significant interaction between sex and resilience was observed in alcohol preference following stress exposure ($F_{1,18}$=7.18, p=0.02; Fig 2-4C). Susceptible males showed increased alcohol preference compared to Resilient males ($F_{1,18}$=10.20, p=0.005), while no differences were observed in females ($F_{1,18}$=0.41, p=0.53). A similar trend was seen in alcohol consumption ($F_{1,18}$=3.69, p=0.07; Fig 2-4D). Susceptible males showed increased alcohol consumption

compared to Resilient males ($F_{1,18}$=5.95, p=0.03) while no difference was observed in females ($F_{1,18}$=0.1, p=0.76). These results indicate that in the absence of prior alcohol exposure, stress susceptibility is associated with increased alcohol intake in males but not in females.

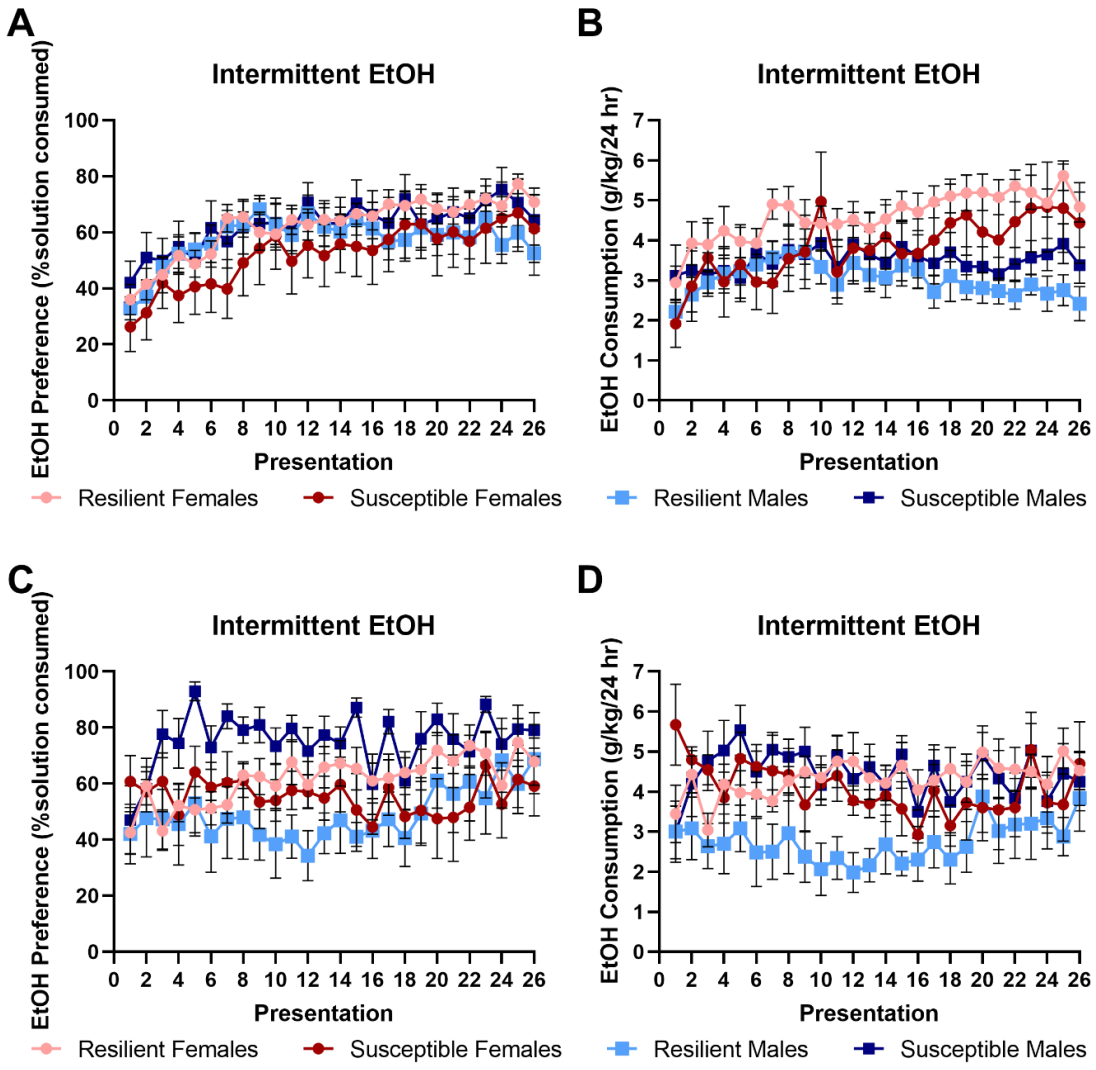


Figure 2-4. Results of intermittent 2-bottle choice following stress exposure (Days 30-86). **A-B.** Results of subject exposed to alcohol (10% EtOH w/v) prior to stress exposure. No differences were observed in alcohol preference **(A)**, although females showed increased consumption compared to males **(B)**. **C-D.** Results of subjects not exposed to alcohol prior to stress exposure. In comparison to Resilient males, Susceptible males showed increased alcohol preference **(C)** and alcohol consumption **(D)**. Error bars represent standard error of the mean.

Baseline anxiety is predictive of susceptibility to SEFL in females

Baseline levels of EtOH consumption and anxiety were retrospectively evaluated as potential predictors of susceptibility to stress. EtOH consumption prior to stress did not appear to be a predictor of stress susceptibility as Susceptible and Resilient subjects did not differ on either alcohol preference ($F_{1,39}$=0.20, p=0.66; Fig 2-5A) or alcohol consumption ($F_{1,39}$=0.07, p=0.79; Fig 2-5B).

In contrast, baseline anxiety was predictive of stress susceptibility in females but not in males. Performance on the light-dark transition test revealed significant interactions between sex and susceptibility on entries into the light compartment ($F_{1,40}$=7.21, p=0.01; Fig 2-5C) and time spent in the light compartment ($F_{1,40}$=8.77, p=0.005; Fig 2-5D). Follow-up simple main effects revealed that Susceptible females made fewer entries (p=0.002) and spent less time in the light compartment (p=0.001) compared to Resilient females. In contrast, Susceptible and Resilient males did not differ on either measure (entries: p=0.62; light time: p=0.58). While Susceptible females also showed numerically increased latency to enter the light compartment, this difference was not significant ($F_{1,40}$=2.24, p=0.14; Fig 2-5E).

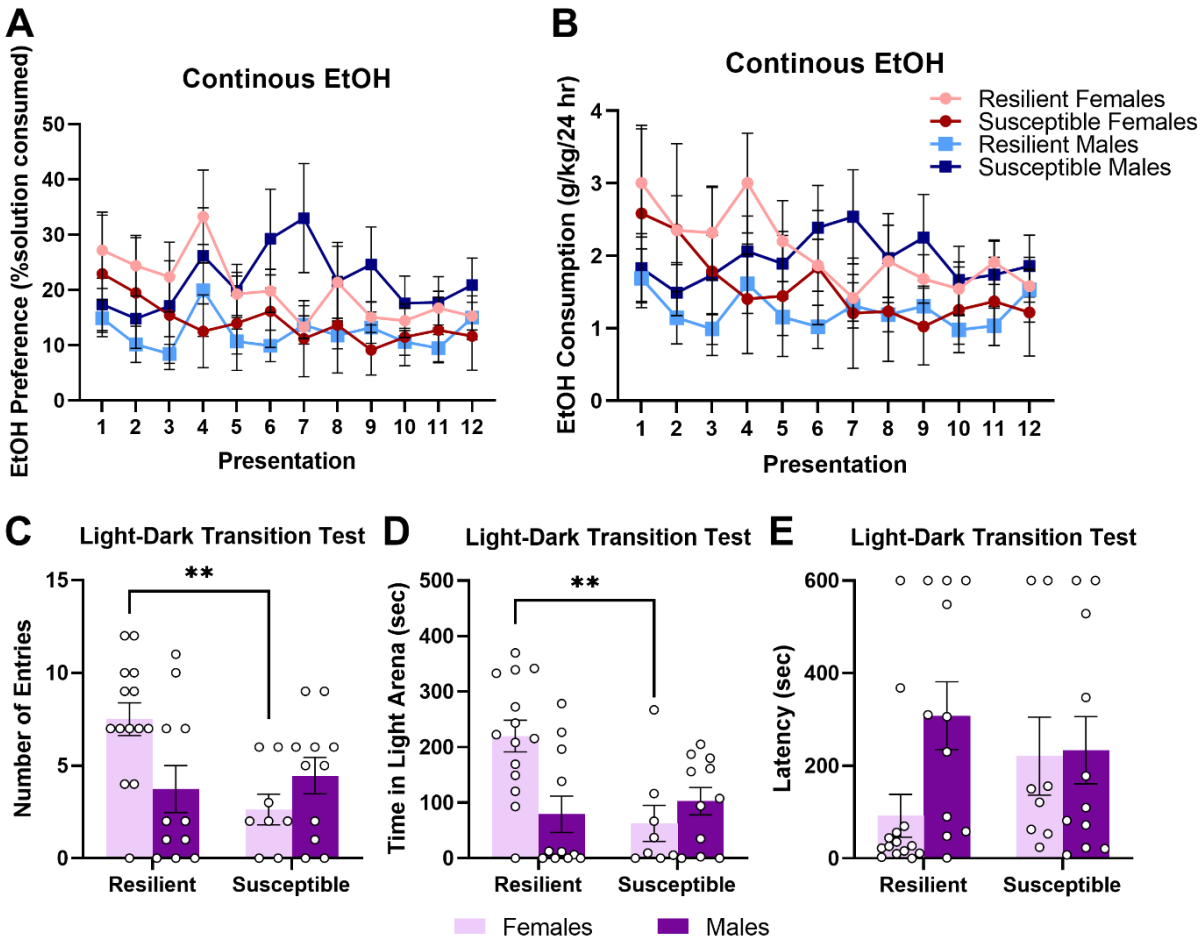


Figure 2-5. Baseline levels of alcohol drinking (10% EtOH w/v) and anxiety-like behavior. **A-B.** No differences in alcohol preference **(A)** or alcohol consumption **(B)** during continuous 2-bottle choice (Days 1-14). **C-E.** Susceptible females show increased anxiety on the light-dark transition test (Day 15) compared to Resilient females. Susceptible females showed reduced entries into the light compartment **(C)** and decreased time in the light compartment **(D)**, though no significant difference in latency to enter the light compartment **(E)**. **p<0.01. Error bars represent standard error of the mean.

Baseline anxiety predicts several fear and anxiety-related behaviors following stress

Given that baseline anxiety was predictive of subsequent performance on the SEFL test, and that Susceptible subjects showed a constellation of behavioral changes relevant to PTSD symptomology, we next looked at whether baseline anxiety predicted post-stress behaviors.

Baseline anxiety predicted fear learning-related behavior in a sex-specific manner. Backward regression was performed to determine whether baseline anxiety levels predicted key measures of fear learning. Baseline anxiety, sex, and their interaction explained a significant amount of variance in performance on the SEFL Test ($F_{3,40}$=2.85, p=0.049, R^2=0.18). The interaction term was found to be significant (β=18.55, p<0.05), and follow-up correlations indicated that baseline anxiety in females was correlated with fear during the SEFL Test in females (r=-0.59, n=22, p=0.004) but not in males (r=0.15, n=22, p=0.51).

This regression model also explained a significant amount of variance in the number of extinction sessions required to reduce fear by 50% ($F_{3,40}$=4.29, p=0.01, R^2=0.24). The interaction term was again significant (β =1.28, p<0.01) with lower anxiety associated with slower extinction in males (r=0.66, n=22, p=0.001) while there was no relationship between baseline anxiety and extinction in females (r=-0.20, n=22, p=0.38). However, this regression model was insufficient to explain a significant amount of variance in fear to the generalization context ($F_{3,40}$=0.98, p=0.41, R^2=0.07) or the white noise context test ($F_{3,40}$=1.91, p=0.14, R^2=0.13).

In contrast to the effect on fear learning, baseline anxiety generally predicted post-stress anxiety in a sex-independent manner. Baseline anxiety and sex explained a significant amount of variance in average locomotion during the open field test ($F_{2,41}$=4.65, p=0.02, R^2=0.19) and the performance on the light-dark transition test ($F_{2,41}$=8.06, p=0.001, R^2=0.28), though not the elevated plus maze ($F_{2,36}$=2.39, p=0.11, R^2=0.12). Baseline anxiety was found to significantly predict performance during the open field test (β =0.73, p<0.05) and the light-dark transition test following stress (β =0.56, p<0.001). These findings indicate that despite the large battery of behavioral assays used, we were able to obtain relatively stable assessments of anxiety in each animal.

Experiment 2: Enhancement of fear to a novel aversive stimulus following extinction of fear generalization

Experiment 1 revealed that susceptible subjects showed a constellation of behavioral changes relevant to PTSD symptomology, including enhanced fear learning to a novel aversive acoustic stimulus. However, interpretation of this particular result was complicated by the fact that susceptible subjects showed increased fear prior to the acoustic stimulus, indicating some degree of fear generalization to the acoustic stimulus context. Thus, it was possible that the elevated fear following the acoustic stimulus reflected fear generalization, rather than an enhancement of learning to a novel stimulus. In Experiment 2 we tested the hypothesis that following extinction of fear generalization to the novel context, susceptible subjects would show enhanced fear learning to the acoustic stimulus.

Methods

Animals

To assess whether susceptible subjects showed enhanced fear to a novel aversive stimulus, 45 adult male and female Long-Evans rats (Envigo) approximately 10-11 weeks old at the start of the experiment were used. Animals were individually housed under a 12-hour light/dark cycle. Food and water were available *ad libitum* in the home cage. Animals were handled daily for 60 seconds each day for 1 week prior to the start of the experiment. The Chancellor's Animal Research Committee at UCLA approved all procedures involving animals.

Stress-enhanced fear learning

Training took place in two sets of four identical fear conditioning chambers housed in sound-attenuating shells Med Associates Inc). Context A (stress context) contained flat grid

floors, was lit by a white house light and scented with 50% Windex solution. Ventilation fans provided background noise. Context B (fear conditioning context) contained a black triangular insert and floors composed of vertically alternating grid bars, was lit by a near-infrared light and scented with 1% acetic acid. Each set of grids was wired to a shock generator and scrambler. Stimulus delivery was controlled and freezing automatically scored using VideoFreeze software (Med Associates Inc).

Subjects were first transported to Context A in their homecages where they received a stressor consisting of 4 1-sec, 1-mA unsignalled footshocks (Fig 2-6A). Fear to the stress context was assessed by returning subjects to Context A the following day for 8 minutes without footshock. The next day subjects were transported to Context B in a black plastic tub divided into 4 quadrants. Following a 3-minute baseline period, all subjects received a single 1-sec, 1-mA footshock and were removed 30 seconds later. All subjects were returned to Context B the following day for 8 minutes to assess freezing to the fear conditioning context.

Fear extinction and aversive acoustic stimulus

Acoustic stimuli were presented in a third set of four identical fear conditioning chambers. Context C contained a curved white plastic wall and white plastic floor inserts and was scented with 1:30 Simple Green solution. Acoustic stimuli were delivered using Goldwood GT-1005 wide dispersion piezo tweeters mounted to the wall of the chambers and connected to an amplifier.

Subjects first underwent 4 days of extinction training to reduce differences in baseline fear to Context C. Subjects were exposed to Context C for 30 minutes per day without footshock delivery. Fear extinction was measured by freezing during the first 5 minutes of each session.

Training occurred the day after completion of fear extinction when no differences were observed between Susceptible and Resilient groups. Following a three-minute baseline period, all subjects received a 100-msec, 110-dB burst of white noise and were removed 30 seconds later. One day later subjects were returned to Context D for 8 minutes to assess fear to the context.

Results

SEFL results

Fear to the stress context was evaluated one day after stress exposure. Susceptible and Resilient subjects did not differ in fear to Context A ($F_{1,41}$=0.99, p=0.33; Fig 2-6B). Freezing scores were relatively low in both groups, possibly due to within-session extinction caused by the long inter-stimulus interval during footshock delivery. Susceptible and Resilient did not differ in baseline fear to Context B ($F_{1,41}$=1.38, p=0.25; Fig 2-6C). During the SEFL test, we found that while distinct Susceptible and Resilient groups again emerged there were no differences in freezing between males and females ($F_{1,41}$=0.01, p=0.94; Fig 2-6D).

Aversive acoustic stimulus results

To eliminate baseline differences in fear to Context C, subjects received 4 days of fear extinction. A Session by Resilience interaction was observed ($F_{3,123}$=3.13, p=0.03; Fig 2-6E). While Susceptible subjects initially showed increased fear to acoustic stimulus context ($p<0.05$), by the final extinction session they extinguished to the level of Resilient subjects ($p>0.05$).

On the day of acoustic stimulus delivery, Susceptible and Resilient subjects did not differ in fear during the three minutes prior to stimulus onset ($F_{1,41}$=0.31, p=0.58; Fig 2-6F). Importantly, Susceptible subjects showed significantly elevated fear to the context the following

day ($F_{1,41}$=5.12, p=0.03; Fig 6G). These results verify that Susceptible subjects show increased fear to a context paired with a novel aversive stimulus.

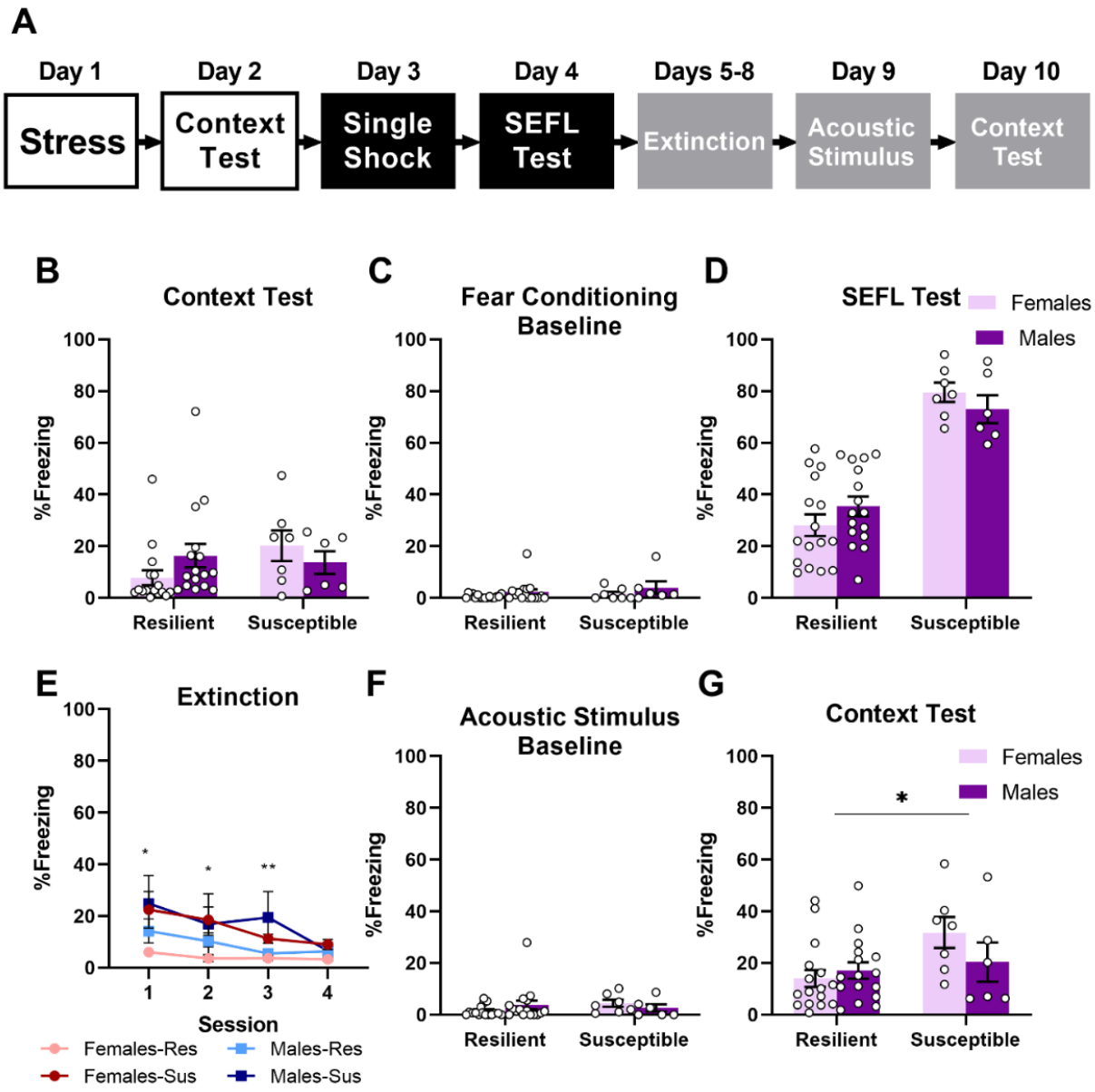


Figure 2-6. Experiment 2 results. **A.** Experiment timeline. **B.** Susceptible and Resilient subjects do not differ in fear to stress context (Day 2). **C.** No differences in fear during the 3 minutes prior to footshock delivery (Day 3). **D.** No sex differences during SEFL Test (Day 4). **E.** Extinction (Days 5-8). Data points show freezing during the first 5 minutes of each session. **F.** No differences in fear prior to acoustic stimulus delivery (Day 9). **G.** Susceptible subjects show increased fear to acoustic stimulus context (Day 10). *p<0.05, **p<0.01. Error bars represent standard error of the mean.

Discussion

One of the challenges in understanding and treating PTSD is understanding why some individuals develop the disorder following trauma exposure while others do not. While several rodent models of stress exposure have been developed to study PTSD, a need remains for models that can probe the individual variability in responses to stress in both male and female subjects (Richter-Levin et al., 2019; Shansky, 2015). Here we demonstrate that a modified version of the stress-enhanced fear learning (SEFL) procedure can be used to study the factors that promote susceptibility versus resilience to the effects of stress. Following exposure to an acute stressor consisting of 4 footshocks over 90 minutes, distinct populations emerge with approximately 60% of subjects classified as Resilient and 40% as Susceptible.

While subjects were classified based on their performance on the SEFL test, these subjects showed alterations in a variety of behaviors relevant to PTSD symptomology, summarized in Table 2-2. 43% of the rats exceeded our classification criterion following the 4-shock stress, while 79% passed the criterion when 15 shocks were administered. This indicates that the magnitude of the stressful event is one important factor in determining whether PTSD-like symptomatology will develop following trauma. However, given the bimodal split in the 4-shock condition, other pre-existing factors are also important determinants of post-stress reactivity. In females, prestress anxiety is correlated with which subjects will develop these post-stress symptoms. The current procedures and measures may provide a useful tool for probing these individual differences.

A key feature of PTSD is exaggerated responses to mild stressors that are reminiscent of the original trauma, which is captured by the SEFL test (Bremner et al., 1995; Dykman et al., 1997; Rau et al., 2005). However, PTSD is also characterized by several other changes in fear

behavior. Overgeneralization of fear from the original trauma situation to safe situations is believed to play a key role in PTSD symptomology, and PTSD patients show increased fear generalization from shock-paired cues to safe cues (Kaczkurkin et al., 2017). Here we demonstrate that addition to showing exaggerated fear to both shock and aversive acoustic stimuli, Susceptible subjects show increased generalization to a novel context.

Task	*Effects of Susceptibility*
Pre-stress	
Continuous access 2-bottle choice	No effect
Light-dark transition test	Reduced entries and time spent in light compartment (females only)
Fear measures	
Generalization test	Increased fear generalization
SEFL test	Increased fear conditioning
Extinction	Impaired fear extinction
Aversive acoustic stimulus	Increased fear conditioning
Anxiety measures	
Open field test	Reduced mobility, blunted response to light onset (males only)
Elevated plus maze	No effect
Light-dark transition test	Reduced entries into light compartment
Alcohol measures	
Intermittent access 2-bottle choice	Increased alcohol consumption and preference (males only)

Table 2-2. Summary of results indicating distinct Susceptible phenotypes. Results were observed in both males and females unless otherwise indicated.

