Title

Characterizing the Behavioral and Neural Mechanisms Underpinning Stress-Enhanced Fear Learning, a Rodent Model of Post-Traumatic Stress Disorder

Author

Tribble, Jennifer Elizabeth

Publication Date

2018

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Characterizing the Behavioral and Neural Mechanisms Underpinning Stress-Enhanced Fear Learning, a Rodent Model of Post-Traumatic Stress Disorder

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Neuroscience

by

Jennifer Elizabeth Tribble

2018

ABSTRACT OF THE DISSERTATION

Characterizing the Behavioral and Neural Mechanisms Underpinning Stress-Enhanced Fear Learning, a Rodent Model of Post-Traumatic Stress Disorder

by

Jennifer Elizabeth Tribble

Doctor of Philosophy in Neuroscience

University of California, Los Angeles, 2018

Professor Michael S. Fanselow, Chair

Post-Traumatic Stress Disorder (PTSD) is a complex, multi-faceted disease that affects a subset of individuals who undergo a traumatic experience. To understand the variety of psychological symptoms and their underpinning neural mechanisms, our laboratory has developed a rodent model of the disease in which animals experience an acute traumatic experience (15 unsignaled footshocks) that leads to a variety of changes in fear, anxiety, and depression (PTSD-like phenotypes). Through using a behavioral model of the disease, we can use pharmacological, behavioral, or genetic manipulations to dissect the neural mechanisms underlying the variety of changes in behavior seen following the acute stressor. In this dissertation, I seek to understand how these different symptoms (exaggerated subsequent fear conditioning, anxiety, and depression) manifest selectively and are driven by distinct neural mechanisms, each of which are sensitive to the effects of stress. As we begin to disentangle the variety of changes that occur in the brain following stress and how these changes in the brain

manifest behaviorally, we can ultimately hope to better diagnose and treat individuals who are suffering from the debilitating symptoms associated with PTSD.

In Chapter 2, I test for the effects of pair housing animals prior to, during, and following the acute stressor. Rodents are extremely social animals, and isolation housing has been shown to magnify or sufficiently cause effects of stress. Therefore, it was critical to understand whether isolation housing is necessary for developing the PTSD-like symptoms observed after an acute stressor. Interestingly, I found that housing condition (isolation versus pair) had no effect on subsequent fear and anxiety phenotypes in adult, male rats undergoing an acute stressor.

In Chapter 3, I test for the role of kappa opioid receptors (KORs) in the subsequent expression of PTSD-like phenotypes following an acute stressor. The KOR antagonist JDTic was administered immediately after trauma, then animals were tested for exaggerated fear conditioning, and anxiety and depression assays. JDTic administration did not perturb the enhanced fear conditioning phenotype observed following stress, but it did mitigate anxiety behavior on the elevated plus maze (EPM). JDTic administered to stressed animals caused an increase in time spent in the open arms of the EPM across an 8 minute session. Shockingly, JDTic administered to unstressed animals caused an anxiogenic phenotype as seen by a failure to habituate to the EPM across an 8 minute session. Animals were also tested in the open field test and forced swim test, but there was no effect of JDTic on these measures. Together, these data indicate that KORs have a selective role in the anxiety-like phenotypes seen in rats following an acute stressor. These data are one example of how different neural circuits could contribute differentially to the array of phenotypes observed following acute stress.

In Chapter 4, I use a variety of techniques to assess the neural mechanisms underlying the enhanced fear phenotype observed in our PTSD model. Specifically, I test for changes in excitatory receptors expressed in brain regions associated with fear and anxiety through Western blotting, RT-PCR, and a genetic manipulation to test the contribution of NMDA-R's to the sensitized fear response. Taken together, these studies begin to describe the neural and molecular changes that lead to the robust enhancement in subsequent fear conditioning observed in stressed animals.

Collectively, these studies serve as a beginning of exploring the many behavioral and neural mechanisms underlying our rodent model of PTSD. Future and subsequent studies may build off of this work to further characterize the PTSD-like phenotypes of our model, and relate these behavioral changes to the specific neural changes that guide them.

The dissertation of Jennifer Elizabeth Tribble is approved.

Dean Buonomano

David L. Glanzman

Pamela J. Kennedy

Alcino Jose Silva

Michael S. Fanselow, Committee Chair

University of California, Los Angeles

2018

"Science is more than a body of knowledge. It is a way of thinking, a way of skeptically interrogating the universe with a fine understanding of human fallibility."

– Carl Sagan

To my family, who encouraged me every step of the way.

To my mentors, who taught me how to be a scientist and professional.

To my friends, near and far, who supported me and lifted me up.

To my cat, who I just wanted to include in here.

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ACKNOWLEDGEMENTS

When I decided to apply to graduate school in 2013, I truly had no idea what I was in for. The past five years have been the most formative years of my life, and I truly cannot thank enough the people in my life who got me here.

First I would like to acknowledge my advisor, Michael Fanselow. Michael, thank you for giving me the intellectual and professional freedom to discover myself as a scientist and as an individual. Your mentorship has given me the confidence to navigate every problem I encounter.

To my committee, thank you for your support and intellectual contributions. Your intelligence and passion for neuroscience continues to inspire me. I would particularly like to thank Pamela Kennedy for her personal and professional support throughout graduate school. I truly left every conversation we had feeling better than going into it. To Jenny Lee, thank you for being the *best* SAO on campus! Your support as a student advisor keeps us all afloat. To Felix Schweizer, thank you for your leadership with the BRI and the NSIDP. Your positivity is contagious and your enthusiasm for graduate education makes the NSIDP such an amazing program.