Impaired fear suppression is also believed to play an important role in the maintenance of PTSD. Exposure therapy, a common treatment for PTSD, is a form of extinction learning in that subjects are repeatedly exposed to fear or anxiety-producing stimuli in a safe environment (Craske et al., 2008). However, one challenge in treating this disorder is that exposure therapy is

often ineffective, and subjects show impaired fear extinction in laboratory settings (Craske et al., 2008; Hembree et al., 2003; Milad et al., 2009). Our results indicate that Susceptible subjects also demonstrate this impairment in fear extinction, though it should be noted that directly comparing extinction rates across Susceptible and Resilient subjects is complicated by the fact that these groups differ in initial freezing levels prior to extinction. To address this issue, we examined the number of extinction sessions needed to suppress freezing to 50% of the initial level of freezing at the start of the extinction session. Using this measure, we still found that susceptible rats took longer to extinguish than rats classified as resilient.

One key aspect of the SEFL procedure is that it can be readily performed in both males and females, while models of stress exposure such as social defeat stress are limited to males (Berton et al., 2006; Golden et al., 2011). Given that women are twice as likely to develop PTSD compared to men even when controlling for trauma type (Kessler et al., 1995; Breslau et al, 1998; Kessler et al, 2017), we anticipated that a greater proportion of females would be classified as Susceptible compared to males. However, we found no differences in the rates of susceptibility in males compared to females, suggesting that biological differences alone may be insufficient to explain the disparity in rates of PTSD across genders.

While we found no sex differences in overall rates of stress susceptibility, we did observe sex-specific differences in its behavioral manifestation. During the Open Field Test, both Susceptible and Resilient females retreated from the light which is in agreement with a previous report indicating that footshock exposure does not impact light avoidance on this task in female rats (Godsil et al., 2005). In contrast, Resilient males also showed light avoidance while Susceptible males showed no change in time spent in Zone 4 following light onset.

A major challenge in treating PTSD is the high levels of comorbidity between PTSD and alcohol abuse (Kessler et al., 1995; Mills et al., 2006; Perkonigg et al., 2000). Notably, rates of alcohol abuse are higher in men than in women, as are rates of comorbidity between PTSD and alcohol abuse (Kessler et al., 1995). Mirroring this pattern, we found that stress susceptibility impacted alcohol consumption in a sex-specific manner with susceptibility associated with increased alcohol consumption in males but not in females. Examination of the results indicates that both Susceptible and Resilient females appear to show elevated levels of alcohol intake compared to Resilient males, which corresponds with previous reports that females show elevated alcohol drinking compared to males (Lancaster et al, 1996; Priddy et al, 2017). Lastly, substance use disorders (SUDs) are more likely to follow than precede PTSD (McFarlane, 1998), which was supported by our finding that no differences in alcohol consumption were observed in Susceptible subjects prior to stress exposure.

Identifying the underlying factors that promote stress susceptibility is critical for understanding why some individuals are at risk for developing PTSD. This procedure offers a powerful tool for directly probing the contribution of proposed risk factors to the development of PTSD-related behaviors. Preexisting anxiety disorders, which are also more common in women than in men, have been shown to increase the risk for subsequent PTSD (Breslau et al., 1997; Kessler et al., 2005; McLean et al., 2011). In support of this, we found that pre-stress anxiety levels were predictive of future stress susceptibility in females, but not in males. Increased activation of the hypothalamus-pituitary-adrenal (HPA) axis in females has been hypothesized to underlie the elevated rates of PTSD and anxiety disorders in women and contribute to stress susceptibility (Kudielka & Wüst, 2010; Rao & Androulakis, 2017). It is therefore plausible that increased baseline anxiety in Susceptible females was reflective of uniquely high levels of HPA

axis activation in these subjects. Interestingly, a recent study reported that baseline anxiety was not associated with stress susceptibility as assessed by changes in social behavior and fear extinction following stress exposure (Colucci et al., 2020). However, this study employed only male rats, highlighting the need for stress exposure models that can be employed in both males and females.

Within female rodents, estrous cycle has been reported to alter fear conditioning and extinction (Milad et al, 2009b; Cushman et al, 2014). We did not collect estrous cycle data in the present experiments as this would require additional handling of the females for cytology sample collection that cannot be adequately controlled for in male rats (Hubscher et al, 2005). However, given that baseline anxiety-related behavior was uniquely predictive of stress susceptibility in females, it will be worthwhile to evaluate the potential role of estrous cycle in future studies.

An additional limitation of this study is the use of a single classification criterion to determine whether subjects were susceptible or resilient, while PTSD diagnosis includes symptoms not necessarily related to trauma-related memories (APA, 2013). However, one strength of our present criterion is that it allowed us to utilize the decades of research using the SEFL model to establish an *a priori* criterion for stress susceptibility, rather than relying on post-hoc classification. Given our findings that Susceptible subjects showed a variety of changes in PTSD-related behaviors, in future studies it will be worth incorporating such measures to identify "PTSD-like" animals, and to investigate whether different groups of subjects show changes in specific types of behaviors, e.g. high-fear animals versus high-anxiety animals.

The SEFL effect lasts a minimum of 3 months and may very well be permanent (Rau et al., 2005; Rau & Fanselow, 2009). An interesting, albeit speculative, question is why does stress

produce such long-lasting and pronounced PTSD symptomatology? One possible answer is that it represents a functional adaptation that helps promote survival in a dangerous world. Another is that it is a fully pathological state. Such pathology might occur because while fear is an adaptation to short term emergencies such as predation (Bolles, 1970), prolonged stress pushes this system into overdrive with detrimental results. Some insight into this issue may be provided by the sex differences we observed. Previously, we reported that with the standard 15-shock procedure the overall levels of SEFL were not different between sexes (Poulos et al., 2015). In this current experiment the 4-shock stressor produced no sex differences in the overall level of SEFL or in the proportion of each sex exhibiting the phenotype. However, there were sex differences in the factors that predicted stress susceptibility. These sex differences suggest that while the levels of SEFL susceptibility and magnitude are equivalent between sexes, the mechanisms through which animals reach that level may differ. That the same levels and proportions are obtained via different mechanisms suggests that there is convergent evolution toward these levels. Such convergent evolution is consistent with the hypothesis that PTSD is an adaptation to dangerous environments.

Conclusion

In conclusion, here we demonstrate that a modified version of the stress-enhanced fear learning procedure can be used to produce distinct susceptible and resilient populations that exhibit a constellation of behaviors relevant to PTSD symptomology in both males and females. We show that while some aspects of stress susceptibility are shared across sexes, other aspects including the ability of baseline anxiety to predict future susceptibility manifest differently in males and females. This procedure is a valuable tool for probing the biological mechanisms that support stress resilience.

Supplementary Materials

Distribution of SEFL Test scores in males versus females

We investigated whether there were any sex differences in the distribution of SEFL test scores among the unstressed controls or among subjects exposed to the 15-shock stress. The Kolmogorov-Smirnov test was used to compare the distributions of scores of No Stress males (n=137, Mean=18.6%, SD=19.7%) and No Stress females (n=45, Mean=18.9%, SD=19.6%), and to compare the distribution of 15-Shock males (n=98, Mean=75.5%, SD=22.4%) and 15-Shock females (n=32, Mean=71.4%, SD=20.4%). We found no sex differences in either the No Stress condition (D=0.18, p=0.22; Figs 2-7A-B) or in the 15-footshock condition (D=0.22, p=0.19; Figs 2-7C-D). We further found that the susceptibility cutoff was the same when calculated separately for females (58%) and males (58%). We performed a similar analysis on the subjects from the current experiment that were exposed to the 4-footshock stress. SEFL Test scores from females (n=22, Mean=54.8, SD=23.6) and males (n=22, Mean=61.9, SD=27.4) are plotted in Figures 2-7E-F. No difference in these distributions was observed (D=0.23, p=0.63).

Evaluation of resilience criterion based on subjects exposed 15-shock stress

Our criterion for classifying subjects as “Susceptible” or “Resilient” was based on identifying individuals that showed abnormally high levels of fear compared to that of unstressed controls. Given that some individuals experience trauma but do not go on to develop PTSD, an alternative approach would be to determine a cutoff for resilience based on the performance of stress-exposed subjects. To address this possibility, we compiled the SEFL test scores obtained from subjects exposed to the standard 15-footshock stress (n=130). We classified subjects as “Resilient” if they showed performance on the SEFL test that was at least two standard deviations

below the means of subjects exposed to the standard 15-footshock stress, which based on this dataset was 30.5% freezing.

We found that this alternative approach did not perform as well for several reasons. First, while only a small proportion of people who experience trauma go on to develop PTSD, this criterion would result in 86.4% of subjects exposed to the 4-footshock stress being classified as "Susceptible". Second, the 58% criterion corresponds to the separation within the bimodal distribution that was observed in the 4-footshock subjects, while the 30.5% criterion does not (Fig 2-1C). Thus, we determined that the 58% criterion based on the unstressed controls was most appropriate.

Fear, anxiety, and alcohol intake in Resilient and Susceptible subjects compared to No Stress subjects

To illustrate how performance of Susceptible and Resilient animals compared to that of unstressed controls on the battery of fear, anxiety and alcohol intake assessments used in this experiment, 62 adult male and female Long-Evans rats (Envigo) concurrently received identical treatment to as those described in Methods, but on Day 16 of the procedure received 90 minutes of context exposure without footshock. 42 subjects received alcohol prior to stress, while 20 subjects did not. Means and standard deviations for each task for Resilient, Susceptible and No Stress groups are shown in Table 2-3. For tasks involving repeated measures, the average value across all time points was computed.

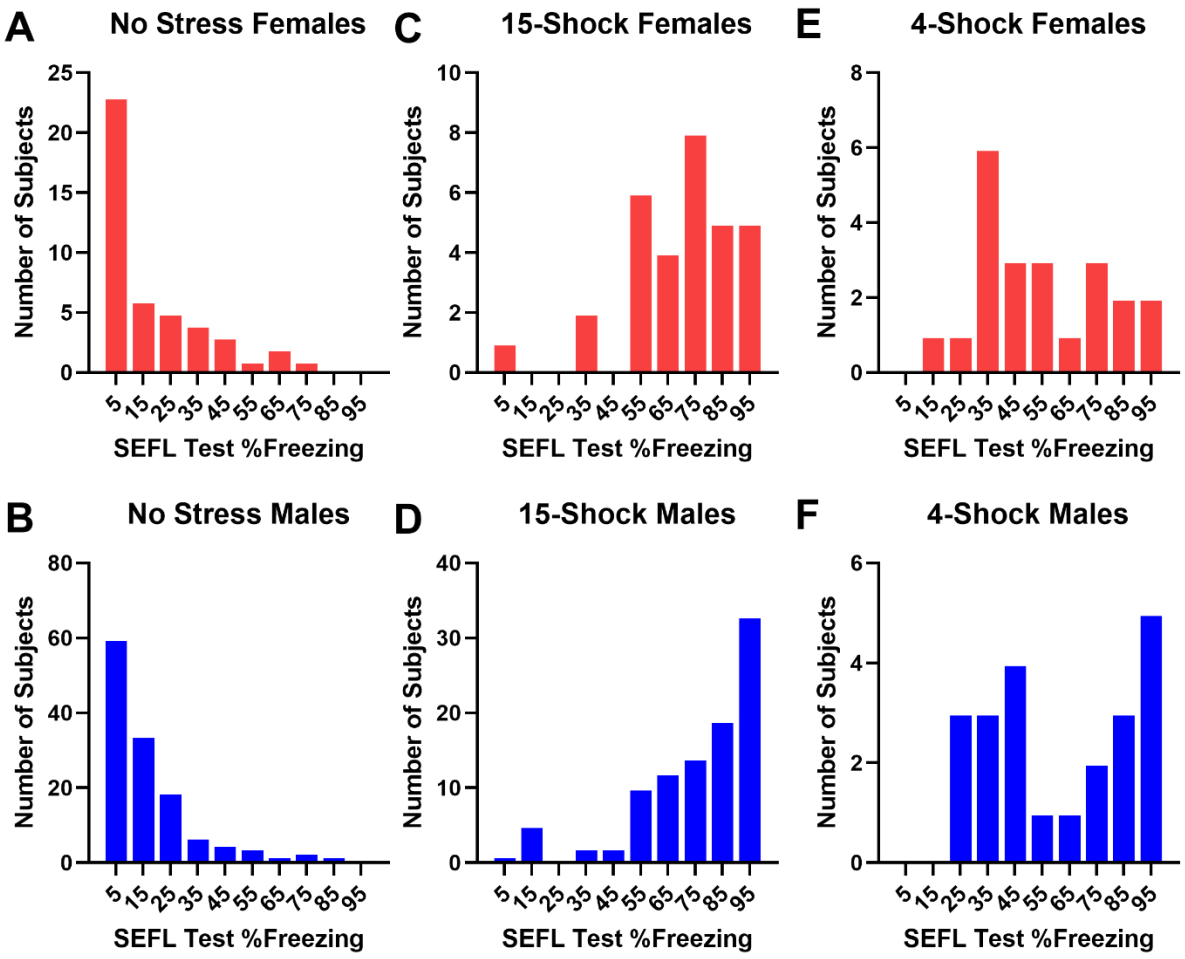


Figure 2-7. Distribution of SEFL Test scores in males versus females following different levels of stress. **A-B.** Distribution of SEFL test scores in (A) unstressed females (n=45) and (B) unstressed males (n=137). No differences were observed in the distribution of scores (statistic). **C-D.** Distribution of SEFL test scores in (C) females exposed to standard 15-footshock stress (n=32) and (D) males exposed to standard 15-footshock stress (n=98). No differences were observed in the distribution of scores (statistic). **E-F.** Distribution of SEFL test scores in females exposed to 4-footshock stress (n=22) and males exposed to 4-footshock stress (n=22). No differences were observed in the distribution of scores (statistic). Tick marks indicate center of 10% bins.

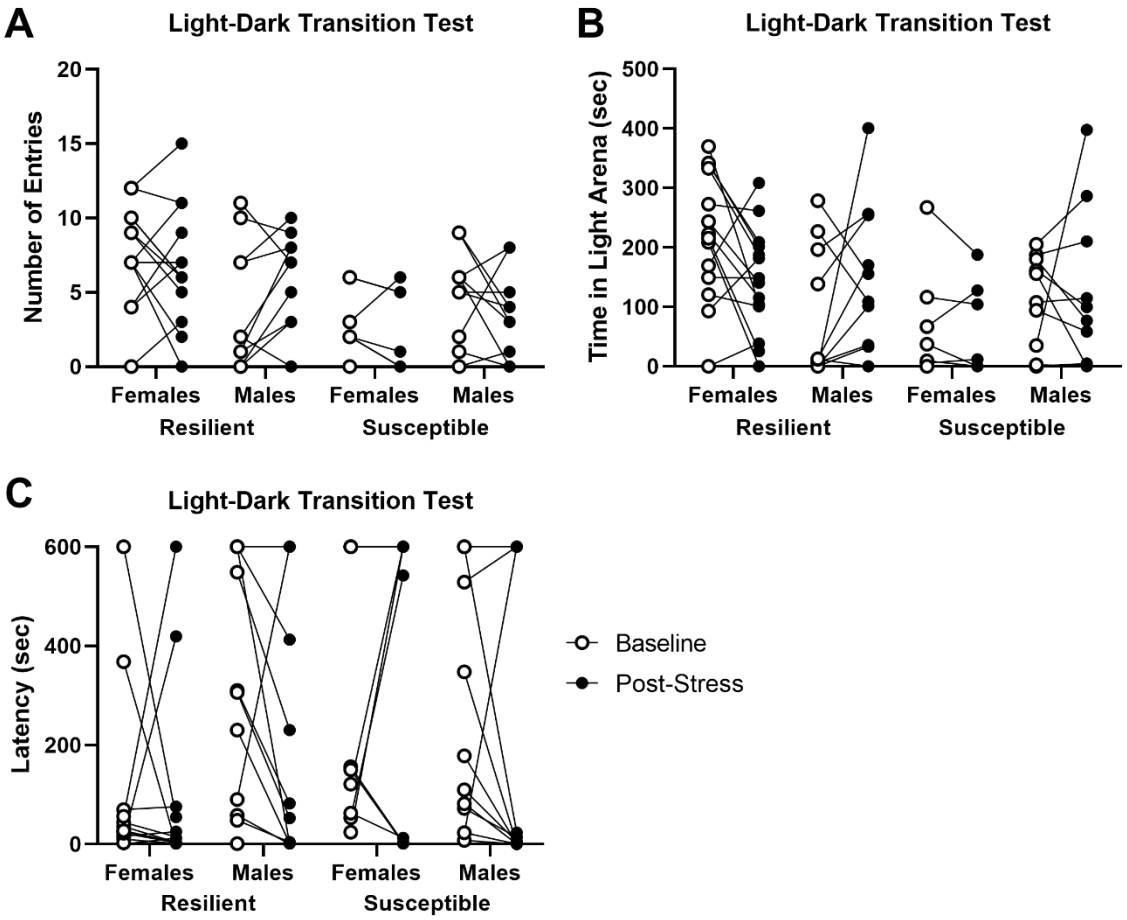


Figure 2-8. Performance on light-dark transition test prior to and following stress. No interactions between stress exposure and resilience were observed on **(A)** number of entries into the light compartment, **(B)** time spent in the light arena or **(C)** latency to enter the light arena. Each line represents an individual subject.

Task	*Resilient Mean(SD)*	*Susceptible Mean(SD)*	*No Stress Mean(SD)*
Pre-stress			
CA 2-BC (EtOH consumption, mg/kg)	1.7(1.0)	1.8(1.7)	1.7(1.2)
CA 2-BC (EtOH preference)	20.8(12.2)	13.1(13.4)	16.5(10.9)
Light-dark transition test (entries)			
Females	7.5(3.3)	2.6(2.3)	6.5(3.2)
Males	3.7(4.2)	4.5(3.3)	4(3.5)
Light-dark transition test (light time, sec)			
Females	220(107.3)	62.8(92.1)	178.8(108.8)
Males	79.3(108.7)	102.7(81.1)	94.1(88.4)
Light-dark transition test (latency, sec)			
Females	92.1(173.3)	221(238.5)	143.4(179.2)
Males	308.4(243.2)	233.6(240.2)	293.5(241)
Fear measures			
Generalization test (%freezing)	5.7(6.9)	27.4(25.2)	1.9(1.4)
SEFL Test (%freezing)	38.5(10.9)	84.4(11.5)	18.2(15.6)
Extinction (final session %freezing)	7.6(8.8)	29.9(28.4)	5.3(7.3)
Extinction (trials to 50%)	2.1(1.1)	3.4(1.4)	2.5(1.7)
Acoustic stimulus test (%freezing)	19.2(15.8)	49.1(21.6)	7.8(8.9)
Anxiety measures			
Open field test (velocity, cm/sec)	7.5(1.8)	5.2(2.3)	7.3(1.8)
Elevated plus maze (open arms time, sec)	62.9(42.7)	43(24.9)	47.8(35.1)
Light-dark transition test (entries)	6.2(3.7)	2.8(2.8)	6(4.4)
Light-dark transition test (light time, sec)	141.6(104.4)	88.4(113.2)	136.1(112.2)
Light-dark transition test (latency, sec)	128.1(213)	252.8(298.2)	176(243.4)
Alcohol intake			
IA 2-BC (EtOH consumption, mg/kg)			
Females	4.3(1)	4.1(0.9)	4.3(0.8)
Males	2.8(1.3)	4.5(1.3)	4.2(1.5)
IA 2-BC (EtOH preference)			
Females	61.6(12.6)	55.7(15)	61.4(13.4)
Males	47.2(19.4)	75.4(11.8)	73.3(24.8)

Table 2-3. Comparison of Susceptible and Resilient subjects with No Stress subjects run through the same procedures. Intermittent access 2-bottle choice (IA 2-BC) measures are from subjects that did not receive alcohol prior to stress. All other measures are from subjects that did receive alcohol prior to stress.

References

APA. (2013). *Diagnostic and statistical manual of mental disorders (DSM-5)*. American Psychiatric Pub.

Ballaz, S. J., Akil, H., & Watson, S. J. (2007). Previous experience affects subsequent anxiety-like responses in rats bred for novelty seeking. *Behav Neurosci*, *121*(5), 1113-1118. https://doi.org/10.1037/0735-7044.121.5.1113

Banasikowski, T. J., Cloutier, C. J., Ossenkopp, K. P., & Kavaliers, M. (2015). Repeated exposure of male mice to low doses of lipopolysaccharide: dose and time dependent development of behavioral sensitization and tolerance in an automated light-dark anxiety test. *Behav Brain Res*, *286*, 241-248. https://doi.org/10.1016/j.bbr.2015.03.004

Berton, O., McClung, C. A., Dileone, R. J., Krishnan, V., Renthal, W., Russo, S. J., Graham, D., Tsankova, N. M., Bolanos, C. A., Rios, M., Monteggia, L. M., Self, D. W., & Nestler, E. J. (2006). Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. *Science*, *311*(5762), 864-868. https://doi.org/10.1126/science.1120972

Bolles, R. C. (1970). Species-specific defense reactions and avoidance learning. *Psychological review*, *77*(1).

Bourin, M., & Hascoët, M. (2003). The mouse light/dark box test. *Eur J Pharmacol*, *463*(1-3), 55-65. https://doi.org/10.1016/s0014-2999(03)01274-3

Brancato, A., Plescia, F., Lavanco, G., Cavallaro, A., & Cannizzaro, C. (2016). Continuous and Intermittent Alcohol Free-Choice from Pre-gestational Time to Lactation: Focus on Drinking Trajectories and Maternal Behavior. *Front Behav Neurosci*, *10*, 31. https://doi.org/10.3389/fnbeh.2016.00031

Bremner, J. D., Krystal, J. H., Southwick, S. M., & Charney, D. S. (1995). Functional neuroanatomical correlates of the effects of stress on memory. *J Trauma Stress*, *8*(4), 527-553.

Breslau, N., Davis, G. C., Andreski, P., Peterson, E. L., & Schultz, L. R. (1997). Sex differences in posttraumatic stress disorder. *Arch Gen Psychiatry*, *54*(11), 1044-1048. https://doi.org/10.1001/archpsyc.1997.01830230082012

Brunello, N., Davidson, J. R., Deahl, M., Kessler, R. C., Mendlewicz, J., Racagni, G., Shalev, A. Y., & Zohar, J. (2001). Posttraumatic stress disorder: diagnosis and epidemiology, comorbidity and social consequences, biology and treatment. *Neuropsychobiology*, *43*(3), 150-162. https://doi.org/10.1159/000054884

Cohen, H., Zohar, J., Gidron, Y., Matar, M. A., Belkind, D., Loewenthal, U., Kozlovsky, N., & Kaplan, Z. (2006). Blunted HPA axis response to stress influences susceptibility to

posttraumatic stress response in rats. *Biol Psychiatry*, *59*(12), 1208-1218. https://doi.org/10.1016/j.biopsych.2005.12.003

Colucci, P., Marchetta, E., Mancini, G. F., Alva, P., Chiarotti, F., Hasan, M. T., & Campolongo, P. (2020). Predicting susceptibility and resilience in an animal model of post-traumatic stress disorder (PTSD). *Transl Psychiatry*, *10*(1), 243. https://doi.org/10.1038/s41398-020-00929-9

Covington, H. E., Lobo, M. K., Maze, I., Vialou, V., Hyman, J. M., Zaman, S., LaPlant, Q., Mouzon, E., Ghose, S., Tamminga, C. A., Neve, R. L., Deisseroth, K., & Nestler, E. J. (2010). Antidepressant effect of optogenetic stimulation of the medial prefrontal cortex. *J Neurosci*, *30*(48), 16082-16090. https://doi.org/10.1523/JNEUROSCI.1731-10.2010

Craske, M. G., Kircanski, K., Zelikowsky, M., Mystkowski, J., Chowdhury, N., & Baker, A. (2008). Optimizing inhibitory learning during exposure therapy. *Behav Res Ther*, *46*(1), 5-27. https://doi.org/10.1016/j.brat.2007.10.003

Crawley, J., & Goodwin, F. K. (1980). Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. *Pharmacol Biochem Behav*, *13*(2), 167-170. https://doi.org/10.1016/0091-3057(80)90067-2

Cushman, J. D., Moore, M. D., Olsen, R. W., & Fanselow, M. S. (2014). The role of the δ GABA (A) receptor in ovarian cycle-linked changes in hippocampus-dependent learning and memory. Neurochemical research, 39(6), 1140-1146.

Dykman, R. A., Ackerman, P. T., & Newton, J. E. (1997). Posttraumatic stress disorder: a sensitization reaction. *Integr Physiol Behav Sci*, *32*(1), 9-18.

Godsil, B. P., Blackmore, M. A., & Fanselow, M. S. (2005). Modulation of an activity response with associative and nonassociative fear in the rat: a lighting differential influences the form of defensive behavior evoked after fear conditioning. *Learn Behav*, *33*(4), 454-463.

Godsil, B. P., & Fanselow, M. S. (2004). Light stimulus change evokes an activity response in the rat. *Learn Behav*, *32*(3), 299-310.

Golden, S. A., Covington, H. E., Berton, O., & Russo, S. J. (2011). A standardized protocol for repeated social defeat stress in mice. *Nat Protoc*, *6*(8), 1183-1191. https://doi.org/10.1038/nprot.2011.361

Goldstein, R. B., Smith, S. M., Chou, S. P., Saha, T. D., Jung, J., Zhang, H., Pickering, R. P., Ruan, W. J., Huang, B., & Grant, B. F. (2016). The epidemiology of DSM-5 posttraumatic stress disorder in the United States: results from the National Epidemiologic Survey on Alcohol and Related Conditions-III. *Soc Psychiatry Psychiatr Epidemiol*, *51*(8), 1137-1148. https://doi.org/10.1007/s00127-016-1208-5

Gruene, T. M., Flick, K., Stefano, A., Shea, S. D., & Shansky, R. M. (2015). Sexually divergent expression of active and passive conditioned fear responses in rats. *Elife*, *4*, e11352.

Hembree, E. A., Foa, E. B., Dorfan, N. M., Street, G. P., Kowalski, J., & Tu, X. (2003). Do patients drop out prematurely from exposure therapy for PTSD? *J Trauma Stress*, *16*(6), 555-562. https://doi.org/10.1023/B:JOTS.0000004078.93012.7d

Hubscher, C. H., Brooks, D. L., & Johnson, J. R. (2005). A quantitative method for assessing stages of the rat estrous cycle. *Biotechnic & Histochemistry*, *80*(2), 79-87.

Jeong, M. J., Lee, C., Sung, K., Jung, J. H., Pyo, J. H., & Kim, J. H. (2020). Fear response-based prediction for stress susceptibility to PTSD-like phenotypes. *Mol Brain*, *13*(1), 134. https://doi.org/10.1186/s13041-020-00667-5

Kaczkurkin, A. N., Burton, P. C., Chazin, S. M., Manbeck, A. B., Espensen-Sturges, T., Cooper, S. E., Sponheim, S. R., & Lissek, S. (2017). Neural Substrates of Overgeneralized Conditioned Fear in PTSD. *Am J Psychiatry*, *174*(2), 125-134. https://doi.org/10.1176/appi.ajp.2016.15121549

Kessler, R. C., Berglund, P., Demler, O., Jin, R., Merikangas, K. R., & Walters, E. E. (2005). Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. *Arch Gen Psychiatry*, *62*(6), 593-602. https://doi.org/10.1001/archpsyc.62.6.593

Kessler, R. C., Sonnega, A., Bromet, E., Hughes, M., & Nelson, C. B. (1995). Posttraumatic stress disorder in the National Comorbidity Survey. *Arch Gen Psychiatry*, *52*(12), 1048-1060.

Kessler, R. C., Aguilar-Gaxiola, S., Alonso, J., Benjet, C., Bromet, E. J., Cardoso, G., ... & Koenen, K. C. (2017). Trauma and PTSD in the WHO world mental health surveys. *European journal of psychotraumatology*, *8*(sup5), 1353383.

Kudielka, B. M., & Wüst, S. (2010). Human models in acute and chronic stress: assessing determinants of individual hypothalamus-pituitary-adrenal axis activity and reactivity. *Stress*, *13*(1), 1-14. https://doi.org/10.3109/10253890902874913

Lancaster, F. E., Brown, T. D., Coker, K. L., Elliott, J. A., & Wren, S. B. (1996). Sex differences in alcohol preference and drinking patterns emerge during the early postpubertal period in sprague-dawley rats. Alcoholism: *Clinical and Experimental Research*, *20*(6), 1043-1049.

Lanius, R. A., Bluhm, R., Lanius, U., & Pain, C. (2006). A review of neuroimaging studies in PTSD: heterogeneity of response to symptom provocation. *J Psychiatr Res*, *40*(8), 709-729. https://doi.org/10.1016/j.jpsychires.2005.07.007

Lanius, R. A., Williamson, P. C., Boksman, K., Densmore, M., Gupta, M., Neufeld, R. W., Gati, J. S., & Menon, R. S. (2002). Brain activation during script-driven imagery induced dissociative responses in PTSD: a functional magnetic resonance imaging investigation. *Biol Psychiatry*, *52*(4), 305-311.

Lanius, R. A., Williamson, P. C., Densmore, M., Boksman, K., Gupta, M. A., Neufeld, R. W., Gati, J. S., & Menon, R. S. (2001). Neural correlates of traumatic memories in posttraumatic stress disorder: a functional MRI investigation. *Am J Psychiatry*, *158*(11), 1920-1922. https://doi.org/10.1176/appi.ajp.158.11.1920

Long, V. A., & Fanselow, M. S. (2012). Stress-enhanced fear learning in rats is resistant to the effects of immediate massed extinction. *Stress*, *15*(6), 627-636. https://doi.org/10.3109/10253890.2011.650251

McFarlane, A. C. (1998). Epidemiological evidence about the relationship between PTSD and alcohol abuse: the nature of the association. *Addict Behav*, *23*(6), 813-825. https://doi.org/10.1016/s0306-4603(98)00098-7

McLean, C. P., Asnaani, A., Litz, B. T., & Hofmann, S. G. (2011). Gender differences in anxiety disorders: prevalence, course of illness, comorbidity and burden of illness. *J Psychiatr Res*, *45*(8), 1027-1035. https://doi.org/10.1016/j.jpsychires.2011.03.006

Meyer, E. M., Long, V., Fanselow, M. S., & Spigelman, I. (2013). Stress increases voluntary alcohol intake, but does not alter established drinking habits in a rat model of posttraumatic stress disorder. *Alcohol Clin Exp Res*, *37*(4), 566-574. https://doi.org/10.1111/acer.12012

Milad, M. R., Pitman, R. K., Ellis, C. B., Gold, A. L., Shin, L. M., Lasko, N. B., Zeidan, M. A., Handwerger, K., Orr, S. P., & Rauch, S. L. (2009). Neurobiological basis of failure to recall extinction memory in posttraumatic stress disorder. *Biol Psychiatry*, *66*(12), 1075-1082. https://doi.org/10.1016/j.biopsych.2009.06.026

Milad, M. R., Igoe, S. A., Lebron-Milad, K., & Novales, J. E. (2009b). Estrous cycle phase and gonadal hormones influence conditioned fear extinction. Neuroscience, 164(3), 887-895.

Mills, K. L., Teesson, M., Ross, J., & Peters, L. (2006). Trauma, PTSD, and substance use disorders: findings from the Australian National Survey of Mental Health and Well-Being. *Am J Psychiatry*, *163*(4), 652-658. https://doi.org/10.1176/appi.ajp.163.4.652

Onaivi, E. S., & Martin, B. R. (1989). Neuropharmacological and physiological validation of a computer-controlled two-compartment black and white box for the assessment of anxiety. *Prog Neuropsychopharmacol Biol Psychiatry*, *13*(6), 963-976. https://doi.org/10.1016/0278-5846(89)90047-x

Pennington, Z. T., Anderson, A. S., & Fanselow, M. S. (2017). The ventromedial prefrontal cortex in a model of traumatic stress: fear inhibition or contextual processing? *Learn Mem*, *24*(9), 400-406. https://doi.org/10.1101/lm.046110.117

Perkonigg, A., Kessler, R. C., Storz, S., & Wittchen, H.-U. (2000). Traumatic events and post-traumatic stress disorder in the community: prevalence, risk factors and comorbidity. *Acta Psychiatr Scand*, *101*(1), 46-59.

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*(1), 45-57. https://doi.org/10.1038/npp.2015.224

Poulos, A. M., Reger, M., Mehta, N., Zhuravka, I., Sterlace, S. S., Gannam, C., Hovda, D. A., Giza, C. C., & Fanselow, M. S. (2014). Amnesia for early life stress does not preclude the adult development of posttraumatic stress disorder symptoms in rats. *Biol Psychiatry*, *76*(4), 306-314. https://doi.org/10.1016/j.biopsych.2013.10.007

Poulos, A. M., Zhuravka, I., Long, V., Gannam, C., & Fanselow, M. (2015). Sensitization of fear learning to mild unconditional stimuli in male and female rats. *Behav Neurosci*, *129*(1), 62-67. https://doi.org/10.1037/bne0000033

Priddy, B. M., Carmack, S. A., Thomas, L. C., Vendruscolo, J. C., Koob, G. F., & Vendruscolo, L. F. (2017). Sex, strain, and estrous cycle influences on alcohol drinking in rats. *Pharmacology Biochemistry and Behavior*, *152*, 61-67.

Pruim, R., Kaplan, D. T., & Horton, N. J. (2017). The mosaic Package: Helping Students to 'Think with Data' Using R. *The R Journal*, *9*(1), 77-102.

R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/

Rao, R. T., & Androulakis, I. P. (2017). Modeling the Sex Differences and Interindividual Variability in the Activity of the Hypothalamic-Pituitary-Adrenal Axis. *Endocrinology*, *158*(11), 4017-4037. https://doi.org/10.1210/en.2017-00544

Rau, V., DeCola, J. P., & Fanselow, M. S. (2005). Stress-induced enhancement of fear learning: an animal model of posttraumatic stress disorder. *Neurosci Biobehav Rev*, *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

Revelle, W. (2019). psych: Procedures for Psychological, Psychometric, and Personality Research. In. Northwestern University, Evanston, Illinois, USA.

Richter-Levin, G., Stork, O., & Schmidt, M. V. (2019). Animal models of PTSD: a challenge to be met. *Mol Psychiatry*, *24*(8), 1135-1156. https://doi.org/10.1038/s41380-018-0272-5

Ritov, G., Boltyansky, B., & Richter-Levin, G. (2016). A novel approach to PTSD modeling in rats reveals alternating patterns of limbic activity in different types of stress reaction. *Mol Psychiatry*, *21*(5), 630-641. https://doi.org/10.1038/mp.2015.169

Rodgers, R. J., & Dalvi, A. (1997). Anxiety, defence and the elevated plus-maze. *Neurosci Biobehav Rev*, *21*(6), 801-810. https://doi.org/10.1016/s0149-7634(96)00058-9

Shansky, R. M. (2015). Sex differences in PTSD resilience and susceptibility: Challenges for animal models of fear learning. *Neurobiol Stress*, *1*, 60-65. https://doi.org/10.1016/j.ynstr.2014.09.005

Simms, J. A., Steensland, P., Medina, B., Abernathy, K. E., Chandler, L. J., Wise, R., & Bartlett, S. E. (2008). Intermittent access to 20% ethanol induces high ethanol consumption in Long-Evans and Wistar rats. *Alcohol Clin Exp Res*, *32*(10), 1816-1823. https://doi.org/10.1111/j.1530-0277.2008.00753.x

Willner, P., Towell, A., Sampson, D., Sophokleous, S., & Muscat, R. (1987). Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant. *Psychopharmacology (Berl)*, *93*(3), 358-364. https://doi.org/10.1007/BF00187257

Wise, R. A. (1973). Voluntary ethanol intake in rats following exposure to ethanol on various schedules. *Psychopharmacologia*, *29*(3), 203-210. https://doi.org/10.1007/BF00414034

CHAPTER 3

The role of Ca^{2+}-permeable AMPA receptors in stress-enhanced fear learning

Abstract

Improper regulation of fear plays a key role in the development and symptomology of post-traumatic stress disorder (PTSD). In particular, PTSD patients show an exaggerated ability to form new fear memories. Stress-enhanced fear learning (SEFL) is a rodent model of stress exposure that captures this sensitization of fear learning, but the mechanisms that support this change are unclear. α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors typically contain a GluA2 subunit which renders them impermeable to Ca^{2+} entry, which is critical for activating the molecular machinery that underlies associative learning. However, Ca^{2+}-permeable AMPA receptors (CP-AMPARs) are entirely composed of GluA1 subunits and lack a GluA2 subunit, allowing Ca^{2+} entry and thereby offering an alternative mechanism for long-term potentiation to occur. We investigated the potential role of CP-AMPARs in supporting enhanced fear learning following stress. We found that viral-mediated overexpression of the GluA1 subunit of the AMPA receptor within the basolateral amygdala (BLA) was insufficient to enhance fear learning to a single footshock in the absence of stress exposure. We further tested the effects of targeted genetic deletion of the GluA2 subunit in the BLA of floxed GluA2 mice. We found that GluA2 deletion was also insufficient to enhance fear learning in the absence of any prior stress. However, when mice were exposed to an additional footshock in a novel context, GluA2 deletion caused increased fear learning and anxiety in male mice but not females. These findings suggest that CP-AMPARs within the BLA may support enhancements in fear learning following stress in males but not in females.

Introduction

The ability to form and store fear memories is crucial for survival. While this ability enables us to learn about and respond to threats, excessive levels of fear can have adverse effects on mental health and well-being. One characteristic of post-traumatic stress disorder (PTSD) is an exaggerated response to mild stressors that are reminiscent of the original trauma, leading to inappropriate fear responses (Bremner et al., 1995; Dykman et al., 1997). The increased arousal and avoidance of trauma-related stimuli that characterizes PTSD can lead to exhaustion, sleep disturbances, and a constricted lifestyle (Eberly & Engdahl, 1991).

Our laboratory has established a rodent model of stress exposure termed stress-enhanced fear learning (SEFL) that uses aspects of Pavlovian fear conditioning to capture this exaggerated fear response (Long & Fanselow, 2012; Perusini et al., 2016; Rau et al., 2005; Rau & Fanselow, 2009). In standard Pavlovian fear conditioning a neutral conditional stimulus (CS), such as a context, is paired with an aversive unconditional stimulus (US), such as a footshock. Following the formation of a CS-US association, the CS will elicit a fear response. In the SEFL model, animals are exposed to an intense acute stressor (typically 10-15 unsignaled footshocks) in a distinct context, followed by a single footshock in a different context. Following stress exposure animals show exaggerated fear to the single footshock context. This enhancement of fear learning is the defining feature of the SEFL model and appears to reflect a nonassociative sensitization of the fear learning process. Previous studies from our laboratory show that extinction of fear to the stress context does not mitigate SEFL (Long & Fanselow, 2012), and manipulations that produce amnesia of the stress leave SEFL unaffected (Poulos et al., 2014; Rau et al., 2005), indicating that SEFL is not simply due to generalization of fear from the stress context to a novel context.

It is likely that the fear sensitization demonstrated in both SEFL and PTSD patients is due to changes in the neural network that support fear conditioning. The basolateral amygdala (BLA) is a key component of this network (Fendt & Fanselow, 1999). During fear conditioning, sensory information converges onto pyramidal neurons of the BLA. While the inputs carrying information about the neutral stimulus are initially too weak to activate the BLA, during fear conditioning these connections become strengthened such that presentations of the previously neutral stimulus acquire the ability to activate the BLA (Kim & Jung, 2006; Rogan et al., 1997). This synaptic strengthening occurs via long-term potentiation (LTP), a process typically triggered by Ca^{2+} influx through N-methyl-D-aspartate (NMDA) glutamate receptors (Maren, 1999). Consequently, blockade of NDMA receptors during conditioning prevents the acquisition of fear (Fanselow & Kim, 1994; Kim et al., 1992). However, increasing evidence suggests that these BLA connections can be strengthened though an alternative, NMDA receptor-independent process.

α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are ionotropic glutamate receptors that mediate fast excitatory transmission within the central nervous system (Lee et al., 2003; Malinow, 2003). The majority of AMPA receptors typically exist as heteromers of GluA1 and GluA2 or GluA2 and GluA3, with the GluA2 subunit rendering the ion channel impermeable to Ca^{2+} entry (Plant et al., 2006; Wenthold et al., 1996). However, increases in GluA1 levels have been shown to result in the formation of AMPA receptors composed entirely of GluA1 subunits that lack the GluA2 subunit (Hollmann et al., 1991; Wiltgen et al., 2010). These Ca^{2+}-permeable AMPA receptors (CP-AMPARs) allow Ca^{2+} entry, and electrophysiological analyses of mice with a deletion of the GluA2 subunit in the CA1 region of the hippocampus have revealed that these mice exhibit a form of LTP that is dependent

on CP-AMPARs and independent of NMDA receptors (Wiltgen et al., 2010). Furthermore, we have previously observed increased levels of GluA1 protein in the BLA following SEFL, and corticosterone synthesis inhibition prevents both this increase in GluA1 and the behavioral changes associated with SEFL (Perusini et al., 2016).

These previous findings lead to the possibility that stress-induced increases in BLA GluA1 levels lead to increased formation of CP-AMPARs, which in turn augment canonical BLA plasticity and ultimately enhance fear learning. The experiments conducted here were designed to test this possibility.

More specifically, in the following experiments we tested the hypothesis that promoting CP-AMPAR formation by increasing the ratio of GluA1 to GluA2 subunits within the BLA is sufficient to enhance fear learning in the absence of stress. In Experiment 1, we tested whether increasing the expression of GluA1 within the BLA is sufficient to enhance fear learning. In Experiments 2 and 3, we verified that floxed GluA2 mice demonstrate SEFL (Experiment 2) and tested whether targeted genetic deletion of GluA2 within the BLA of these mice is sufficient to enhance fear learning (Experiment 3).

Experiment 1: Effects of GluA1 overexpression on fear learning in the absence of stress.

Our general hypothesis is that increases in GluA1 levels within the BLA support enhancements in fear learning following stress. In Experiment 1 we specifically tested the hypothesis that overexpressing GluA1 using a herpes simplex virus (HSV) viral vector within the BLA would be sufficient to enhance fear learning in the absence of stress.

Materials and Methods

Subjects

31 naïve adult male C57Bl/6J mice (The Jackson Laboratory, Bar Harbor, ME, USA) approximately 8 weeks old at the start of the experiment were used. Mice were randomly assigned to GluA1 or GFP conditions. Animals were individually housed under a 12-hour light/dark cycle. Environmental enrichment in the form of nesting material was provided. Food and water were available *ad libitum* in the home cage. Animals were handled daily for 60 seconds each day for 1 week prior to the start of the experiment. The UCLA Institutional Animal Care and Use Committee approved all procedures involving animals.

Apparatus

All fear conditioning took place in four identical fear conditioning chambers housed in sound-attenuating shells (Med-Associates Inc, St. Albans, VT). Each chamber contained grid floors composed of stainless steel bars wired to a shock generator and scrambler. Chambers were cleaned and scented with 50% Windex between each session and ventilation fans provided background noise. Shock delivery was controlled and freezing automatically scored using VideoFreeze software (Med Associates Inc, St. Albans, VT, USA).

Surgical procedures

Following anesthesia induction with isoflurane and administration of the analgesic carprofen (5 mg/kg, s.c.), the skull was exposed and holes were drilled above the injection site. 10 ul Hamilton syringes were loaded with either HSV-GluA1-GFP (GluA1 condition) or HSV-GFP control virus (GFP condition) and lowered to the following BLA coordinates relative to bregma: AP=−1.7 mm, ML=±3.25 mm, DV=−4.75 mm. 0.3 μl of virus was infused at a rate of

0.1 μl/min and injectors were left in place for 10 mins before being raised. The incision site was closed with sterile nylon sutures. Animals were given an additional injection of carprofen (5 mg/kg, s.c.) 24 hours after surgery and maintained on the antibiotic Enroflox for five days following surgery. Behavioral testing began after 3 days of recovery from surgery.

Behavioral testing procedures.

On day 1, **s**ubjects were individually loaded into fear conditioning chambers. Following a three-minute baseline period, subjects received a single 2-sec, 0.4-mA footshock and were removed two minutes later. The next day, subjects were returned to the conditioning chamber for 8 minutes to assess context fear. Fear was assessed via freezing, a reliably and ethologically valid measure of fear in rodents (Fanselow & Bolles, 1979). Shock reactivity was measured by activity during footshock presentation.

Immunohistochemistry.

Immediately following behavioral testing, subjects were euthanized via isoflurane overdose. Brains were extracted and fixed in 4% paraformaldehyde at 4°C for 24 hours, followed by 24 hours in escalating doses of 10%, 20% and 30% sucrose in 1X PBS. Brains were frozen and sectioned in 40 μm slices. Slices containing the BLA across the anterior-posterior axis were collected in 1X PBS. The immunohistochemistry protocol to stain for GluA1 protein is as follows. Slices were first washed 3 times in 1X PBS. Slices were then blocked in 5% natural goat serum and 0.3% Triton X-100 in 1X PBS for one hour and incubated in primary antibody overnight at 4°C (Abcam Rabbit Anti-Glutamate Receptor 1 Antibody, #ab31232, diluted 1:1000 in blocking solution). Slices were then was three times in 1X PBS and incubated in secondary

antibody for 2 hours at room temperature (Goat Anti-Rabbit IgG Alexa Fluor 568 #ab175471, 1:500 dilution in blocking solution). Slices were washed three times in 1X PBS, mounted onto microscope slides and coverslipped using Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA). Slices were imaged at 10x magnification with a Keyence BZ-X710 fluorescent microscope. The spread of viral expression was measured by GFP expression, and subjects without expression within the BLA or substantial expression outside the BLA were excluded. The BLA was traced using the Paxinos and Franklin mouse brain atlas, and the number of GluA1-positive and DAPI-stained cells were counted using ImageJ software.

Data Analysis

Data were analyzed using IBM SPSS Statistics software. Each measure was analyzed using one-way ANOVA with virus condition (GluA1 or GFP) as the independent variable. Data were plotted using GraphPad Prism and diagrams of viral expression were created using Adobe Illustrator.

Results

Fear conditioning (Day 1)

All subjects showed minimal fear during the 3-minute period prior to footshock delivery on day 1, which did not differ between groups ($F_{1,20}$=0.79, p=0.39; Fig 3-1A). Groups did not differ in either reactivity to the footshock itself ($F_{1,20}$=0.1, p=0.76; Fig 3-1B), or in freezing during the 2-minute period following footshock delivery ($F_{1,20}$=1.10, p=0.31; Fig 3-1C).

Context test (Day 2)

When returned to the fear conditioning context one day after training, both groups showed low levels of fear that did not differ from each other ($F_{1,20}$=1.25, p=0.28; Fig 3-1D), indicating that GluA1 overexpression was insufficient to enhance fear learning in the absence of stress.

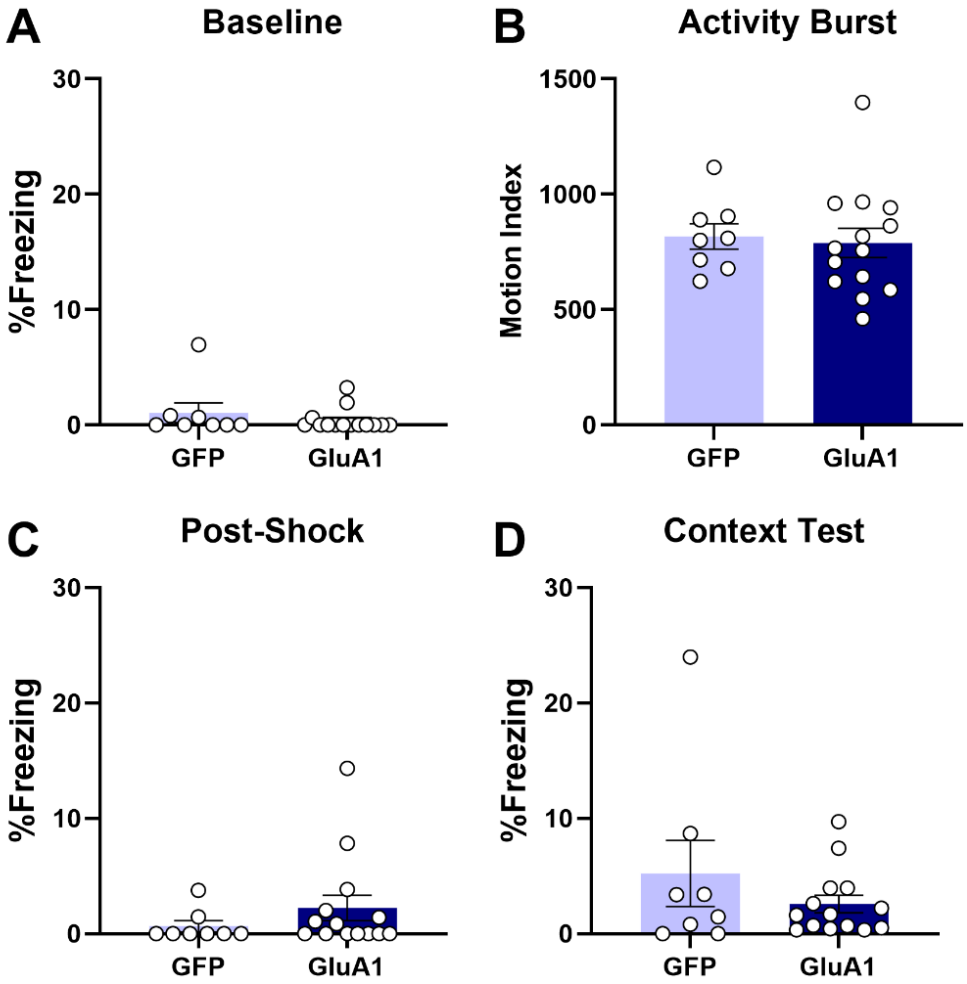


Figure 3-1. Experiment 1 fear conditioning results. **A.** No differences in 3-minute baseline fear prior to footshock (Day 1). **B.** No differences in shock reactivity (Day 1). **C.** No differences in 30-second period immediately following footshock (Day 1). **D.** No differences in fear during 8-minute context test (Day 2). Error bars represent standard error of the mean.

Histology and immunohistochemistry

Following behavioral testing, GluA1 overexpression was verified using immunohistochemistry and accurate viral placement was verified using GFP expression (Figs 3-2A-B). 9 animals were excluded due to inaccurate virus placement or tissue damage. Increased expression of GluA1 within the BLA was verified by comparing the number of GluA1-positive neurons to the total number of DAPI-labeled cells. GluA1 subjects showed an increased ratio of GluA1-positive to DAPI-labeled cells relative to GFP controls, indicating that the GluA1 virus successfully increased GluA1 expression ($F_{1,20}$=13.76, p=0.001; Fig 3-2A). It should be noted that relatively little GluA1 immunofluorescence was observed among control subjects, which is comparable to previous observations in our laboratory (Pennington et al., 2020). The spread of GFP expression is illustrated in Fig 3-2B (coronal section diagrams based on Paxinos and Franklin mouse brain atlas). Representative images from GFP and GluA1 mice are shown in Figs 3-2C-J.

Collectively, these results indicate that GluA1 overexpression within the BLA was insufficient to enhance fear learning in the absence of stress. However, there were a few limitations to the experimental approach that may have hindered our ability to detect any changes in fear learning. First, while viral overexpression of GluA1 did produce a significant increase in GluA1 immunofluorescence, it is not clear that this increase was sufficient to drive the production of CP-AMPARs. Second, the short-acting nature of HSVs limited our ability to probe further behavioral changes that may have arisen as a result of this manipulation. In subsequent experiments we utilized a more long-lasting manipulation, the targeted genetic deletion of GluA2 subunits within the BLA, to promote the formation of CP-AMPARs, and then tested the effects of the longer-lasting manipulation on fear learning.

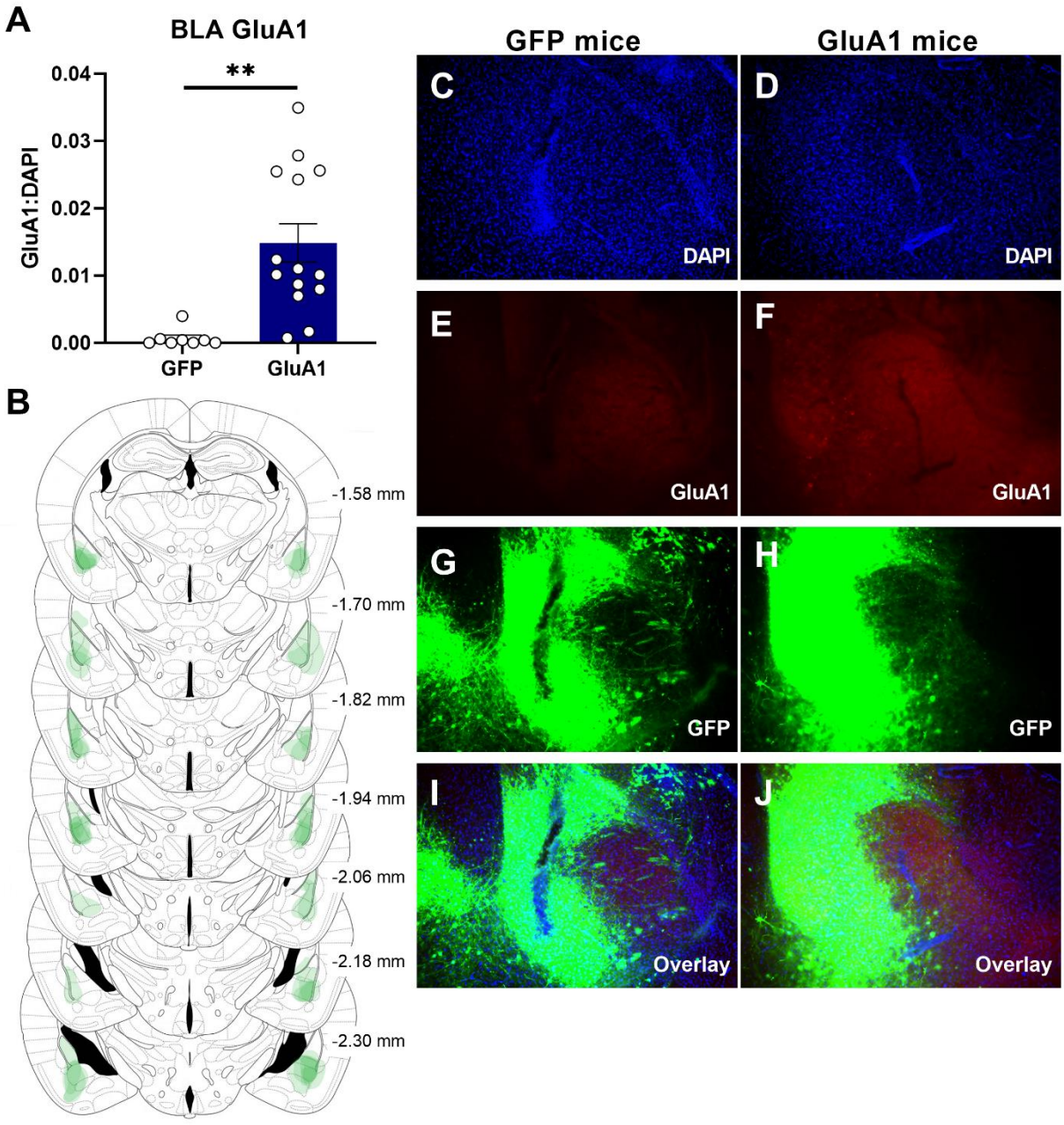


Figure 3-2. Experiment 1 histology results. **A.** Ratio of GluA1-positive cells to DAPI-positive cells within the BLA. **B.** Spread of GFP expression in coronal sections. Each layer represents maximal GFP expression from an individual animal. Distance from bregma (mm) is shown. **C-J.** Representative images of DAPI-labeled cells (C-D), GluA1 staining (E-F), GFP expression (G-H) and overlay (I-J). Images taken at 10x magnification. **p<0.01. Error bars represent standard error of the mean.

Experiment 2: Stress-enhanced fear learning in floxed GluA2 mice

To further test the potential role of CP-AMPARs within the BLA in supporting stress-enhanced fear learning, we aimed to test whether promoting the formation of these receptors through the deletion of GluA2 subunits would be sufficient to enhance fear learning. The first step in this test was to verify that floxed GluA2 mice demonstrate SEFL. Experiment 2, therefore, hypothesized that that stress produces a robust enhancement in fear learning among floxed GluA2 mice.

Materials and Methods

Subjects

Floxed GluA2 mice on a 129S6 background were generated as described in Wiltgen et al (2010) and bred in-house. In these mice infusion of a Cre-expressing virus causes a permanent deletion of the *gria2* gene that encodes the GluA2 subunit of the AMPA receptor. 22 mice (10 females and 12 males) approximately 10 weeks old at the start of the experiment were assigned to Stress and No Stress conditions. Animals were individually housed under a 12 hour light/dark cycle. Environmental enrichment in the form of nesting material was provided. Food and water were available *ad libitum* in the home cage. Animals were handled daily for 60 seconds each day for 1 week prior to the start of the experiment. The UCLA Institutional Animal Care and Use Committee approved all procedures involving animals.

Apparatus

All fear conditioning took place in two sets of four identical fear conditioning chambers housed in sound-attenuating shells (Med Associates Inc). Each set of chambers served as a distinct context which were differentiated by floor texture, shape, lighting and odor. Context A

(stress context) contained flat grid floors, was lit by a white house light and scented with 50% Windex solution. Ventilation fans provided background noise. Context B (fear conditioning context) contained a curved white plastic wall and floors composed of staggered grid bars and was scented with 1% acetic acid. All grid floors were wired to shock generators and scramblers. Shock delivery was controlled and freezing automatically scored using VideoFreeze software (Med Associates Inc). The open field apparatus consisted of a 60 cm x 60 cm arena with gray plastic walls and white Plexiglas floor, divided into four 30 cm x 30 cm quadrants. The room was dimly lit by a lamp in the corner of the room and a white noise generator provided background noise. Activity was recorded and automatically scored using EthoVision software (Noldus, Wageningen, Netherlands).

Behavioral testing procedures

On day 1, subjects were transported to Context A in their home cages. Stress condition subjects received either 10 unsignalled 1-sec, 1-mA footshocks over 60 minutes (6 minute average ISI), while No Stress subjects received equivalent context exposure without footshock. The following day subjects were returned to Context A for 8 minutes to assess fear to the stress context. Fear was assessed via freezing, a reliably and ethologically valid measure of fear in rodents (Fanselow & Bolles, 1979). On day 3, subjects were transported to Context B in clean, empty cages. Following a three-minute baseline period, all subjects received a single 2-sec, 1-mA footshock and were removed 30 sec later. Shock reactivity was measured by total activity during footshock presentation. Subjects were returned to Context B one day after conditioning for 8 minutes to assess whether stress exposure enhanced fear learning to a subsequent mild stressor.

Previous findings from our laboratory indicate that the SEFL procedure produces increased anxiety in addition to enhanced fear learning (Perusini et al., 2016). One day after completion of the SEFL procedure, anxiety was assessed using the open field test, a classic test of anxiety in rodents (Walsh & Cummins, 1976). Four mice were run at a time, with one mouse placed in each quadrant of the open field arena and allowed to freely explore for 10 minutes. Anxiety was assessed by the amount of exploratory behavior and the time spent in the center of the open field arena.

Data Analysis

Data were analyzed using IBM SPSS Statistics software. Each measure was analyzed using 2-way ANOVA with condition (Stress versus No Stress) as the first factor and sex (males versus female) as the second factor. Data were plotted using GraphPad Prism.

Results

SEFL procedure

When returned to Context A on day 2, Stress mice showed greater fear compared to No Stress controls ($F_{1,18}$=192.79, p<0.001; Fig 3-3A), indicating successful acquisition of fear to the stress context. No effects of Sex ($F_{1,18}$=1.87, p=0.19), or Stress*Sex interaction ($F_{1,18}$=1.45, p=0.24) were observed.

Generalization from Context A to Context B was assessed by freezing during the 3-minute baseline period prior to footshock delivery. All groups showed minimal levels of fear that were not affected by Condition ($F_{1,18}$=3.93, p=0.06; Fig 3-3B). No effects of Sex ($F_{1,18}$=0.03, p=0.86) or Stress*Sex interaction ($F_{1,18}$=0.38, p=0.55) were observed. This ensured that any

subsequent differences in fear learning would reflect changes in fear learning and not simply generalization of fear from the stress context.

Stress subjects showed decreased shock reactivity compared to No Stress controls ($F_{1,18}$=5.46, p=0.03; Fig 3-3C), but there was no effect of Sex ($F_{1,18}$=3.19, p=0.09) or Condition*Sex interaction ($F_{1,18}$=0.48, p=0.5). Despite showing reduced reactivity to the footshock, Stress subjects showed marginally greater freezing immediately following footshock delivery ($F_{1,18}$=3.43, p=0.08; Fig 3-3D). There was no effect of Sex ($F_{1,18}$=1.13, p=0.3), or Group*Sex interaction ($F_{1,18}$=0.1, p=0.75).

When returned to Context B to assess the ability of stress exposure to enhance fear learning, Stress subjects showed increased fear compared to No Stress controls ($F_{1,18}$=42.12, p<0.001; Fig 3-3E) indicating that stress exposure did indeed enhance fear learning. No effects of Sex ($F_{1,18}$=0.11, p=0.75) or Stress*Sex interaction ($F_{1,18}$=0.09, p=0.77) were observed.

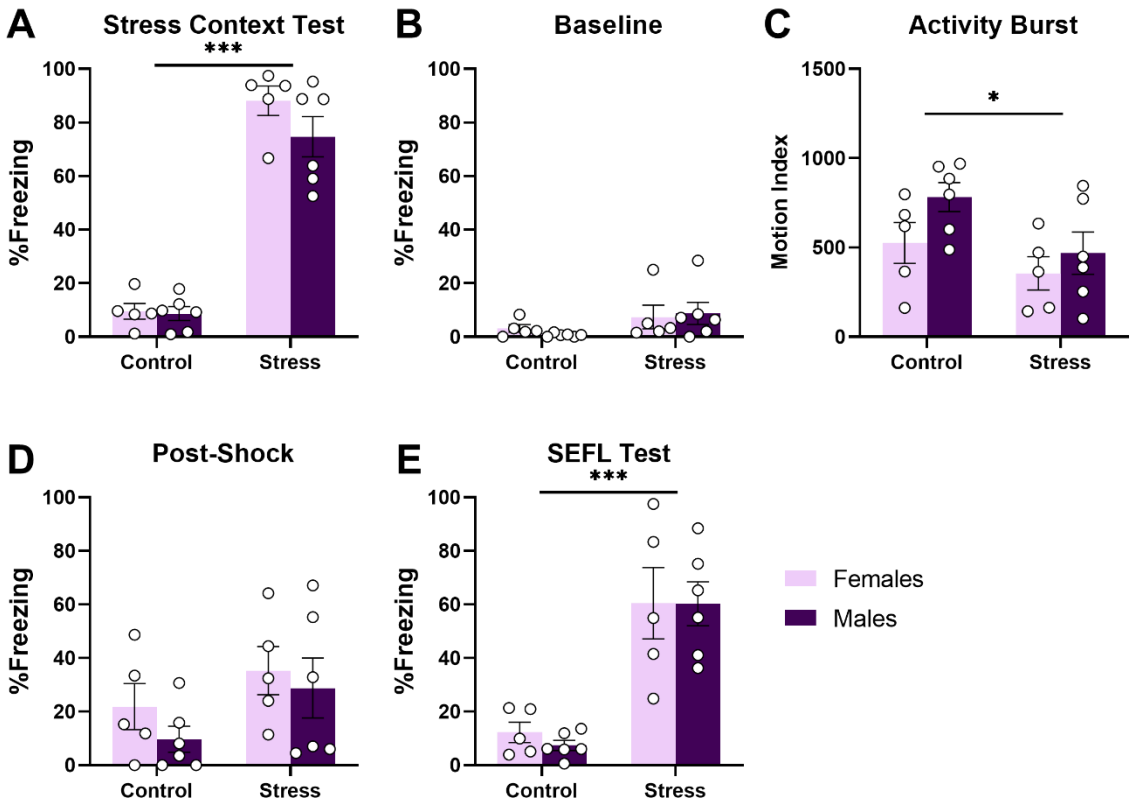


Figure 3-3. Experiment 2 SEFL results. **A.** Stress subjects show increased fear to stress context test (Day 2). **B.** No differences in baseline fear during the 3-minutes prior to footshock delivery were observed (Day 3). **C.** Stress subjects showed reduced shock reactivity to the single footshock (Day 3). **D.** No differences in fear immediately following footshock delivery were observed (Day 3). **E.** Stress subjects show increased fear to single footshock context (Day 4). *p<0.05. ***p<0.001. Error bars represent standard error of the mean.

Open field test

On the final day of testing, anxiety was measured using the open field test. Stress subjects showed reduced exploratory behavior compared to No Stress controls ($F_{1,18}$=5.11, p=0.04; Fig 3-4A), though there was no effect of Sex ($F_{1,18}$=1.93, p=0.18) or Condition*Sex interaction ($F_{1,18}$=0.001, p=0.99). Consistent with previous observations, stress exposure did not impact time spent in the center of the open field apparatus ($F_{1,18}$=1.2, p=0.29; Fig 3-4B). No

effects of Sex (F1,18=1.19, p=0.29) or Condition*Sex interaction ($F_{1,18}$=0.8, p=0.38) were observed.

Collectively, these results indicate that floxed GluA2 mice demonstrate the same behavioral changes following stress that have previously been observed in rats and C57Bl/6 mice. Thus, this experiment confirmed that floxed GluA2 mice demonstrate SEFL.

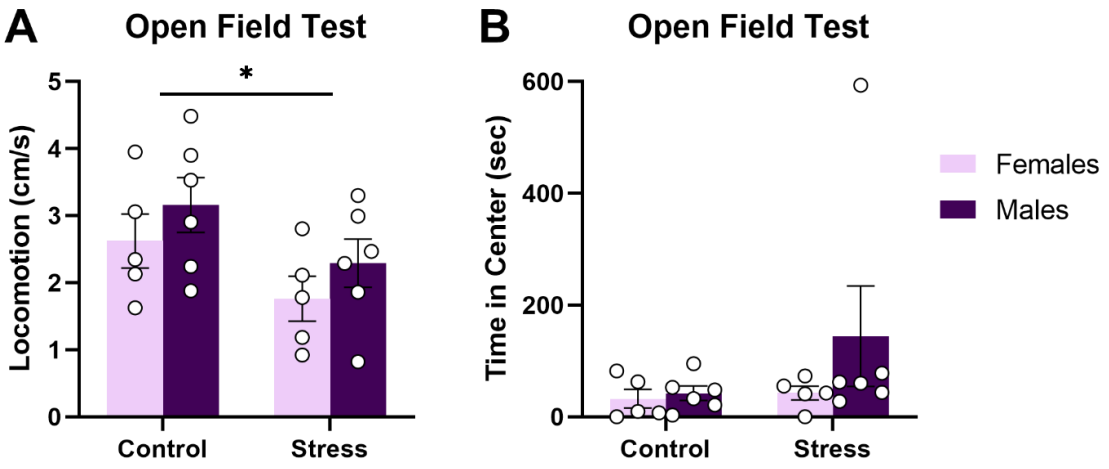


Figure 3-4. Experiment 2 open field test results (Day 5). **A.** Stress subjects show reduced exploratory behavior compared to No Stress controls. **B.** No differences in time spent in the center of the open field test were observed. *p<0.05. Error bars represent standard error of the mean.

Experiment 3: Impact of BLA GluA2 deletion on fear learning

In Experiment 2 we demonstrated SEFL in floxed GluA2 mice. In Experiment 3 we tested the hypothesis that promoting the formation of CP-AMPARs by genetic deletion of the GluA2 subunit using a Cre-expressing adeno-associated virus (AAV) would be sufficient to enhance fear learning in the absence of stress.

Materials and Methods

Subjects

Floxed GluA2 mice on a 129S6 background were generated as described previously (Wiltgen et al., 2010) and bred in-house. 60 mice (31 females and 29 males) approximately 9-10 weeks old at the start of the experiment were assigned to either Cre or GFP control virus conditions. Animals were individually housed under a 12 hour light/dark cycle. Food and water were available *ad libitum* in the home cage. Animals were handled daily for 60 seconds for 1 week prior to the start of the experiment. The Chancellor's Animal Research Committee at UCLA approved all procedures involving animals.

Apparatus

All fear conditioning took place in two sets of four identical fear conditioning chambers housed in sound-attenuating shells (Med Associates Inc). Each set of chambers served as a distinct context which were differentiated by floor texture, shape, lighting and odor. Context A contained a curved white plastic wall and floors composed of staggered grid bars, was lit by a near-infrared light and scented with 1% acetic acid. Context B contained flat grid floors, was lit by a white house light and scented with 50% Windex solution. Ventilation fans provided background noise. All grid floors were wired to shock generators and scramblers. Shock delivery was controlled and freezing automatically scored using VideoFreeze software (Med Associates Inc). The open field apparatus consisted of a 60 cm x 60 cm arena with gray plastic walls and white Plexiglas floor, divided into four 30 cm x 30 cm quadrants. The room was dimly lit by a lamp in the corner of the room and a white noise generator provided background noise. Activity was recorded and automatically scored using EthoVision software (Noldus).

Surgical procedures

Following anesthesia induction with isoflurane and administration of the analgesic carprofen (5 mg/kg, s.c.), the skull was exposed and holes were drilled above the injection site. 10 µl Hamilton syringes were loaded with either AAV2-hSyn-GFP-CRE (Cre condition) or AAV2-hSyn-GFP control virus (GFP condition; UNC Vector Core) and lowered to the following BLA coordinates relative to bregma: AP=−1.7 mm, ML=±3.25 mm, DV=−4.75 mm. 0.3 µl of virus was infused at a rate of 0.1 µl/min and injectors were left in place for 10 mins before being raised. The incision site was closed with sterile nylon sutures. Animals were given an additional injection of carprofen (5 mg/kg, s.c.) 24 hours after surgery and maintained on the antibiotic enroflox for five days following surgery. Behavioral testing began after at least 4 weeks of recovery surgery to allow for sufficient viral expression.

Behavioral testing procedures

Context A fear conditioning (Days 1-2). Subjects were transported to Context A in their home cages. Following a 3-minute baseline period, all subjects received a single 2-sec, 1-mA footshock and were removed 30 seconds later. Fear was assessed via freezing and shock reactivity was measured by activity during footshock presentation. The next day subjects were returned to Context A for 8 minutes to test whether GluA2 deletion was sufficient to enhance fear learning in the absence of stress.

Context B fear conditioning (Days 3-4). To test whether a combination of GluA2 deletion and a prior fear learning event would be sufficient to enhance subsequent fear learning, all subjects received a second mild stressor in a novel context. Subjects were transported to Context B in

clean, empty cages. Following a three-minute baseline period, all subjects received a single 2-sec, 1-mA footshock and were removed 30 sec later. The next day subjects were returned to Context B for 8 minutes to assess whether GluA2 deletion combined with a prior fear learning experience would be sufficient to enhance fear learning to a second mild stressor.

Open field test (Day 5). The open field test was used to determine whether GluA2 deletion could promote increased anxiety in the absence of stress. Four mice were run at a time, with one mouse placed in each quadrant of the open field arena and allowed to freely explore for 10 minutes. Anxiety was assessed by the amount of exploratory behavior and the time spent in the center of the open field arena.

Histology and immunohistochemistry

Following behavioral testing, viral placement was assessed by GFP expression and GluA2 deletion was verified using immunohistochemistry. One day after behavioral testing, subjects were euthanized via isoflurane overdose. Brains were extracted and fixed in 4% paraformaldehyde at 4°C for 24 hours, followed by 24 hours in 15% sucrose and 30% sucrose in 1X PBS. Brains were frozen and sectioned in 40 µm slices. Slices containing the BLA were collected in 1X PBS. The immunohistochemistry protocol to stain for GluA2 protein is as follows. Slices were first washed 3 times in 1X PBS. Slices were then blocked in 5% natural goat serum and 0.3% Triton X-100 in 1X PBS for one hour and incubated in primary antibody overnight at 4°C (Millipore Sigma Rabbit Anti-GluA2 antibody #AB1768-I, diluted 1:1000 in blocking solution). Slices were then washed three times in 1X PBS and incubated in secondary antibody for 2 hours at room temperature (Goat Anti-Rabbit IgG Alexa Fluor 568 #ab175471, 1:500 dilution in blocking solution). Slices were washed three times in 1X PBS, mounted onto

microscope slides and coverslipped using Vectashield with DAPI (Vector Labs). Slices were imaged with a Keyence BZ-X710 fluorescent microscope. The spread of viral expression was measured by GFP expression imaged at 4x magnification, and subjects without expression within the BLA or expression outside the BLA were excluded. To quantify GluA2 staining, slices were imaged at 20x magnification. The BLA was traced using the Paxinos and Franklin mouse brain atlas, and the number of GluA2-positive and DAPI-stained cells were counted using ImageJ software.

Data Analysis

Data were analyzed using IBM SPSS Statistics software. The effects of virus on males and females for each measure were analyzed using the Mann-Whitney U Test. Data were plotted using GraphPad Prism and diagrams of viral expression were created using Adobe Illustrator.

Results

Context A fear conditioning

To assess whether GluA2 deletion would produce enhanced fear learning in the absence of stress, subjects received a single footshock in Context A. During the 3-minute period prior to the single footshock, there were no effects of virus on either females ($U=30$, $n_1=12$, $n_2=8$, $p=0.18$; Fig 3-5A) or males ($U=36.5$, $n_1=10$, $n_2=8$, $p=0.76$). Shock reactivity during the single footshock was also not affected in either females ($U=30$, $n_1=12$, $n_2=8$, $p=0.18$; Fig 3-5B) or males ($U=36$, $n_1=10$, $n_2=8$, $p=0.76$). Freezing during the 30-second period following footshock was not affected in either females ($U=58$, $n_1=12$, $n_2=8$, $p=0.47$; Fig 3-5C) or males ($U=41$, $n_1=10$, $n_2=8$, $p=1$). One day after conditioning subjects were returned to Context A for 8 minutes

to assess fear to the context. Virus type did not impact context fear in either females (U=57, n_1=12, n_2=8, p=0.52; Fig 3-5D) or males (U=26.5, n_1=10, n_2=8, p=0.24). GluA2 deletion within the BLA was therefore insufficient to enhance fear learning in the absence of stress exposure

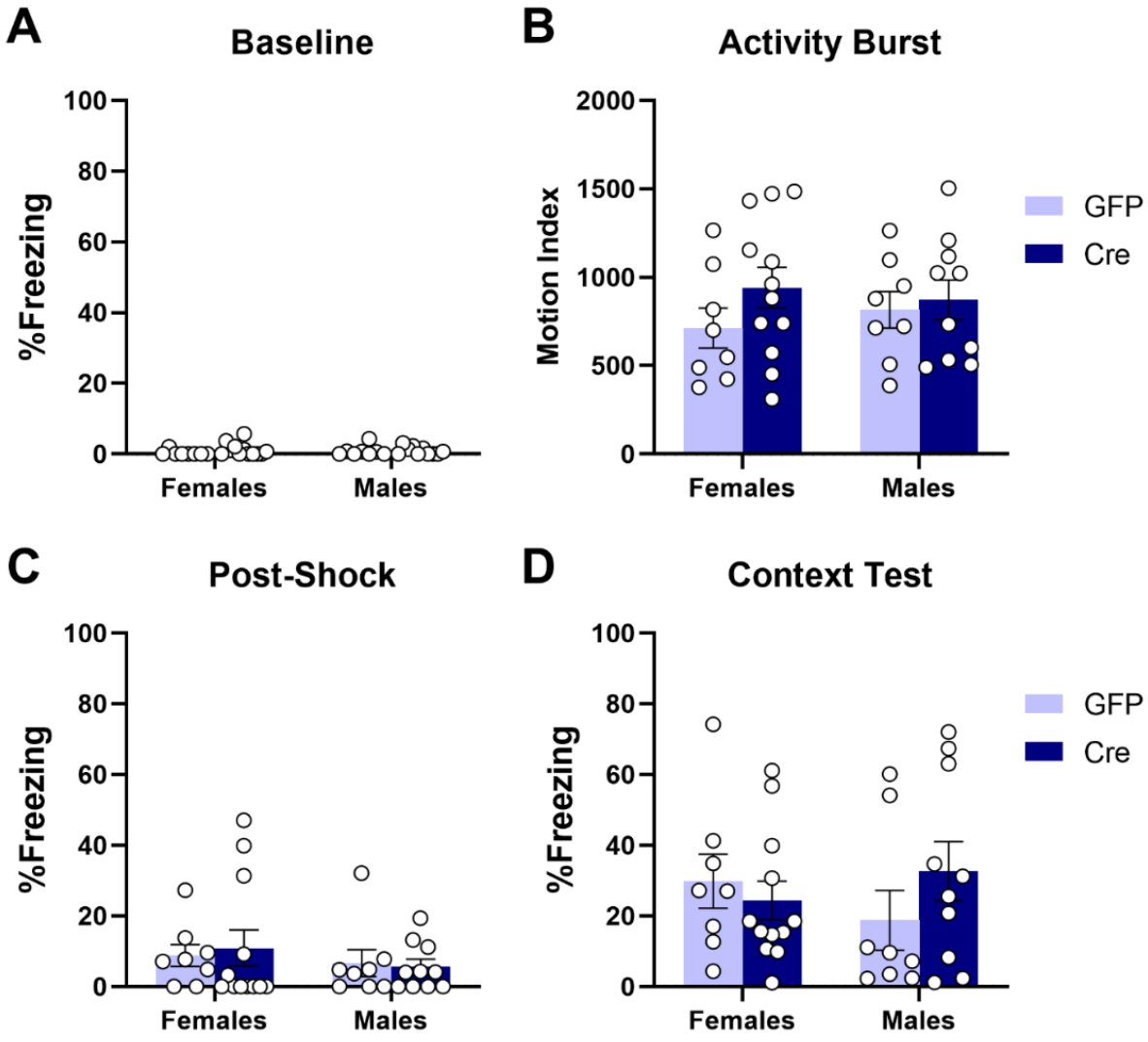


Figure 3-5. Experiment 3 Context A fear conditioning results (Days 1-2). **A.** No differences in baseline fear prior to footshock (Day 1). **B.** No differences in shock reactivity during footshock (Day 1). **C.** No differences in fear during the 30 seconds immediately following footshock (Day 1). **D.** No differences during 8-minute context test (Day 2). Error bars represent standard error of the mean.

Context B fear conditioning

To assess the possibility that GluA2 deletion combined with a prior history of mild stress exposure would enhance fear learning, subjects received a second fear conditioning in a novel context. Prior to footshock exposure, Cre males showed a small but significant elevation in baseline fear compared to GFP males ($U=16$, $n_1=10$, $n_2=8$, $p=0.03$; Fig 3-6A) while no baseline differences were observed in females ($U=27$, $n_1=12$, $n_2=8$, $p=0.12$). No differences in shock reactivity were observed in females ($U=57$, $n_1=12$, $n_2=8$, $p=0.52$; Fig 3-6B) or males ($U=53$, $n_1=10$, $n_2=8$, $p=0.27$). During the 30-second period immediately following footshock Cre males again showed a significant elevation in freezing compared to GFP males ($U=16$, $n_1=10$, $n_2=8$, $p=0.03$; Fig 3-6C), while no differences were observed in females ($U=40$, $n_1=12$, $n_2=8$, $p=0.57$).

Subjects were returned to Context B one day after conditioning for 8 minutes to assess context fear. Cre males showed marginally greater freezing compared to GFP males ($U=19$, $n_1=10$, $n_2=8$, $p=0.07$; Fig 3-6D), while no differences were observed in females ($U=48$, $n_1=12$, $n_2=8$, $p=1$). These results indicate that while GluA2 deletion alone was insufficient to enhance fear learning, GluA2 deletion combined with previous experience of a mild stressor (the single footshock in Context A) was sufficient to enhance subsequent fear learning in male mice, but not females.

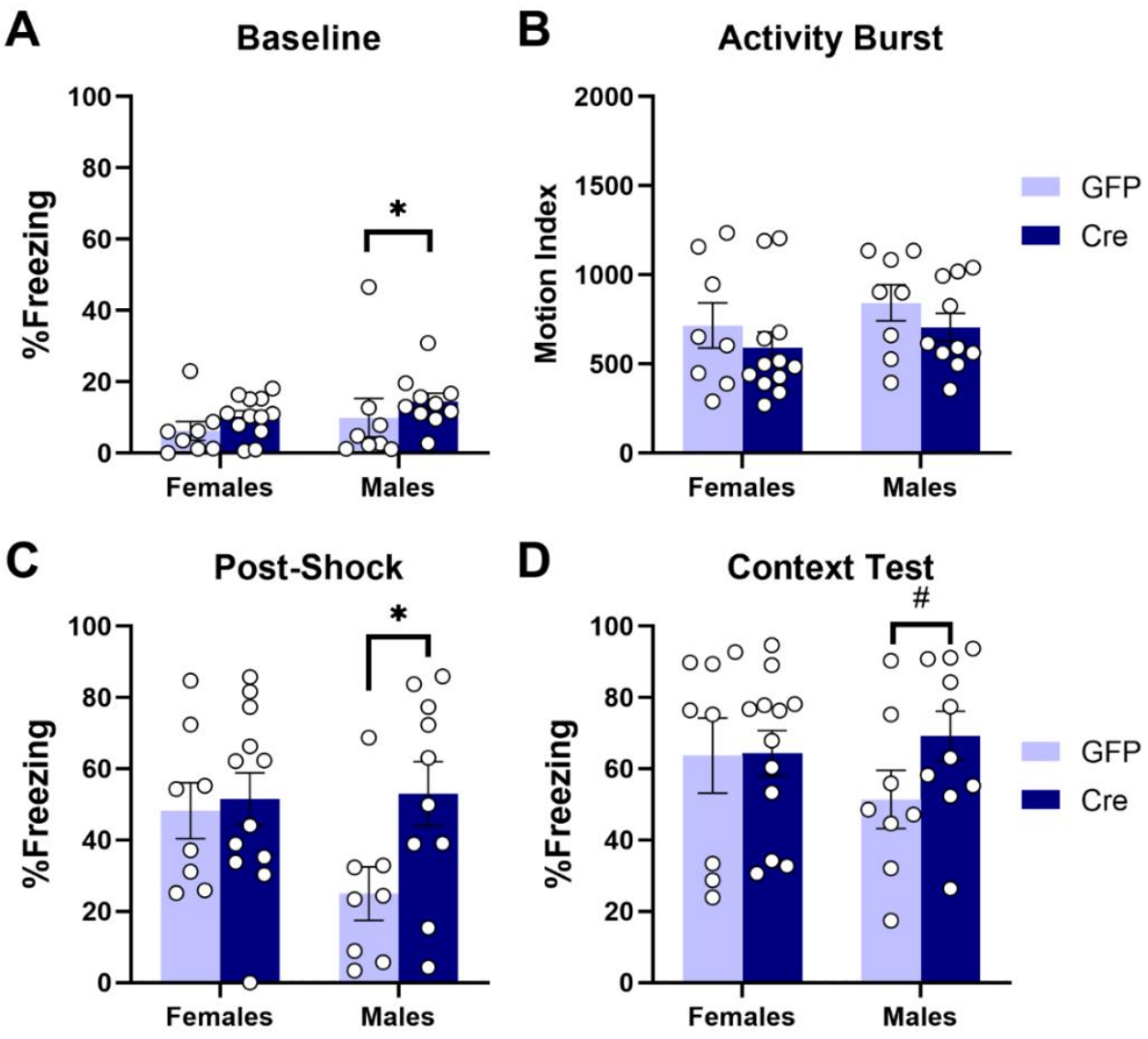


Figure 3-6. Experiment 3 Context B fear conditioning results (Days 3-4). **A.** Increased baseline fear prior to footshock in Cre males compared to GFP males (Day 3). **B.** No differences in shock reactivity (Day 3). **C.** Cre males showed increased freezing immediately following footshock delivery (Day 3). **D.** Cre males showed marginally greater freezing when returned to Context B the following day (Day 4). #p<0.1, *p<0.05. Error bars represent standard error of the mean.

Open field test

As the SEFL procedure has been shown to increase anxiety-related behavior, we next investigated whether GluA2 deletion impacted performance on the open field test. Cre males

showed marginally decreased locomotion compared to GFP males (U=61.5, n_1=10, n_2=8, p=0.06; Fig 3-7A) while no differences were observed in females (U=51, n_1=12, n_2=8, p=0.85). No differences in time spent in the center of the open field were observed in either males (U=33, n_1=10, n_2=8, p=0.57; Fig 3-7B) or females (U=39, n_1=12, n_2=8, p=0.52).

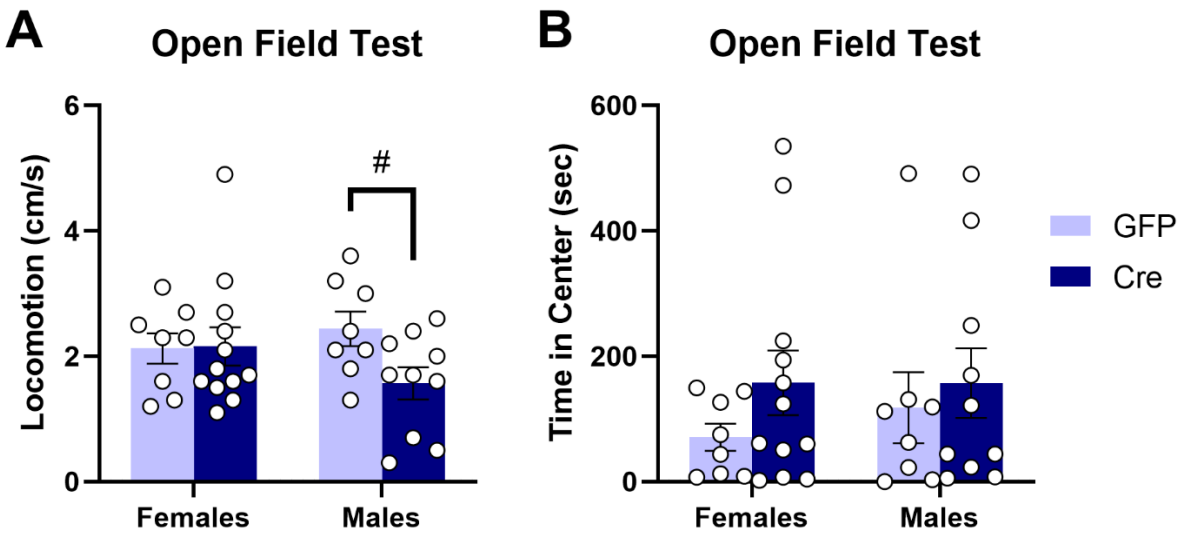


Figure 3-7. Experiment 3 open field test results (Day 5). **A.** Cre males show marginally decreased exploratory behavior compared to GFP males. **B.** No differences in time spent in the center of the open field. #p<0.1. Error bars represent standard error of the mean.

Histology

Following behavioral testing, GluA2 deletion was verified using immunohistochemistry and accurate viral placement was verified using GFP expression. 20 animals were excluded due to inaccurate virus placement or poor viral expression. Decreased expression of GluA2 within the BLA was verified by comparing the number of GluA2-positive neurons to the total number of DAPI-labeled cells within a subset of subjects (Fig 3-8A). Cre subjects showed a decreased

ratio of GluA2-positive to DAPI-labeled cells relative to GFP controls, indicating that the Cre virus successfully decreased GluA2 expression in ($U=25.5$, $n_1=12$, $n_2=13$, $p=0.003$). The spread of GFP expression is illustrated in Fig 3-8B (coronal section diagrams based on Paxinos and Franklin mouse brain atlas). Representative images from GFP and Cre mice are shown in Figs 3-8C-J.

The results of Experiment 3 corroborate and extend the conclusions of Experiment 1. Promoting the formation of CP-AMPARs in the BLA through viral mediated GluA2 deletion was insufficient to enhance fear learning in the absence of any prior stress exposure. However, when exposed to an additional footshock in a novel context, GluA2 deletion produced elevated fear learning and increased anxiety on the open field test in males, but not females. These results indicate that GluA2 deletion paired with a relatively mild stressor may be sufficient to enhance fear learning, but this effect is limited to males.

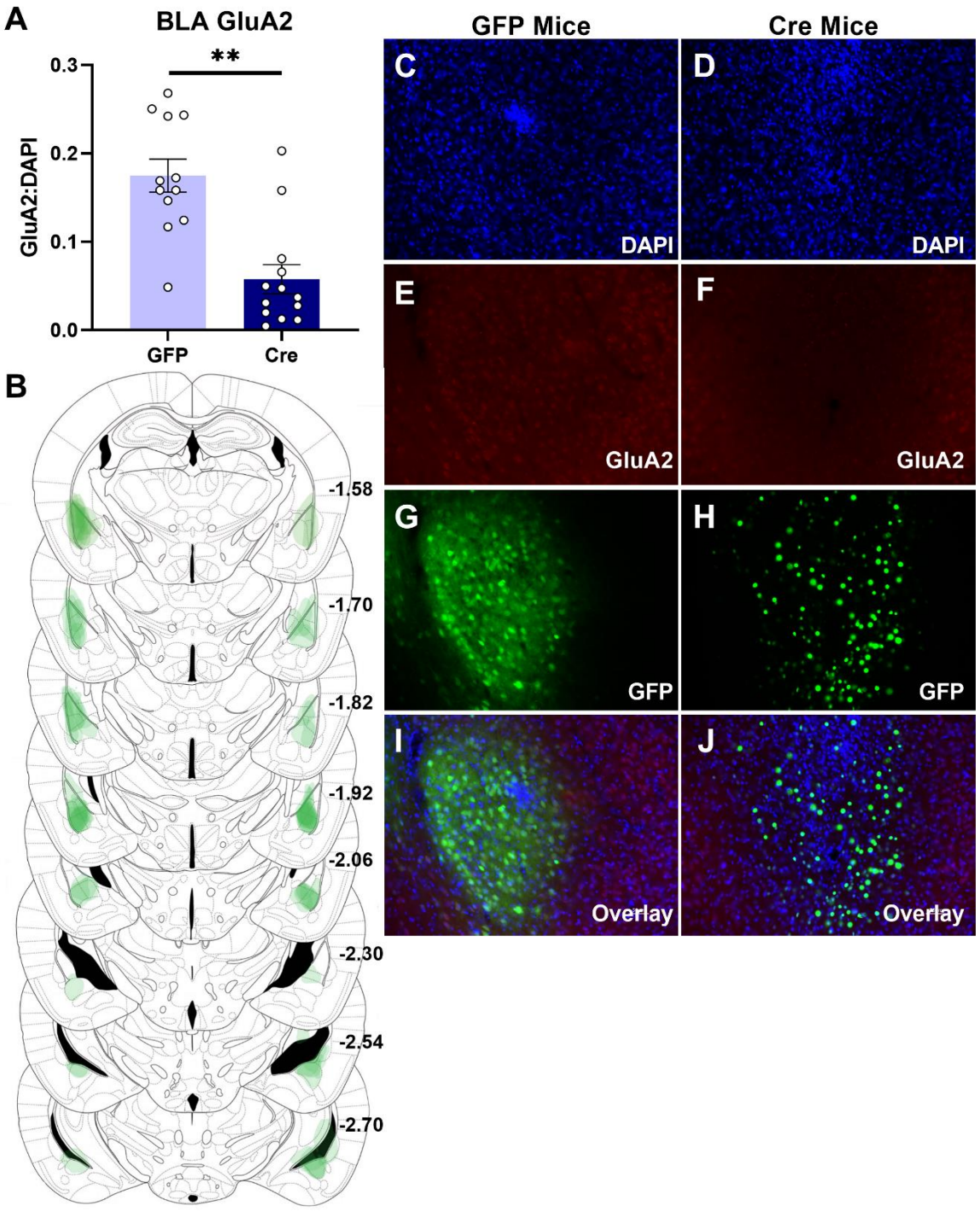


Figure 3-8. Experiment 3 histology results. **A.** Ratio of GluA2-positive cells to DAPI-positive cells within the BLA. **B.** Spread of GFP expression in coronal sections. Each layer represents maximal GFP expression from an individual animal. Distance from bregma (mm) is shown. **C-J.** Representative images of DAPI-labeled cells (C-D), GluA2 staining (E-F), GFP expression (G-H) and overlay (I-J). Images taken at 20x magnification. **p<0.01. Error bars represent standard error of the mean.

Discussion

Sensitization of fear learning is a defining feature of PTSD (Bremner et al., 1995; Dykman et al., 1997), but the neural changes following stress that support this enhancement of fear learning remain unclear. In previous research, we showed that stress exposure causes a long-lasting increase in GluA1 protein within the BLA, and that inhibition of corticosterone synthesis prevents both this elevation in GluA1 and the enhancement in fear learning (Perusini et al., 2016). Given that increased GluA1 levels can lead to formation of CP-AMPARs that support an NMDAR-independent form of LTP (Hollmann et al., 1991; Wiltgen et al., 2010), we hypothesized that SEFL is supported by increased formation of CP-AMPARs within the BLA.

In the experiments reported here we investigated whether increasing the ratio of GluA1 to GluA2 subunits within the BLA is sufficient to enhance fear learning in the absence of stress. We first tested whether GluA1 overexpression enhances fear learning in the absence of stress. In Experiment 1 we infused an GluA1-expressing HSV into the BLA of C57Bl/6 mice. Contrary to our expectations, we found GluA1 overexpression did not appear to produce any changes in fear learning to a single footshock. However, owing to the short-acting nature of HSVs we had little opportunity to explore any additional behavioral effects of this manipulation, such as whether GluA1 overexpression would enhance susceptibility to a subsequent stressor. We therefore explored more long-lasting alternatives methods in the subsequent experiments.

Experiments 2 and 3 utilized floxed GluA2 mice which allowed for permanent targeted deletion of the GluA2 subunit through the infusion of a Cre-expressing virus. This approach allowed us to promote the formation of CP-AMPARs by limiting the availability of GluA2 subunits within the BLA. The floxed GluA2 mice were generated on an 129S6 background, in

contrast to our previous SEFL studies that used C57Bl/6 mice (Pennington et al., 2020; Rajbhandari et al., 2018). Given the fact that substantial differences in fear learning between mouse strains have been reported (Balogh & Wehner, 2003; Smith et al., 2007; Stiedl et al., 1999), and that 129S6 mice typically show higher freezing than C57Bl/6 mice (Smith et al., 2007), we first verified that 129S6 mice would show enhanced fear learning following stress.

Experiment 2 demonstrated that these floxed GluA2 mice showed enhanced fear learning following stress. While the overall freezing levels in the floxed GluA2 mice were elevated compared to previous reports in C57Bl/6 mice (Pennington et al., 2020; Rajbhandari et al., 2018), a strong SEFL effect was observed for both males and females, with no differences between males and females. These mice also displayed several other behaviors typically observed during SEFL, including high levels of fear to the stress context, decreased reactivity to the single footshock and decreased exploratory behavior during the open field test.

In Experiment 3, we investigated whether GluA2 deletion within the BLA would enhance fear learning in the absence of stress. During the first fear conditioning event in Context A, there was no enhancement in freezing either during the 30 seconds immediately following the footshock or during the context test the following day. These results, combined with the lack of a fear learning enhancement following GluA1 overexpression in Experiment 1, indicated that increasing the ratio of GluA1 to GluA2 alone is insufficient to enhance fear learning in the absence of additional stress.

Following exposure to a single footshock in Context A, we exposed subjects to a single footshock to a novel context (Context B). While no differences had been observed in Context A, GluA2 deletion appeared to impact fear to Context B in a sex-specific manner. In male subjects a

small but significant difference in baseline fear suggested that while no differences had been observed during the Context A test, GluA2 deletion had slightly enhanced fear generalization. Increased freezing was also observed both immediately following footshock delivery and during the context test the following day. Unlike the effects observed in males, no differences in freezing were observed in females at any time points. Similarly, GluA2 deletion was associated with reduced locomotion during the open field test in males but not females. While no differences were observed in time spent in the center of the open field, this corresponds with the results of Experiment 2 and other work from our laboratory indicating that SEFL reduces exploratory behavior but does not alter time spent in the center of the arena (Perusini et al., 2016). Collectively, these results indicate that GluA2 deletion enhances fear learning and anxiety in males that have a history of prior footshock exposure, but not females.

These sex-specific effects of GluA2 deletion are noteworthy since males and females showed similar performance during the SEFL procedure in Experiment 2. These results suggest that while males and females show comparable behavioral changes following stress, these changes may occur through different mechanisms. Interestingly, previous studies have indicated that stress differentially impacts GluA1 and GluA2 levels in males and females. Early life stress combined with cocaine exposure has been shown to increase the ratio of GluA1 to GluA2 in the NAc in males but not in females (Castro-Zavala et al., 2020), while early life stress decreases BLA GluA2 levels in males but not females (Guadagno et al., 2020). There may also be sex differences in the role of GluA1 in fear conditioning, as contextual fear conditioning is impaired in GluA1-knockout male mice, but not in GluA1-knockout female mice (Dachtler et al., 2011).

The fact that GluA2 deletion impacts performance on a second fear conditioning event without impacting the first fear conditioning were unexpected but still support a role of CP-

AMPARs in supporting enhanced fear learning following stress. Stress involves a host of physiological changes throughout the central nervous system, and the BLA is sensitive to the effects of multiple endocrine signals released during stress including glucocorticoids, norepinephrine and corticotropin-releasing factor (Roozendaal et al., 2009). It is therefore plausible that a single manipulation is unlikely to reproduce this host of effects. For example, we have previously observed that while corticosterone synthesis is necessary for SEFL, systemic corticosterone injections alone are insufficient to enhance fear learning in the absence of stress (Perusini et al., 2016).

Two-hit and multiple-hit accounts of stress susceptibility posit that stress-related disorders do not necessarily arise from a single experience. Instead, they may develop due the combined effects of factors including genetic predisposition and subsequent stress experiences (Daskalakis et al., 2013; Imanaka et al., 2006; Lesse et al., 2017; Maynard et al., 2001). We have previously shown that exposure to a single footshock does not enhance fear learning to a subsequent footshock in a novel context (Rau & Fanselow, 2009). It is therefore conceivable that the effects we observed in Experiment 3 reflected an increase in stress susceptibility caused by increased levels of CP-AMPARs combined with exposure to a mild, sub-threshold stressor (the single footshock in Context A).

There were several limitations that must be acknowledged. First, we assumed that increasing the availability of GluA1 subunits relative to GluA2 subunits would result in the production of CP-AMPARs. However, we did not verify that this was in fact the case. It would be worth taking advantage of the fact that CP-AMPARs produce a distinct electrophysiological signature to verify that GluA1 overexpression and GluA2 deletion do indeed produce increases

in CP-AMPAR-mediated current (Wiltgen et al., 2010). While we observed no effects of GluA2 deletion in females, both GFP and Cre-treated females showed relatively high freezing levels in Context B suggesting that perhaps differences were present but obscured by a ceiling effect. While Rau et al (2009) found that a single footshock is insufficient to enhance subsequent fear learning in rats, this may not be the case for this particular mouse strain as all groups appeared to show greater fear during the Context B test compared to the Context A test. In future studies it will be worthwhile to titrate the intensity of each stressor to ensure that any enhancements can be clearly observed in both males and females. Lastly, the effects of GluA2 deletion on freezing during the Context B test and locomotion during the open field test approached but did not quite reach statistical significance, likely due to the variability of behavior and the relatively small sample size. In future studies it will be important to replicate these effects in a larger cohort of subjects.

Conclusion

In these experiments we investigated whether promoting the formation of CP-AMPARs in the BLA through manipulation of GluA1 and GluA2 subunit availability would be sufficient to enhance fear learning in the absence of stress. We found that neither GluA1 overexpression nor GluA2 deletion alone was sufficient to enhance fear learning to a single footshock (immediate effect). However, GluA2 deletion combined with a history of sub-threshold stress exposure appeared to enhance fear learning to a subsequent footshock in males but not females. These findings indicate that males and females may show similar behavioral changes following stress exposure that are supported by different biological mechanisms.

References

Balogh, S. A., & Wehner, J. M. (2003). Inbred mouse strain differences in the establishment of long-term fear memory. *Behav Brain Res*, *140*(1-2), 97-106. https://doi.org/10.1016/s0166-4328(02)00279-6

Bremner, J. D., Krystal, J. H., Southwick, S. M., & Charney, D. S. (1995). Functional neuroanatomical correlates of the effects of stress on memory. *J Trauma Stress*, *8*(4), 527-553.

Castro-Zavala, A., Martín-Sánchez, A., & Valverde, O. (2020). Sex differences in the vulnerability to cocaine's addictive effects after early-life stress in mice. *Eur Neuropsychopharmacol*, *32*, 12-24. https://doi.org/10.1016/j.euroneuro.2019.12.112

Dachtler, J., Fox, K. D., & Good, M. A. (2011). Gender specific requirement of GluR1 receptors in contextual conditioning but not spatial learning. *Neurobiol Learn Mem*, *96*(3), 461-467. https://doi.org/10.1016/j.nlm.2011.07.001

Daskalakis, N. P., Bagot, R. C., Parker, K. J., Vinkers, C. H., & de Kloet, E. R. (2013). The three-hit concept of vulnerability and resilience: toward understanding adaptation to early-life adversity outcome. *Psychoneuroendocrinology*, *38*(9), 1858-1873. https://doi.org/10.1016/j.psyneuen.2013.06.008

Dykman, R. A., Ackerman, P. T., & Newton, J. E. (1997). Posttraumatic stress disorder: a sensitization reaction. *Integr Physiol Behav Sci*, *32*(1), 9-18.

Eberly, R. E., & Engdahl, B. E. (1991). Prevalence of somatic and psychiatric disorders among former prisoners of war. *Hosp Community Psychiatry*, *42*(8), 807-813.

Fanselow, M. S., & Bolles, R. C. (1979). Naloxone and shock-elicited freezing in the rat. *J Comp Physiol Psychol*, *93*(4), 736-744.

Fanselow, M. S., & Kim, J. J. (1994). Acquisition of contextual Pavlovian fear conditioning is blocked by application of an NMDA receptor antagonist D,L-2-amino-5-phosphonovaleric acid to the basolateral amygdala. *Behav Neurosci*, *108*(1), 210-212.

Fendt, A., & Fanselow, M. S. (1999). The neuroanatomical and neurochemical basis of conditioned fear. *Neuroscience & Biobehavioral Reviews*, *23*(5), 734-760.

Guadagno, A., Verlezza, S., Long, H., Wong, T. P., & Walker, C. D. (2020). It Is All in the Right Amygdala: Increased Synaptic Plasticity and Perineuronal Nets in Male, But Not Female, Juvenile Rat Pups after Exposure to Early-Life Stress. *J Neurosci*, *40*(43), 8276-8291. https://doi.org/10.1523/JNEUROSCI.1029-20.2020

Hollmann, M., Hartley, M., & Heinemann, S. (1991). Ca2+ permeability of KA-AMPA--gated glutamate receptor channels depends on subunit composition. *Science*, *252*(5007), 851-853.

Imanaka, A., Morinobu, S., Toki, S., & Yamawaki, S. (2006). Importance of early environment in the development of post-traumatic stress disorder-like behaviors. *Behav Brain Res*, *173*(1), 129-137. https://doi.org/10.1016/j.bbr.2006.06.012

Kim, J. J., Fanselow, M. S., DeCola, J. P., & Landeira-Fernandez, J. (1992). Selective impairment of long-term but not short-term conditional fear by the N-methyl-D-aspartate antagonist APV. *Behavioral neuroscience*, *106*(4), 1.

Kim, J. J., & Jung, M. W. (2006). Neural circuits and mechanisms involved in Pavlovian fear conditioning: a critical review. *Neurosci Biobehav Rev*, *30*(2), 188-202. https://doi.org/10.1016/j.neubiorev.2005.06.005

Lee, H. K., Takamiya, K., Han, J. S., Man, H., Kim, C. H., Rumbaugh, G., Yu, S., Ding, L., He, C., Petralia, R. S., Wenthold, R. J., Gallagher, M., & Huganir, R. L. (2003). Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory. *Cell*, *112*(5), 631-643. https://doi.org/10.1016/s0092-8674(03)00122-3

Lesse, A., Rether, K., Gröger, N., Braun, K., & Bock, J. (2017). Chronic Postnatal Stress Induces Depressive-like Behavior in Male Mice and Programs second-Hit Stress-Induced Gene Expression Patterns of OxtR and AvpR1a in Adulthood. *Mol Neurobiol*, *54*(6), 4813-4819. https://doi.org/10.1007/s12035-016-0043-8

Long, V. A., & Fanselow, M. S. (2012). Stress-enhanced fear learning in rats is resistant to the effects of immediate massed extinction. *Stress*, *15*(6), 627-636. https://doi.org/10.3109/10253890.2011.650251

Malinow, R. (2003). AMPA receptor trafficking and long-term potentiation. *Philos Trans R Soc Lond B Biol Sci*, *358*(1432), 707-714. https://doi.org/10.1098/rstb.2002.1233

Maren, S. (1999). Long-term potentiation in the amygdala: a mechanism for emotional learning and memory. *Trends Neurosci*, *22*(12), 561-567.

Maynard, T. M., Sikich, L., Lieberman, J. A., & LaMantia, A. S. (2001). Neural development, cell-cell signaling, and the "two-hit" hypothesis of schizophrenia. *Schizophr Bull*, *27*(3), 457-476. https://doi.org/10.1093/oxfordjournals.schbul.a006887

Paxinos, G., & Franklin, K. B. (2019). *Paxinos and Franklin's the mouse brain in stereotaxic coordinates.* Academic press.

Pennington, Z. T., Trott, J. M., Rajbhandari, A. K., Li, K., Walwyn, W. M., Evans, C. J., & Fanselow, M. S. (2020). Chronic opioid pretreatment potentiates the sensitization of fear

learning by trauma. *Neuropsychopharmacology*, *45*(3), 482-490. https://doi.org/10.1038/s41386-019-0559-5

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*(1), 45-57. https://doi.org/10.1038/npp.2015.224

Plant, K., Pelkey, K. A., Bortolotto, Z. A., Morita, D., Terashima, A., McBain, C. J., Collingridge, G. L., & Isaac, J. T. (2006). Transient incorporation of native GluR2-lacking AMPA receptors during hippocampal long-term potentiation. *Nat Neurosci*, *9*(5), 602-604. https://doi.org/10.1038/nn1678

Poulos, A. M., Reger, M., Mehta, N., Zhuravka, I., Sterlace, S. S., Gannam, C., Hovda, D. A., Giza, C. C., & Fanselow, M. S. (2014). Amnesia for early life stress does not preclude the adult development of posttraumatic stress disorder symptoms in rats. *Biol Psychiatry*, *76*(4), 306-314. https://doi.org/10.1016/j.biopsych.2013.10.007

Rajbhandari, A. K., Gonzalez, S. T., & Fanselow, M. S. (2018). Stress-Enhanced Fear Learning, a Robust Rodent Model of Post-Traumatic Stress Disorder. *J Vis Exp*(140). 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. *Neurosci Biobehav Rev*, *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

Rogan, M. T., Stäubli, U. V., & LeDoux, J. E. (1997). Fear conditioning induces associative long-term potentiation in the amygdala. *Nature*, *390*(6660), 604-607. https://doi.org/10.1038/37601

Roozendaal, B., McEwen, B. S., & Chattarji, S. (2009). Stress, memory and the amygdala. *Nat Rev Neurosci*, *10*(6), 423-433. https://doi.org/10.1038/nrn2651

Smith, D. R., Gallagher, M., & Stanton, M. E. (2007). Genetic background differences and nonassociative effects in mouse trace fear conditioning. *Learn Mem*, *14*(9), 597-605. https://doi.org/10.1101/lm.614807

Stiedl, O., Radulovic, J., Lohmann, R., Birkenfeld, K., Palve, M., Kammermeier, J., Sananbenesi, F., & Spiess, J. (1999). Strain and substrain differences in context- and tone-dependent fear conditioning of inbred mice. *Behav Brain Res*, *104*(1-2), 1-12. https://doi.org/10.1016/s0166-4328(99)00047-9

Walsh, R. N., & Cummins, R. A. (1976). The open-field test: a critical review. *Psychological bulletin*, *83*(3), 482.

Wenthold, R. J., Petralia, R. S., Blahos J, I. I., & Niedzielski, A. S. (1996). Evidence for multiple AMPA receptor complexes in hippocampal CA1/CA2 neurons. *J Neurosci*, *16*(6), 1982-1989.

Wiltgen, B. J., Royle, G. A., Gray, E. E., Abdipranoto, A., Thangthaeng, N., Jacobs, N., Saab, F., Tonegawa, S., Heinemann, S. F., O'Dell, T. J., Fanselow, M. S., & Vissel, B. (2010). A role for calcium-permeable AMPA receptors in synaptic plasticity and learning. *PLoS One*, *5*(9). https://doi.org/10.1371/journal.pone.0012818

DISCUSSION

Improper regulation of fear is a key feature of post-traumatic stress disorder (PTSD) (Bremner et al., 1995; Dykman et al., 1997), and the increased arousal and avoidance of trauma-related stimuli that accompany PTSD can severely impact quality of life (Eberly & Engdahl, 1991). PTSD is also highly comorbid with substance use disorders (SUDs), with alcohol abuse being the most common (Kessler et al., 1997; Kessler et al., 1995; Mills et al., 2006; Perkonigg et al., 2000). However, despite the burden of these disorders the mechanisms through which trauma alters fear learning remain elusive. Similarly, only a small percentage of people who experience trauma go on to develop PTSD but the factors that mediate stress susceptibility are unclear (Brunello et al., 2001; Kessler et al., 2017; Kessler et al., 1995). In this dissertation I developed a novel model of stress exposure to study the factors that promote stress susceptibility and resilience, and found that while males and females show similar changes in fear and anxiety-related behaviors following stress exposure, these behavioral changes may be mediated by different biological mechanisms.

Sex differences in stress reactivity

One critical question is why PTSD and SUDs occur at such different rates in men and women. Although men are more likely than women to experience trauma, PTSD is twice as common in women than in men even when controlling for the type of trauma experienced (Breslau et al., 1997; Kessler et al., 2017; Kessler et al., 1995). Despite this fact, the vast majority of rodent studies investigating the effects of stress have been conducted exclusively in males. The first goal of this dissertation was to explore how males and females differ in stress reactivity and determine whether the increased prevalence of PTSD in women is captured by a

rodent model of stress exposure. I found that despite the increased prevalence of PTSD among women compared to men, male and female rodents generally showed comparable changes in fear and anxiety-related behavior following stress exposure.

Sex differences in fear learning following stress

Our laboratory developed a rodent model of stress exposure termed stress-enhanced fear learning (SEFL) that captures the exaggerated fear learning observed in PTSD patients (Perusini et al., 2016; Poulos et al., 2015; Rau et al., 2005; Rau & Fanselow, 2009). The key feature of the SEFL model is that following exposure to an intense acute stressor (15 unsignalled footshocks over 90 minutes for rats, 10 footshocks over 60 minutes for mice), subjects show sensitized fear learning to a single footshock presented in a novel context. This procedure also produces a number of additional changes relevant to PTSD symptomology including increased anxiety and alcohol drinking (Meyer et al., 2013; Perusini et al., 2016). However, as much of our work using this model has been conducted in males it was unknown whether females would show the same behavioral changes following stress.

We first examined whether males and females differed in fear learning following stress exposure. Because women are more likely to develop PTSD and because women with PTSD show greater changes in fear learning under laboratory conditions (Gamwell et al., 2015; Inslicht et al., 2013), we hypothesized that females would show greater enhancements in fear learning compared to males, as well as increased fear generalization and impaired fear extinction. However, the results did not support this hypothesis. The magnitude of the SEFL effect did not differ in males and female rats following the standard 15-footshock stress (Figs 1-2 and 1-8), nor were differences in fear generalization or extinction observed between males and females (Figs

1-2 and 1-8). Similarly, no sex differences in fear learning were observed among floxed GluA2 mice following the standard 10-footshock stress (Fig 3-3).

While these effects appear to be at odds with epidemiological data from the human population, they are in agreement with a small but growing body of literature examining sex differences in rodent models of stress exposure. In the absence of stress exposure, female rodents tend to show comparable (Graham et al., 2009; Markus & Zecevic, 1997) or reduced (Pryce et al., 1999) fear learning to discrete tone CSs, while freezing during tests of contextual fear learning is often slightly lower in females (Daviu et al., 2014; Maren et al., 1994; Markus & Zecevic, 1997; Poulos et al., 2015). Similarly, stress exposure has been shown to either equally enhance fear learning in males and females (Poulos et al., 2015), or enhance fear learning and impair fear extinction in males but not females (Baran et al., 2009; Keller et al., 2015; Wood & Shors, 1998).

Sex differences in anxiety-related behavior following stress

While no sex differences in fear learning were observed, we did find evidence that males and females differed in anxiety-related behavior. Female rats showed decreased anxiety both prior to stress exposure as indicated by increased exploration of the light compartment of the light-dark transition test (Fig 1-1) and following stress exposure as indicated by increased locomotion during the open field test (Figs 1-3 and 1-9). While stress reduced general exploratory behavior during the open field test, the magnitude of this effect did not differ between males and females (Figs 1-3 and 1-9). These results are in agreement with other observations of reduced anxiety-like behaviors in female rodents (Johnston & File, 1991; Scholl et al., 2019; Zimmerberg & Farley, 1993). Interestingly, no sex differences were observed in the

performance of the floxed GluA2 mice on the open field test following stress (Fig 3-4), but this may be due to the fact that these mice were generated on an 129S6 background, which tend to show heightened fear and anxiety compared to other mouse strains (Curley et al., 2012; Smith et al., 2007; Stiedl et al., 1999).

Stress exposure did differentially impact the response of males and females to an anxiogenic light stimulus. While both stressed and unstressed females showed avoidance of the light, this response appeared to be blunted in stressed males who showed no indications of avoiding the light stimulus (Fig 1-3). This effect appeared to be driven by males possessing an exaggerated preference for particular locations within the arena. Among the subjects that initially preferred Zone 1 (closest to the light), the unstressed males and both groups of females moved away from this location following light onset, spending increased time in Zone 4 (furthest from the lamps). Stressed males, however, spent significantly less time in Zone 4 compared to the other groups during and following light onset (Figs 1-3 and 1-13).

One potential interpretation of this effect is that it reflects an impairment in cognitive flexibility. Stress has been shown to impair higher-order cognitive functions that allow animals to adapt to changes environmental demands, and such impairments are associated with many types of psychopathology (Holmes & Wellman, 2009). For example, stress exposure has been shown to impair performance on reversal learning and attentional set-shifting tasks, in which subjects must alter their behavior when reward-related contingencies are reversed (Bondi et al., 2008; Hill et al., 2005; Szuran et al., 2000). It is conceivable that a similar phenomenon was observed during the open field test, in which stressed males showed an impaired ability to adapt to Zone 1 initially being the preferred location, then becoming aversive following light onset.

Interestingly, a recent study demonstrated that acute stress impairs performance on the Wisconsin Card Sorting Tasks, an assessment of cognitive flexibility, in men but not women (Shields et al., 2016)

This resilience of females against this particular effect of stress, and the generally decreased anxiety observed in females, may seem counterintuitive given the increased prevalence of PTSD and anxiety disorders among women (Brunello et al., 2001; Kessler et al., 2017; Kessler et al., 2005; Kessler et al., 1995). However, as in the realm of fear conditioning there is substantial evidence that female rodents generally show more activity and less anxiety-like behavior on classic tests such as the open field and elevated plus maze (Beck & Luine, 2002; Bowman et al., 2009; Johnston & File, 1991). It has been proposed that since many of these classic tests were developed and validated exclusively in males, these tests do not accurately assess anxiety related behavior in females and are instead more reflective of general activity (Fernandes et al., 1999). However, results from these current studies and others demonstrating increased anxiety-related behaviors using these tasks following stress exposure in both males and females do not support such an explanation (Luine et al., 2017).

An alternative explanation is the role of gonadal hormones. Estradiol, an endogenous estrogen, is synthesized in the ovaries and plays an important role in mediating stress and anxiety-related behavior (Heck & Handa, 2019). Estradiol has been shown to have anxiolytic effects on the open field test and elevated plus maze (Filova et al., 2015; Walf et al., 2008; Walf & Frye, 2007, 2010). Furthermore, female rats have shown reduced anxiety-like behavior during the proestrus phases of the estrous cycle, when estradiol levels are high, compared to during the diestrus phase, when estradiol levels are low (Marcondes et al., 2001; Mora et al., 1996; Walf &

Frye, 2007). It has therefore been proposed that the high circulating estradiol levels in female rodents reduce anxiety and support stress resilience (Luine et al., 2017). This will be discussed in greater detail below.

Sex differences in alcohol drinking following stress

An important challenge in treating PTSD is the high rates of comorbidity between PTSD and SUDs, of which alcohol abuse is the most common (Kessler et al., 1997; Kessler et al., 1995; Mills et al., 2006; Perkonigg et al., 2000). Our laboratory has previously demonstrated that the SEFL procedure enhances voluntary alcohol consumption in male rats (Meyer et al., 2013), but it was unknown whether this procedure would produce a similar effect in females. Initially, while we found generally elevated alcohol drinking in females compared to males, we saw no effects of stress on alcohol drinking in either males or females when subjects had received continuous-alcohol 2-bottle choice (CA 2-BC) access prior to stress (Fig 1-5). One potential explanation for this finding was that the alcohol exposure prior to stress had impaired the ability of stress exposure to produce any subsequent changes. Meyer et al (2013) demonstrated that intermittent-access 2-bottle choice (IA 2-BC) prior to stress exposure prevents any further effects of stress on alcohol intake. We had anticipated that CA 2-BC was unlikely to have the same blunting effect, as this procedure results in relatively low drinking levels while IA 2-BC produces greater, escalating amounts of drinking (Brancato et al., 2016; Simms et al., 2008; Wise, 1973). When we tested this possibility, we found no effects of stress in subjects that received identical treatment except for receiving no alcohol prior to stress (Fig 1-10).

These results implied that a different aspect of the testing procedure was impairing the ability of stress to alter subsequent alcohol drinking. The second major methodological

difference between the procedures used here and that of Meyer et al (2013) was the extensive battery of behavioral tests employed between stress and alcohol exposure. One possibility was that the additional testing across multiple contexts and apparatuses acted as a form of environmental enrichment, which has been shown to attenuate the effects of stress (Francis et al., 2002; Solinas et al., 2008). To address this possibility, we compared alcohol drinking in stressed and unstressed males that had received either a battery of behavioral tests or remained in their homecage. The results, while inconclusive, suggested that the additional testing may instead have functioned as an additional source of stress as subjects that received both stress and testing showed slightly elevated alcohol intake compared to the other groups (Fig 1-16).

One conclusion that can be drawn from this pattern of results is that the relationship between stress exposure and alcohol drinking in rodents is complex and heavily influenced by the surrounding environmental factors. Indeed, the results of studies investigating the effects of stress during adulthood on alcohol drinking have generally been mixed. While our laboratory and others have found increased alcohol intake following footshock stress exposure (Anisman & Waller, 1974; Füllgrabe et al., 2007; Kinney & Schmidt, 1979; Meyer et al., 2013; Vengeliene et al., 2003) other studies report either no change (Chester et al., 2008; Choca et al., 1977; Fidler & LoLordo, 1996) or decreased alcohol drinking (Champagne & Kirouac, 1987; Darnaudéry et al., 2007) following stress. It has been suggested that this variability may be due to an array of factors, such as variations in testing procedure including the nature and intensity of the stressor, the predictability of footshock or the time between stress and alcohol access, as well as the animals' perceptions of the stressor and its ability to mount an effective coping mechanism (Becker et al., 2011). More work is therefore needed to elucidate the factors that govern the influence of stress on alcohol drinking.

The SEFL model as a tool for studying stress susceptibility

The second main goal of this dissertation was to demonstrate that the SEFL model could be used as a tool for studying stress susceptibility. While approximately one third of the population will experience trauma at some point during their lifetime, only 10-20% of these individuals will go on to develop PTSD and the factors that promote stress susceptibility are poorly understood (Brunello et al., 2001; Kessler et al., 2017; Kessler et al., 1995). Animal models that capture some of the behavioral symptoms of PTSD offer a powerful tool to study the mechanisms of this disorder, yet there has been little investigation of the individual differences that may promote stress susceptibility versus resilience within these models (Richter-Levin et al., 2019; Shansky, 2015). Furthermore, much of the work that has explored stress susceptibility and resilience has been limited to males. For example, the social defeat stress model is one of the most commonly used for studying stress susceptibility versus resilience, yet because females do not show the same type of aggression this procedure can only be used in males (Berton et al., 2006; Golden et al., 2011).

As the SEFL model has been shown to reliably enhance fear learning in both male and female rodents, in Chapter 2 I explored whether the SEFL model could be used to investigate stress susceptibility and resilience. I demonstrated that a reduced-intensity version of the SEFL procedure (4 footshocks instead of the standard 10-15) allowed the emergence of distinct susceptible and resilient populations that differed on a constellation of behaviors relevant to PTSD symptomology (Fig 2-1).

Establishing a susceptibility criterion

The first challenge was to establish a criterion for classifying subjects as susceptible or resilient. Many studies investigating stress susceptibility rely on post-hoc methods such as quantifying the behavioral outcome of interest in the data set under study (e.g. increased freezing or reduced social interaction) and defining the 20-30% of subjects at one extreme as susceptible and the 20-30% at the other extreme as resilient (Colucci et al., 2020; Covington et al., 2010; Jeong et al., 2020; Ritov et al., 2016). These methods of classification are limited as they are based just on the current sample and similar procedures are rarely applied to unstressed controls. In Chapter 2 we improved upon these methods by establishing an *a priori* criterion based on prior sampling of a large population of subjects showing a normal fear response.

We were able to take advantage of our many studies utilizing the SEFL procedure to compile SEFL test performance from a large number of unstressed control subjects. Subjects in the present experiments that showed freezing that was more than two standard deviations above the means of these unstressed controls during the SEFL test (58%) were classified as "Susceptible" while all other subjects were classified as "Resilient". A pronounced bimodal distribution was observed among subjects exposed to the 4-footshock stress, with 43% of subjects exceeding our classification criterion (Fig 2-1). These results confirmed that this modified version of the SEFL procedure is effective at revealing distinct susceptible and resilient groups and serves as a valuable tool for studying the biological and behavioral differences between these groups. While we had hypothesized that females would be more likely to be classified as Susceptible compared to males, we found no sex differences in the proportion of subjects classified as Susceptible (Fig 2-1; Fig 2-7). While these results are contrary to the increased rates of PTSD observed in women compared to men, they reinforce the findings of Chapter 1 that male and female rats generally do not differ in fear learning.

Susceptible subjects showed changes in fear learning, generalization and extinction

While Susceptible subjects were classified based on their performance during the SEFL test, they also differed on other aspects of fear learning. In addition to showing enhanced fear learning to a novel aversive stimulus (Fig 2-6), Susceptible subjects showed increased fear generalization and impaired fear extinction compared to Resilient subjects (Fig 2-2). These results are of significance given that the inability to suppress fear and the overgeneralization of fear from the original trauma to safe situations play a key role in PTSD symptomology and weaken the effectiveness of treatments like exposure therapy (Craske et al., 2008; Hembree et al., 2003; Kaczkurkin et al., 2017; Milad, Pitman, et al., 2009). Given that these different aspects of fear learning are all assessed via freezing, it is possible that they are each tapping into some more holistic aspect of each individual animal, i.e. high-freezing animals versus low-freezing animals. However, the consistency of behavior across these different tasks is noteworthy, and it is conceivable that multiple aspects of PTSD may also manifest from a common source.

Anxiety-related behaviors are influenced by stress susceptibility and sex

Susceptible subjects also showed changes in anxiety-related behavior which followed the patterns of effects caused by stress exposure observed in Chapter 1. Susceptible groups showed increased anxiety following stress as indicated by reduced exploratory behavior during the open field test and light-dark transition test, although the effects of stress susceptibility were not different in males and females (Fig 2-3). However, stress susceptibility did manifest differently in males and females in their response to the appearance of an anxiogenic light stimulus during the open field test. Mirroring the pattern of results observed in Chapter 1, Resilient males and

both groups of females showed avoidance of Zone 1 following light onset but this response was blunted in Susceptible males (Fig 2-3).

Stress susceptibility associated with increased alcohol drinking in males but not females

Stress susceptibility differentially impacted alcohol drinking in males and females. Among subjects that had not received alcohol prior to stress, Susceptible males showed elevated alcohol drinking following stress exposure compared to Resilient males, while no differences were observed in females (Fig 2-4). This finding reflects the fact that rates of alcohol abuse are higher among men than women, as are the proportion of men with PTSD that go on to develop SUDs (Kessler et al., 1995). It has long been proposed that the comorbidity between PTSD and SUDs is driven at least in part by a desire to self-medicate with drugs to alleviate PTSD symptoms, and the anxiolytic properties of alcohol are well known (McFarlane, 1998; Strahlendorf & Strahlendorf, 1983). Given the elevated anxiety in Susceptible males, it is conceivable that the increased alcohol drinking in this group is motivated by a drive to alleviate this anxious state.

Baseline anxiety predicts stress susceptibility in females but not males

This modified SEFL procedure offers a powerful tool for probing how pre-existing risk factors may contribute to the development of PTSD-related behaviors, and how these factors may differ between sexes. Preexisting anxiety disorders, which are also more common in women than in men, have been shown to increase the risk for subsequent PTSD (Breslau et al., 1997; Kessler et al., 2005; McLean et al., 2011). In support of this, we found that pre-stress anxiety levels were predictive of future stress susceptibility in females, but not in males (Fig 2-5).

Increased activation of the hypothalamus-pituitary-adrenal (HPA) axis in females has been hypothesized to underlie the elevated rates of PTSD and anxiety disorders in women and contribute to stress susceptibility (Kudielka & Wüst, 2010; Rao & Androulakis, 2017). It is therefore plausible that increased baseline anxiety in Susceptible females was reflective of uniquely high levels of HPA axis activation in these subjects, and that anxiolytic drug treatment prior to stress exposure might reduce rates of stress susceptibility in females.

The role of Ca^{2+}-permeable AMPA receptors in SEFL

Ca^{2+}-permeable AMPA receptors as an alternative source of long-term potentiation

The final objective of this dissertation was to investigate the biological mechanisms that support changes in fear learning following SEFL. We have hypothesized that the likely locus of change in both SEFL and PTSD is the basolateral amygdala (BLA), the central component of the fear learning network which during fear conditioning receives converging sensory information about the conditional stimulus (CS) and aversive unconditional stimulus (US) (Fendt & Fanselow, 1999). PTSD patients show elevated amygdala activity in response to negative stimuli (Brohawn et al., 2010; Rauch et al., 2000; Shin et al., 2006). SEFL also appears to involve changes in BLA function, as this procedure has been shown to increase glucocorticoid receptors within the BLA and inactivation of the BLA during stress exposure prevents SEFL (Perusini et al., 2016; Poulos et al., 2014). However, the precise changes within the BLA that support the long-lasting enhancement in fear learning have remained elusive.

We tested the possibility that augmented synaptic plasticity within the BLA might support these changes. α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors

mediate fast excitatory transmission within the central nervous system and are typically composed of GluA1 and GluA2 subunits or GluA2 and GlA3 subunits (Lee et al., 2003; Malinow, 2003). Because the GluA2 subunit renders the ion channel impermeable to CA^{2+}, the induction of long-term potentiation relies on Ca^{2+} entry through N-methyl-D-aspartate receptors (NMDARs). In previous studies, we demonstrated that stress exposure causes a long-lasting increase in GluA1 protein within the BLA, and that inhibition of corticosterone synthesis prevents both this elevation in GluA1 and the enhancement in fear learning (Perusini et al., 2016). Given that increased GluA1 levels can lead to formation of CA^{2+}-permeable AMPA receptors (CP-AMPARs) that support an NMDAR-independent form of LTP (Hollmann et al., 1991; Wiltgen et al., 2010), we hypothesized that SEFL is supported by increased formation of CP-AMPARs within the BLA.

Ca^{2+}-permeable AMPA receptors may support SEFL in males but not females

In Chapter 1, we investigated potential sex differences in the effects of stress exposure on both behaviors relevant to PTSD and on GluA1 levels within the BLA. Interestingly, while no differences in fear learning were observed between males and females (Fig 1-2; Fig 1-8), we found that stress differentially impacted GluA1 levels across sexes. Stress exposure altered GluA1 levels within the BLA of male rats, decreasing GluA1 levels in subjects that received alcohol prior to stress (Fig 1-6) and increasing GluA1 levels in subjects that had not received alcohol prior to stress (Fig 1-11). The decrease in GluA1 was unexpected given that we have previously seen increased GluA1 following stress in males (Perusini et al., 2016), but could potentially be caused by the extensive battery of behavioral testing and alcohol access these subjects received following stress exposure, as both of these can impact GluA1 phosphorylation

and trafficking. In contrast, no effects of stress exposure on GluA1 levels in females were observed in either experiment (Fig 1-6; Fig 1-11) suggesting that stress-induced changes in GluA1 levels may be unique to males.

In Chapter 3, we found further evidence that that CP-AMPARs support enhanced fear learning in males but not females. In floxed GluA2 mice, we observed no sex differences in performance during the SEFL procedure with both male and female mice showing robust enhancements in fear learning (Fig 3-3) and increased anxiety during the open field test (Fig 3-4). Mirroring the effects observed in Chapter 1, these results indicated that stress produced similar changes in fear and anxiety-related behaviors in male and female mice.

Neither GluA1 overexpression nor GluA2 deletion alone was sufficient to enhance fear learning in the absence of any other stress exposure in either males or females (Fig 3-1; Fig 3-5), indicating that the formation of CP-AMPARs alone may be insufficient to enhance fear learning. However, when subjects received a second footshock in a novel context, GluA2 deletion enhanced freezing to the novel context in males but not females (Fig 3-6). Similarly, GluA2 deletion caused elevated anxiety on the open field test in males but not females (Fig 3-7). These findings indicate that increases in CP-AMPAR formation combined with prior mild stress exposure may enhance stress susceptibility in males, while the enhanced fear learning in females may be supported by a different mechanism.

Potential mechanisms that support enhanced fear learning following stress in females

Interactions between gonadal hormones and the HPA axis

These findings lead to an important question: what are the mechanisms that mediate stress susceptibility in females? One potential explanation lies in the interaction between the HPA axis and gonadal hormones. We demonstrated that baseline anxiety levels are uniquely predictive of future stress susceptibility in female rats but not in males (Fig 2-5). This suggests that among females, individual variability in HPA axis activity may play an important role in mediating stress susceptibility. Compared to males, female rodents show increased activation of the HPA axis under basal conditions and following stress exposure as indicated by increased levels of corticotropin releasing factor (CRF), adrenocorticotropin hormone (ACTH) and corticosterone (Babb et al., 2013; Bangasser et al., 2010; Handa et al., 1994; Heck & Handa, 2019; Leśniewska, Miśkowiak, et al., 1990; Leśniewska, Nowak, et al., 1990; Viau et al., 2005). Binding of glucocorticoids in the hypothalamus and hippocampus play an important role in the termination of the stress response, and females have fewer glucocorticoid receptors in these regions suggesting reduced negative feedback of the HPA axis (MacLusky et al., 1996; Turner & Weaver, 1985). This increased HPA axis reactivity has been proposed to contribute to the increased rates of PTSD among women (Kudielka & Wüst, 2010; Rao & Androulakis, 2017).

These differences in HPA axis activity are thought to be driven by gonadal hormones, which are primarily estrogens such as estradiol in females and androgens such as testosterone in males, although both are present in both sexes (Heck & Handa, 2019). In addition to playing an important role in development, gonadal hormones can also mediate HPA axis activity in adulthood. Estradiol levels fluctuate across the 4-day estrous cycle in female rodents, peaking during proestrus and rapidly declining during estrus. During proestrus when estradiol levels are high, female rodents show increased basal and stress-induced release of ACTH and corticosterone compared to estrus and diestrus phases when estradiol levels are low (Carey et al.,

1995; Viau & Meaney, 1991). Similar effects have been seen in ovariectomized females, with estradiol treatment producing a similar sensitization of HPA axis activity (Carey et al., 1995; Herman et al., 2016). Estrogen receptors are expressed throughout key parts of the central nervous system that mediate HPA axis activity, including the paraventricular nucleus (PVN) of the hypothalamus and hippocampus where binding of estradiol may reduce the negative feedback of the HPA axis by glucocorticoids (Handa et al., 1994; Weiser & Handa, 2009). Thus, it has been suggested that increased estrogen levels in females lead to sensitization of the HPA axis, enhancing stress susceptibility.

Effects of estrogens on fear and anxiety

Paradoxically, while estradiol is associated with greater HPA axis activity it is also associated with reduced anxiety-like behavior (Luine et al., 2017). Estradiol has been shown to have anxiolytic effects on the open field test and elevated plus maze (Filova et al., 2015; Walf et al., 2008; Walf & Frye, 2007, 2010) and female rats tested at different stages of the estrous cycle show reduced anxiety-like behavior when estradiol levels are high compared when estradiol levels are low (Marcondes et al., 2001; Mora et al., 1996; Sayin et al., 2014; Walf & Frye, 2007).

There is also evidence that low estrogen levels impair fear extinction. Female rats that underwent fear extinction during metestrus when estradiol levels are low showed impaired fear extinction recall compared to females extinguished during proestrus (Milad, Igoe, et al., 2009; Rey et al., 2014) and estrogen administration has been shown to enhance fear extinction (Zeidan et al., 2011). Similar results have been seen in humans, where women undergoing fear extinction during the high-estrogen phase of the menstrual cycle show enhanced fear extinction (Milad et al., 2010). This suggests that low estrogen levels in women may be a risk factor for developing

conditions like PTSD. In support of this possibility, low estrogen levels are associated with impaired fear extinction among women with PTSD (Glover et al., 2012) and suppression of estrogen levels through hormonal contraceptives has been shown to impair fear extinction (Graham & Milad, 2013). While the explanation for these contradictory results is unclear, they nevertheless suggest that interactions between gonadal hormones and the HPA axis may play an important role in mediating stress susceptibility in females.

It is notable that in the literature, the effects of fear learning appear mixed on the surface but differences emerge when controlling for estrous cycle (Maeng & Milad, 2015). In the present studies of sex differences, we did not monitor estrus cycle because this would require additional handling of the female rats for collection of cytology samples which cannot be controlled for in male subjects (Hubscher et al., 2005). However, it is possible that in females the stage of the estrus cycle during stress exposure played an important role in stress susceptibility, especially given that the stress exposure occurred only a single day after baseline anxiety was assessed. It is important to note that while estrous cycle may be an important mediator of stress reactivity in females, there are likely other factors that play a similar role in males. If estrus cycle were causing females to effectively fluctuate around the means of the males, we would expect to see more variability in females compared to males but this not been observed either in the present studies or in previous work (Poulos et al., 2015).

Conclusions

In conclusion, the findings of this dissertation indicate important sex differences in the mechanisms that underlie behavioral changes following stress exposure. Epidemiological data indicates that gender is an important mediator of stress susceptibility, as PTSD is more common

among women while SUDs are more prevalent among men. In these studies, we found that the 15-footshock stress typically used in rat SEFL experiments generally produced similar changes in fear and anxiety behavior in male and female rats. Using a reduced-intensity stressor, we demonstrated that stress susceptibility manifests differently in male and female rats, with susceptible males showing increased alcohol drinking and susceptible females showing increased baseline anxiety. Lastly, we provided evidence that the similar enhancements in fear learning observed in males and females following stress exposure are supported by different neural mechanisms, with CP-AMPARs supporting enhanced fear learning in males but not females. Collectively, these findings highlight the importance of understanding how sex differences may contribute to stress reactivity and subsequent psychopathology.

References

Anisman, H., & Waller, T. G. (1974). Effects of inescapable shock and shock-produced conflict on self selection of alcohol in rats. *Pharmacol Biochem Behav*, *2*(1), 27-33. https://doi.org/10.1016/0091-3057(74)90131-2

Babb, J. A., Masini, C. V., Day, H. E., & Campeau, S. (2013). Sex differences in activated corticotropin-releasing factor neurons within stress-related neurocircuitry and hypothalamic-pituitary-adrenocortical axis hormones following restraint in rats. *Neuroscience*, *234*, 40-52. https://doi.org/10.1016/j.neuroscience.2012.12.051

Bangasser, D. A., Curtis, A., Reyes, B. A., Bethea, T. T., Parastatidis, I., Ischiropoulos, H., Van Bockstaele, E. J., & Valentino, R. J. (2010). Sex differences in corticotropin-releasing factor receptor signaling and trafficking: potential role in female vulnerability to stress-related psychopathology. *Mol Psychiatry*, *15*(9), 877, 896-904. https://doi.org/10.1038/mp.2010.66

Baran, S. E., Armstrong, C. E., Niren, D. C., Hanna, J. J., & Conrad, C. D. (2009). Chronic stress and sex differences on the recall of fear conditioning and extinction. *Neurobiol Learn Mem*, *91*(3), 323-332. https://doi.org/10.1016/j.nlm.2008.11.005

Beck, K. D., & Luine, V. N. (2002). Sex differences in behavioral and neurochemical profiles after chronic stress: role of housing conditions. *Physiol Behav*, *75*(5), 661-673. https://doi.org/10.1016/s0031-9384(02)00670-4

Becker, H. C., Lopez, M. F., & Doremus-Fitzwater, T. L. (2011). Effects of stress on alcohol drinking: a review of animal studies. *Psychopharmacology (Berl)*, *218*(1), 131-156. https://doi.org/10.1007/s00213-011-2443-9

Berton, O., McClung, C. A., Dileone, R. J., Krishnan, V., Renthal, W., Russo, S. J., Graham, D., Tsankova, N. M., Bolanos, C. A., Rios, M., Monteggia, L. M., Self, D. W., & Nestler, E. J. (2006). Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. *Science*, *311*(5762), 864-868. https://doi.org/10.1126/science.1120972

Bondi, C. O., Rodriguez, G., Gould, G. G., Frazer, A., & Morilak, D. A. (2008). Chronic unpredictable stress induces a cognitive deficit and anxiety-like behavior in rats that is prevented by chronic antidepressant drug treatment. *Neuropsychopharmacology*, *33*(2), 320-331. https://doi.org/10.1038/sj.npp.1301410

Bowman, R. E., Micik, R., Gautreaux, C., Fernandez, L., & Luine, V. N. (2009). Sex-dependent changes in anxiety, memory, and monoamines following one week of stress. *Physiol Behav*, *97*(1), 21-29. https://doi.org/10.1016/j.physbeh.2009.01.012

Brancato, A., Plescia, F., Lavanco, G., Cavallaro, A., & Cannizzaro, C. (2016). Continuous and Intermittent Alcohol Free-Choice from Pre-gestational Time to Lactation: Focus on

Drinking Trajectories and Maternal Behavior. *Front Behav Neurosci, 10*, 31. https://doi.org/10.3389/fnbeh.2016.00031

Bremner, J. D., Krystal, J. H., Southwick, S. M., & Charney, D. S. (1995). Functional neuroanatomical correlates of the effects of stress on memory. *J Trauma Stress, 8*(4), 527-553.

Breslau, N., Davis, G. C., Andreski, P., Peterson, E. L., & Schultz, L. R. (1997). Sex differences in posttraumatic stress disorder. *Arch Gen Psychiatry, 54*(11), 1044-1048. https://doi.org/10.1001/archpsyc.1997.01830230082012

Brohawn, K. H., Offringa, R., Pfaff, D. L., Hughes, K. C., & Shin, L. M. (2010). The neural correlates of emotional memory in posttraumatic stress disorder. *Biol Psychiatry, 68*(11), 1023-1030. https://doi.org/10.1016/j.biopsych.2010.07.018

Brunello, N., Davidson, J. R., Deahl, M., Kessler, R. C., Mendlewicz, J., Racagni, G., Shalev, A. Y., & Zohar, J. (2001). Posttraumatic stress disorder: diagnosis and epidemiology, comorbidity and social consequences, biology and treatment. *Neuropsychobiology, 43*(3), 150-162. https://doi.org/10.1159/000054884

Carey, M. P., Deterd, C. H., de Koning, J., Helmerhorst, F., & de Kloet, E. R. (1995). The influence of ovarian steroids on hypothalamic-pituitary-adrenal regulation in the female rat. *J Endocrinol, 144*(2), 311-321. https://doi.org/10.1677/joe.0.1440311

Champagne, F., & Kirouac, G. (1987). Effects of unavoidable electric shocks on voluntary alcohol consumption in the rat. *Percept Mot Skills, 64*(1), 335-338. https://doi.org/10.2466/pms.1987.64.1.335

Chester, J. A., Barrenha, G. D., Hughes, M. L., & Keuneke, K. J. (2008). Age- and sex-dependent effects of footshock stress on subsequent alcohol drinking and acoustic startle behavior in mice selectively bred for high-alcohol preference. *Alcohol Clin Exp Res, 32*(10), 1782-1794. https://doi.org/10.1111/j.1530-0277.2008.00763.x

Choca, J. P., Wilson, A. S., & Garcia, T. J. (1977). Effects of shock and darkness on alcohol consumption by rats. *J Stud Alcohol, 38*(11), 2184-2187. https://doi.org/10.15288/jsa.1977.38.2184

Colucci, P., Marchetta, E., Mancini, G. F., Alva, P., Chiarotti, F., Hasan, M. T., & Campolongo, P. (2020). Predicting susceptibility and resilience in an animal model of post-traumatic stress disorder (PTSD). *Transl Psychiatry, 10*(1), 243. https://doi.org/10.1038/s41398-020-00929-9

Covington, H. E., Lobo, M. K., Maze, I., Vialou, V., Hyman, J. M., Zaman, S., LaPlant, Q., Mouzon, E., Ghose, S., Tamminga, C. A., Neve, R. L., Deisseroth, K., & Nestler, E. J. (2010). Antidepressant effect of optogenetic stimulation of the medial prefrontal cortex. *J Neurosci, 30*(48), 16082-16090. https://doi.org/10.1523/JNEUROSCI.1731-10.2010

Craske, M. G., Kircanski, K., Zelikowsky, M., Mystkowski, J., Chowdhury, N., & Baker, A. (2008). Optimizing inhibitory learning during exposure therapy. *Behav Res Ther*, *46*(1), 5-27. https://doi.org/10.1016/j.brat.2007.10.003

Curley, J. P., Jensen, C. L., Franks, B., & Champagne, F. A. (2012). Variation in maternal and anxiety-like behavior associated with discrete patterns of oxytocin and vasopressin 1a receptor density in the lateral septum. *Horm Behav*, *61*(3), 454-461. https://doi.org/10.1016/j.yhbeh.2012.01.013

Darnaudéry, M., Louvart, H., Defrance, L., Léonhardt, M., Morley-Fletcher, S., Gruber, S. H., Galietta, G., Mathé, A. A., & Maccari, S. (2007). Impact of an intense stress on ethanol consumption in female rats characterized by their pre-stress preference: modulation by prenatal stress. *Brain Res*, *1131*(1), 181-186. https://doi.org/10.1016/j.brainres.2006.11.005

Daviu, N., Andero, R., Armario, A., & Nadal, R. (2014). Sex differences in the behavioural and hypothalamic-pituitary-adrenal response to contextual fear conditioning in rats. *Horm Behav*, *66*(5), 713-723. https://doi.org/10.1016/j.yhbeh.2014.09.015

Dykman, R. A., Ackerman, P. T., & Newton, J. E. (1997). Posttraumatic stress disorder: a sensitization reaction. *Integr Physiol Behav Sci*, *32*(1), 9-18.

Eberly, R. E., & Engdahl, B. E. (1991). Prevalence of somatic and psychiatric disorders among former prisoners of war. *Hosp Community Psychiatry*, *42*(8), 807-813.

Fendt, A., & Fanselow, M. S. (1999). The neuroanatomical and neurochemical basis of conditioned fear. *Neuroscience & Biobehavioral Reviews*, *23*(5), 734-760.

Fernandes, C., González, M. I., Wilson, C. A., & File, S. E. (1999). Factor analysis shows that female rat behaviour is characterized primarily by activity, male rats are driven by sex and anxiety. *Pharmacol Biochem Behav*, *64*(4), 731-738. https://doi.org/10.1016/s0091-3057(99)00139-2

Fidler, T. L., & LoLordo, V. M. (1996). Failure to find postshock increases in ethanol preference. *Alcohol Clin Exp Res*, *20*(1), 110-121. https://doi.org/10.1111/j.1530-0277.1996.tb01053.x

Filova, B., Malinova, M., Babickova, J., Tothova, L., Ostatnikova, D., Celec, P., & Hodosy, J. (2015). Effects of testosterone and estradiol on anxiety and depressive-like behavior via a non-genomic pathway. *Neurosci Bull*, *31*(3), 288-296. https://doi.org/10.1007/s12264-014-1510-8

Francis, D. D., Diorio, J., Plotsky, P. M., & Meaney, M. J. (2002). Environmental enrichment reverses the effects of maternal separation on stress reactivity. *J Neurosci*, *22*(18), 7840-7843.

Füllgrabe, M. W., Vengeliene, V., & Spanagel, R. (2007). Influence of age at drinking onset on the alcohol deprivation effect and stress-induced drinking in female rats. *Pharmacol Biochem Behav*, *86*(2), 320-326. https://doi.org/10.1016/j.pbb.2006.10.004

Gamwell, K., Nylocks, M., Cross, D., Bradley, B., Norrholm, S. D., & Jovanovic, T. (2015). Fear conditioned responses and PTSD symptoms in children: Sex differences in fear-related symptoms. *Dev Psychobiol*, *57*(7), 799-808. https://doi.org/10.1002/dev.21313

Glover, E. M., Jovanovic, T., Mercer, K. B., Kerley, K., Bradley, B., Ressler, K. J., & Norrholm, S. D. (2012). Estrogen levels are associated with extinction deficits in women with posttraumatic stress disorder. *Biol Psychiatry*, *72*(1), 19-24. https://doi.org/10.1016/j.biopsych.2012.02.031

Golden, S. A., Covington, H. E., Berton, O., & Russo, S. J. (2011). A standardized protocol for repeated social defeat stress in mice. *Nat Protoc*, *6*(8), 1183-1191. https://doi.org/10.1038/nprot.2011.361

Graham, B. M., & Milad, M. R. (2013). Blockade of estrogen by hormonal contraceptives impairs fear extinction in female rats and women. *Biol Psychiatry*, *73*(4), 371-378. https://doi.org/10.1016/j.biopsych.2012.09.018

Graham, L. K., Yoon, T., Lee, H. J., & Kim, J. J. (2009). Strain and sex differences in fear conditioning: 22 kHz

ultrasonic vocalizations and freezing in rats. *Psychology & Neuroscience*, *2*(2), 219-225.

Handa, R. J., Burgess, L. H., Kerr, J. E., & O'Keefe, J. A. (1994). Gonadal steroid hormone receptors and sex differences in the hypothalamo-pituitary-adrenal axis. *Horm Behav*, *28*(4), 464-476. https://doi.org/10.1006/hbeh.1994.1044

Heck, A. L., & Handa, R. J. (2019). Sex differences in the hypothalamic-pituitary-adrenal axis' response to stress: an important role for gonadal hormones. *Neuropsychopharmacology*, *44*(1), 45-58. https://doi.org/10.1038/s41386-018-0167-9

Hembree, E. A., Foa, E. B., Dorfan, N. M., Street, G. P., Kowalski, J., & Tu, X. (2003). Do patients drop out prematurely from exposure therapy for PTSD? *J Trauma Stress*, *16*(6), 555-562. https://doi.org/10.1023/B:JOTS.0000004078.93012.7d

Herman, J. P., McKlveen, J. M., Ghosal, S., Kopp, B., Wulsin, A., Makinson, R., Scheimann, J., & Myers, B. (2016). Regulation of the Hypothalamic-Pituitary-Adrenocortical Stress Response. *Compr Physiol*, *6*(2), 603-621. https://doi.org/10.1002/cphy.c150015

Hill, M. N., Patel, S., Carrier, E. J., Rademacher, D. J., Ormerod, B. K., Hillard, C. J., & Gorzalka, B. B. (2005). Downregulation of endocannabinoid signaling in the hippocampus following chronic unpredictable stress. *Neuropsychopharmacology*, *30*(3), 508-515. https://doi.org/10.1038/sj.npp.1300601

Hollmann, M., Hartley, M., & Heinemann, S. (1991). Ca2+ permeability of KA-AMPA--gated glutamate receptor channels depends on subunit composition. *Science*, *252*(5007), 851-853.

Holmes, A., & Wellman, C. L. (2009). Stress-induced prefrontal reorganization and executive dysfunction in rodents. *Neurosci Biobehav Rev*, *33*(6), 773-783. https://doi.org/10.1016/j.neubiorev.2008.11.005

Hubscher, C. H., Brooks, D. L., & Johnson, J. R. (2005). A quantitative method for assessing stages of the rat estrous cycle. *Biotech Histochem*, *80*(2), 79-87. https://doi.org/10.1080/10520290500138422

Inslicht, S. S., Metzler, T. J., Garcia, N. M., Pineles, S. L., Milad, M. R., Orr, S. P., Marmar, C. R., & Neylan, T. C. (2013). Sex differences in fear conditioning in posttraumatic stress disorder. *J Psychiatr Res*, *47*(1), 64-71. https://doi.org/10.1016/j.jpsychires.2012.08.027

Jeong, M. J., Lee, C., Sung, K., Jung, J. H., Pyo, J. H., & Kim, J. H. (2020). Fear response-based prediction for stress susceptibility to PTSD-like phenotypes. *Mol Brain*, *13*(1), 134. https://doi.org/10.1186/s13041-020-00667-5

Johnston, A. L., & File, S. E. (1991). Sex differences in animal tests of anxiety. *Physiol Behav*, *49*(2), 245-250. https://doi.org/10.1016/0031-9384(91)90039-q

Kaczkurkin, A. N., Burton, P. C., Chazin, S. M., Manbeck, A. B., Espensen-Sturges, T., Cooper, S. E., Sponheim, S. R., & Lissek, S. (2017). Neural Substrates of Overgeneralized Conditioned Fear in PTSD. *Am J Psychiatry*, *174*(2), 125-134. https://doi.org/10.1176/appi.ajp.2016.15121549

Keller, S. M., Schreiber, W. B., Staib, J. M., & Knox, D. (2015). Sex differences in the single prolonged stress model. *Behav Brain Res*, *286*, 29-32. https://doi.org/10.1016/j.bbr.2015.02.034

Kessler, R. C., Aguilar-Gaxiola, S., Alonso, J., Benjet, C., Bromet, E. J., Cardoso, G., Degenhardt, L., de Girolamo, G., Dinolova, R. V., Ferry, F., Florescu, S., Gureje, O., Haro, J. M., Huang, Y., Karam, E. G., Kawakami, N., Lee, S., Lepine, J. P., Levinson, D., Navarro-Mateu, F., Pennell, B. E., Piazza, M., Posada-Villa, J., Scott, K. M., Stein, D. J., Ten Have, M., Torres, Y., Viana, M. C., Petukhova, M. V., Sampson, N. A., Zaslavsky, A. M., & Koenen, K. C. (2017). Trauma and PTSD in the WHO World Mental Health Surveys. *Eur J Psychotraumatol*, *8*(sup5), 1353383. https://doi.org/10.1080/20008198.2017.1353383

Kessler, R. C., Berglund, P., Demler, O., Jin, R., Merikangas, K. R., & Walters, E. E. (2005). Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. *Arch Gen Psychiatry*, *62*(6), 593-602. https://doi.org/10.1001/archpsyc.62.6.593

Kessler, R. C., Crum, R. M., Warner, L. A., Nelson, C. B., Schulenberg, J., & Anthony, J. C. (1997). Lifetime co-occurrence of DSM-III-R alcohol abuse and dependence with other psychiatric disorders in the National Comorbidity Survey. *Arch Gen Psychiatry, 54*(4), 313-321. https://doi.org/10.1001/archpsyc.1997.01830160031005

Kessler, R. C., Sonnega, A., Bromet, E., Hughes, M., & Nelson, C. B. (1995). Posttraumatic stress disorder in the National Comorbidity Survey. *Arch Gen Psychiatry, 52*(12), 1048-1060.

Kinney, L., & Schmidt, H. (1979). Effect of cued and uncued inescapable shock on voluntary alcohol consumption in rats. *Pharmacol Biochem Behav, 11*(6), 601-604. https://doi.org/10.1016/0091-3057(79)90249-1

Kudielka, B. M., & Wüst, S. (2010). Human models in acute and chronic stress: assessing determinants of individual hypothalamus-pituitary-adrenal axis activity and reactivity. *Stress, 13*(1), 1-14. https://doi.org/10.3109/10253890902874913

Lee, H. K., Takamiya, K., Han, J. S., Man, H., Kim, C. H., Rumbaugh, G., Yu, S., Ding, L., He, C., Petralia, R. S., Wenthold, R. J., Gallagher, M., & Huganir, R. L. (2003). Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory. *Cell, 112*(5), 631-643. https://doi.org/10.1016/s0092-8674(03)00122-3

Leśniewska, B., Miśkowiak, B., Nowak, M., & Malendowicz, L. K. (1990). Sex differences in adrenocortical structure and function. XXVII. The effect of ether stress on ACTH and corticosterone in intact, gonadectomized, and testosterone- or estradiol-replaced rats. *Res Exp Med (Berl), 190*(2), 95-103. https://doi.org/10.1007/pl00020011

Leśniewska, B., Nowak, M., & Malendowicz, L. K. (1990). Sex differences in adrenocortical structure and function. XXVIII. ACTH and corticosterone in intact, gonadectomised and gonadal hormone replaced rats. *Horm Metab Res, 22*(7), 378-381.

Luine, V., Gomez, J., Beck, K., & Bowman, R. (2017). Sex differences in chronic stress effects on cognition in rodents. *Pharmacol Biochem Behav, 152*, 13-19. https://doi.org/10.1016/j.pbb.2016.08.005

MacLusky, N. J., Yuan, H., Elliott, J., & Brown, T. J. (1996). Sex differences in corticosteroid binding in the rat brain: an in vitro autoradiographic study. *Brain Res, 708*(1-2), 71-81. https://doi.org/10.1016/0006-8993(95)01310-5

Maeng, L. Y., & Milad, M. R. (2015). Sex differences in anxiety disorders: Interactions between fear, stress, and gonadal hormones. *Horm Behav, 76*, 106-117. https://doi.org/10.1016/j.yhbeh.2015.04.002

Malinow, R. (2003). AMPA receptor trafficking and long-term potentiation. *Philos Trans R Soc Lond B Biol Sci, 358*(1432), 707-714. https://doi.org/10.1098/rstb.2002.1233

Marcondes, F. K., Miguel, K. J., Melo, L. L., & Spadari-Bratfisch, R. C. (2001). Estrous cycle influences the response of female rats in the elevated plus-maze test. *Physiol Behav*, *74*(4-5), 435-440. https://doi.org/10.1016/s0031-9384(01)00593-5

Maren, S., De Oca, B., & Fanselow, M. S. (1994). Sex differences in hippocampal long-term potentiation (LTP) and Pavlovian fear conditioning in rats: positive correlation between LTP and contextual learning. *Brain Res*, *661*(1-2), 25-34. https://doi.org/10.1016/0006-8993(94)91176-2

Markus, E. J., & Zecevic, M. (1997). Sex differences and estrous cycle changes

in hippocampus-dependent fear conditioning. *PSychobiology*, *25*(3), 246-252.

McFarlane, A. C. (1998). Epidemiological evidence about the relationship between PTSD and alcohol abuse: the nature of the association. *Addict Behav*, *23*(6), 813-825. https://doi.org/10.1016/s0306-4603(98)00098-7

McLean, C. P., Asnaani, A., Litz, B. T., & Hofmann, S. G. (2011). Gender differences in anxiety disorders: prevalence, course of illness, comorbidity and burden of illness. *J Psychiatr Res*, *45*(8), 1027-1035. https://doi.org/10.1016/j.jpsychires.2011.03.006

Meyer, E. M., Long, V., Fanselow, M. S., & Spigelman, I. (2013). Stress increases voluntary alcohol intake, but does not alter established drinking habits in a rat model of posttraumatic stress disorder. *Alcohol Clin Exp Res*, *37*(4), 566-574. https://doi.org/10.1111/acer.12012

Milad, M. R., Igoe, S. A., Lebron-Milad, K., & Novales, J. E. (2009). Estrous cycle phase and gonadal hormones influence conditioned fear extinction. *Neuroscience*, *164*(3), 887-895. https://doi.org/10.1016/j.neuroscience.2009.09.011

Milad, M. R., Pitman, R. K., Ellis, C. B., Gold, A. L., Shin, L. M., Lasko, N. B., Zeidan, M. A., Handwerger, K., Orr, S. P., & Rauch, S. L. (2009). Neurobiological basis of failure to recall extinction memory in posttraumatic stress disorder. *Biol Psychiatry*, *66*(12), 1075-1082. https://doi.org/10.1016/j.biopsych.2009.06.026

Milad, M. R., Zeidan, M. A., Contero, A., Pitman, R. K., Klibanski, A., Rauch, S. L., & Goldstein, J. M. (2010). The influence of gonadal hormones on conditioned fear extinction in healthy humans. *Neuroscience*, *168*(3), 652-658. https://doi.org/10.1016/j.neuroscience.2010.04.030

Mills, K. L., Teesson, M., Ross, J., & Peters, L. (2006). Trauma, PTSD, and substance use disorders: findings from the Australian National Survey of Mental Health and Well-Being. *Am J Psychiatry*, *163*(4), 652-658. https://doi.org/10.1176/appi.ajp.163.4.652

Mora, S., Dussaubat, N., & Díaz-Véliz, G. (1996). Effects of the estrous cycle and ovarian hormones on behavioral indices of anxiety in female rats. *Psychoneuroendocrinology*, *21*(7), 609-620. https://doi.org/10.1016/s0306-4530(96)00015-7

Perkonigg, A., Kessler, R. C., Storz, S., & Wittchen, H.-U. (2000). Traumatic events and post-traumatic stress disorder in the community: prevalence, risk factors and comorbidity. *Acta Psychiatr Scand*, *101*(1), 46-59.

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*(1), 45-57. https://doi.org/10.1038/npp.2015.224

Poulos, A. M., Reger, M., Mehta, N., Zhuravka, I., Sterlace, S. S., Gannam, C., Hovda, D. A., Giza, C. C., & Fanselow, M. S. (2014). Amnesia for early life stress does not preclude the adult development of posttraumatic stress disorder symptoms in rats. *Biol Psychiatry*, *76*(4), 306-314. https://doi.org/10.1016/j.biopsych.2013.10.007

Poulos, A. M., Zhuravka, I., Long, V., Gannam, C., & Fanselow, M. (2015). Sensitization of fear learning to mild unconditional stimuli in male and female rats. *Behav Neurosci*, *129*(1), 62-67. https://doi.org/10.1037/bne0000033

Pryce, C. R., Lehmann, J., & Feldon, J. (1999). Effect of sex on fear conditioning is similar for context and discrete CS in Wistar, Lewis and Fischer rat strains. *Pharmacol Biochem Behav*, *64*(4), 753-759. https://doi.org/10.1016/s0091-3057(99)00147-1

Rao, R. T., & Androulakis, I. P. (2017). Modeling the Sex Differences and Interindividual Variability in the Activity of the Hypothalamic-Pituitary-Adrenal Axis. *Endocrinology*, *158*(11), 4017-4037. https://doi.org/10.1210/en.2017-00544

Rau, V., DeCola, J. P., & Fanselow, M. S. (2005). Stress-induced enhancement of fear learning: an animal model of posttraumatic stress disorder. *Neurosci Biobehav Rev*, *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

Rauch, S. L., Whalen, P. J., Shin, L. M., McInerney, S. C., Macklin, M. L., Lasko, N. B., Orr, S. P., & Pitman, R. K. (2000). Exaggerated amygdala response to masked facial stimuli in posttraumatic stress disorder: a functional MRI study. *Biol Psychiatry*, *47*(9), 769-776. https://doi.org/10.1016/s0006-3223(00)00828-3

Rey, C. D., Lipps, J., & Shansky, R. M. (2014). Dopamine D1 receptor activation rescues extinction impairments in low-estrogen female rats and induces cortical layer-specific activation changes in prefrontal-amygdala circuits. *Neuropsychopharmacology*, *39*(5), 1282-1289. https://doi.org/10.1038/npp.2013.338

Richter-Levin, G., Stork, O., & Schmidt, M. V. (2019). Animal models of PTSD: a challenge to be met. *Mol Psychiatry*, *24*(8), 1135-1156. https://doi.org/10.1038/s41380-018-0272-5

Ritov, G., Boltyansky, B., & Richter-Levin, G. (2016). A novel approach to PTSD modeling in rats reveals alternating patterns of limbic activity in different types of stress reaction. *Mol Psychiatry, 21*(5), 630-641. https://doi.org/10.1038/mp.2015.169

Sayin, A., Derinöz, O., Yüksel, N., Şahin, S., & Bolay, H. (2014). The effects of the estrus cycle and citalopram on anxiety-like behaviors and c-fos expression in rats. *Pharmacol Biochem Behav, 124*, 180-187. https://doi.org/10.1016/j.pbb.2014.06.002

Scholl, J. L., Afzal, A., Fox, L. C., Watt, M. J., & Forster, G. L. (2019). Sex differences in anxiety-like behaviors in rats. *Physiol Behav, 211*, 112670. https://doi.org/10.1016/j.physbeh.2019.112670

Shansky, R. M. (2015). Sex differences in PTSD resilience and susceptibility: Challenges for animal models of fear learning. *Neurobiol Stress, 1*, 60-65. https://doi.org/10.1016/j.ynstr.2014.09.005

Shields, G. S., Trainor, B. C., Lam, J. C., & Yonelinas, A. P. (2016). Acute stress impairs cognitive flexibility in men, not women. *Stress, 19*(5), 542-546. https://doi.org/10.1080/10253890.2016.1192603

Shin, L. M., Rauch, S. L., & Pitman, R. K. (2006). Amygdala, medial prefrontal cortex, and hippocampal function in PTSD. *Ann N Y Acad Sci, 1071*, 67-79. https://doi.org/10.1196/annals.1364.007

Simms, J. A., Steensland, P., Medina, B., Abernathy, K. E., Chandler, L. J., Wise, R., & Bartlett, S. E. (2008). Intermittent access to 20% ethanol induces high ethanol consumption in Long-Evans and Wistar rats. *Alcohol Clin Exp Res, 32*(10), 1816-1823. https://doi.org/10.1111/j.1530-0277.2008.00753.x

Smith, D. R., Gallagher, M., & Stanton, M. E. (2007). Genetic background differences and nonassociative effects in mouse trace fear conditioning. *Learn Mem, 14*(9), 597-605. https://doi.org/10.1101/lm.614807

Solinas, M., Chauvet, C., Thiriet, N., El Rawas, R., & Jaber, M. (2008). Reversal of cocaine addiction by environmental enrichment. *Proc Natl Acad Sci U S A, 105*(44), 17145-17150. https://doi.org/10.1073/pnas.0806889105

Stiedl, O., Radulovic, J., Lohmann, R., Birkenfeld, K., Palve, M., Kammermeier, J., Sananbenesi, F., & Spiess, J. (1999). Strain and substrain differences in context- and tone-dependent fear conditioning of inbred mice. *Behav Brain Res, 104*(1-2), 1-12. https://doi.org/10.1016/s0166-4328(99)00047-9

Strahlendorf, H. K., & Strahlendorf, J. C. (1983). Ethanol suppression of locus coeruleus neurons: relevancy to the fetal alcohol syndrome. *Neurobehav Toxicol Teratol, 5*(2), 221-224.

Szuran, T. F., Pliska, V., Pokorny, J., & Welzl, H. (2000). Prenatal stress in rats: effects on plasma corticosterone, hippocampal glucocorticoid receptors, and maze performance. *Physiol Behav*, *71*(3-4), 353-362. https://doi.org/10.1016/s0031-9384(00)00351-6

Turner, B. B., & Weaver, D. A. (1985). Sexual dimorphism of glucocorticoid binding in rat brain. *Brain Res*, *343*(1), 16-23. https://doi.org/10.1016/0006-8993(85)91153-9

Vengeliene, V., Siegmund, S., Singer, M. V., Sinclair, J. D., Li, T. K., & Spanagel, R. (2003). A comparative study on alcohol-preferring rat lines: effects of deprivation and stress phases on voluntary alcohol intake. *Alcohol Clin Exp Res*, *27*(7), 1048-1054. https://doi.org/10.1097/01.ALC.0000075829.81211.0C

Viau, V., Bingham, B., Davis, J., Lee, P., & Wong, M. (2005). Gender and puberty interact on the stress-induced activation of parvocellular neurosecretory neurons and corticotropin-releasing hormone messenger ribonucleic acid expression in the rat. *Endocrinology*, *146*(1), 137-146. https://doi.org/10.1210/en.2004-0846

Viau, V., & Meaney, M. J. (1991). Variations in the hypothalamic-pituitary-adrenal response to stress during the estrous cycle in the rat. *Endocrinology*, *129*(5), 2503-2511. https://doi.org/10.1210/endo-129-5-2503

Walf, A. A., Ciriza, I., Garcia-Segura, L. M., & Frye, C. A. (2008). Antisense oligodeoxynucleotides for estrogen receptor-beta and alpha attenuate estradiol's modulation of affective and sexual behavior, respectively. *Neuropsychopharmacology*, *33*(2), 431-440. https://doi.org/10.1038/sj.npp.1301416

Walf, A. A., & Frye, C. A. (2007). Estradiol decreases anxiety behavior and enhances inhibitory avoidance and gestational stress produces opposite effects. *Stress*, *10*(3), 251-260. https://doi.org/10.1080/00958970701220416

Walf, A. A., & Frye, C. A. (2010). Estradiol reduces anxiety- and depression-like behavior of aged female mice. *Physiol Behav*, *99*(2), 169-174. https://doi.org/10.1016/j.physbeh.2009.09.017

Weiser, M. J., & Handa, R. J. (2009). Estrogen impairs glucocorticoid dependent negative feedback on the hypothalamic-pituitary-adrenal axis via estrogen receptor alpha within the hypothalamus. *Neuroscience*, *159*(2), 883-895. https://doi.org/10.1016/j.neuroscience.2008.12.058

Wiltgen, B. J., Royle, G. A., Gray, E. E., Abdipranoto, A., Thangthaeng, N., Jacobs, N., Saab, F., Tonegawa, S., Heinemann, S. F., O'Dell, T. J., Fanselow, M. S., & Vissel, B. (2010). A role for calcium-permeable AMPA receptors in synaptic plasticity and learning. *PLoS One*, *5*(9). https://doi.org/10.1371/journal.pone.0012818

Wise, R. A. (1973). Voluntary ethanol intake in rats following exposure to ethanol on various schedules. *Psychopharmacologia*, *29*(3), 203-210. https://doi.org/10.1007/BF00414034

Wood, G. E., & Shors, T. J. (1998). Stress facilitates classical conditioning in males, but impairs classical conditioning in females through activational effects of ovarian hormones. *Proc Natl Acad Sci U S A*, *95*(7), 4066-4071. https://doi.org/10.1073/pnas.95.7.4066

Zeidan, M. A., Igoe, S. A., Linnman, C., Vitalo, A., Levine, J. B., Klibanski, A., Goldstein, J. M., & Milad, M. R. (2011). Estradiol modulates medial prefrontal cortex and amygdala activity during fear extinction in women and female rats. *Biol Psychiatry*, *70*(10), 920-927. https://doi.org/10.1016/j.biopsych.2011.05.016

Zimmerberg, B., & Farley, M. J. (1993). Sex differences in anxiety behavior in rats: role of gonadal hormones. *Physiol Behav*, *54*(6), 1119-1124. https://doi.org/10.1016/0031-9384(93)90335-d