To my other mentors, Kristy Sherrer, Colleen Callahan, and notably Annie Maxfield, I cannot thank you enough for your guidance through my graduate education. I am so fortunate to have been surrounded by a group of successful women who fearlessly take on the world. Annie, thank you for supporting me through the tough times and celebrating me during the good times. You are the definition of a friend and mentor.

To my friends at AWiSE, BSC, Knowing Neurons, the Career Center, the Science Policy Group and more, thank you all for your encouragement and friendship. To my SciPol friends across the country, meeting such a vibrant community has been one of the true joys of graduate

school. I cannot wait to see where you all go; the world needs your talent and passion. Kate, Kendra, Wenting, Martina, Catharine, Maria, Zoe, Elizabeth, Richard, Drew, Holly, Brian, Ellen, and so many more – thank you for being a spark in my life.

To the Fanselow lab, past and present, I thank you wholeheartedly for your friendship, mentorship, and support. All of you are amazing and unique individuals, and I am honored to have worked beside you (and, briefly, a squirrel). To Sarah Hersman, thank you for your guidance and mentorship in my early years. To Jenn Perusini, thank you for the lessons in SEFL and GluA1. To Zach Pennington, thank you for teaching me probably 80% of what I know. Your candor will never be forgotten. To Abha Rajbhandari, thank you for the experimental and intellectual support (and healthy desserts!). To Annie Hoffman, thank you for the long talks and support. To Sarah Gonzalez, thank you for your patience in sharing our office, and for being a true friend and labmate. To all my collaborators, particularly Eric Harvey, thank you for the pleasure of working with you. To my undergraduate assistants, Melissa Liu and Dickson Chen, thank you for your dedication and help with experiments.

Thank you to the amazing students of the NSIDP, past and present. I couldn't have asked for a better group of individuals to be surrounded by in my graduate program. I would specifically like to thank my cohort, many of whom I am honored to call such good friends. Janelle, thank you for the always exciting adventures and general debauchery. Courtney, David, Sophie, Cynthia, Andrew, Elliot and Allison, thank you for everything.

This dissertation work was supported by The Staglin Music Festival Center for Brain and Behavioral Health (MSF), the ARCS Foundation Fellowship (JET) and the UCLA-NIH Neural Microcircuits Training Grant (JET).

To my family, thank you for the *many* freak outs and late night phone calls, the life advice, the "how to be an adult" lessons, and supporting me unconditionally. I am so thankful and fortunate to have you in my family. To my mom, words cannot express how lucky I am to call you my mother. You taught me everything from loving Star Wars to parenting a cat. To my dad, thank you for the inspiration you have given me. Not many have had such a successful and happy career as a scientist, and I look up to you every day. To my brother, thank you for the endless surprises and friendly sibling competition. I love you all so much.

To my friends, no matter where you are, you are always in my heart. To Jessica and Virginia, it always feels like middle school with you two (in the best way possible)! Jessica, your passion and kindness inspires me every day. Virginia, you are vivacious and a true gem. To Shelby, thank you for being such a great listener and friend, and from talking me out of bangs more times than I can count. To Elizabeth, thank you for your unconditional friendship, sushi, and sports adventures. To Pascal and Kaycie, thank you for your support, love, and all the happy hours and brunches. To Gilly, thank you for your friendship – and for introducing me to country music. To all my friends, near and far, your support has gotten me through the hard times and allowed me to celebrate the good ones.

"Find a group of people who challenge and inspire you; spend a lot of time with them, and it will change your life."

- Amy Poehler

Chapter 2 includes data, figures, and analysis collected by Jennifer Tribble.

Chapter 3 includes data, figures, and analysis collected in collaboration with Eric Harvey, Melissa Liu and Dickson Chen.

Chapter 4 includes unpublished data collected in collaboration with Abha Rajbhandari and Sarah Gonzalez.

VITA

2009-2013	BS in Chemistry and BS in Microbiology, The University of Texas at Austin
2012-2013	Undergraduate Research Fellow, Drew Lab, The University of Texas at Austin
2013	Lewis Scholar in Microbiology, The University of Texas at Austin
2014-2017	Advancing Women in Science and Engineering Coordinator, UCLA
2014-2018	ARCS Scholar
2015-2017	STEM Graduate Career Consultant, Career Center, UCLA
2015-2018	Teaching Assistant, Departments of Psychology and Neuroscience, UCLA
2016-2017	UCLA-NIH Neural Microcircuits Training Grant Recipient
2017-2018	Biological Sciences Council Treasurer, UCLA
2017-2018	Science Policy Group at UCLA Co-Founder and President, UCLA
2018	Grad Slam Semi-Finalist, UCLA
2018-2019	Early Career Policy Ambassador, Society for Neuroscience

Select Publications and Presentations

Tribble JE and Fanselow MS. Pair Housing Rats Does Not Protect from Behavioral Consequences of an Acute Traumatic Experience. In Preparation.

Rajbhandari AK, **Tribble JE**, and Fanselow MS. Neural Circuits of Fear Memory. Learning and Memory: A Comprehensive Reference, 2nd edition. January 2017.

Fehlhaber K, Frohlich J, **Tribble JE**, Ramanathan A, and Lee J. Knowing Neurons: A creative neuroscience education website by young neuroscientists. Society for Neuroscience Annual Meeting, San Diego. 2016.

Tribble JE, Rios MB, Chen DT, Liu MA, and Fanselow MS. Classifying an emergent hippocampal learning sex difference in pre- and post-pubescent rats. ARCS Luncheon, Los Angeles. 2016.

Tribble JE, Perusini JN, Zelikowsky M, Fanselow MS. 2014. Effects of single- versus pair-housed rats on fear sensitization and baseline anxiety following acute traumatic stress.

Hoffman AN, Rajbhandari AK, **Tribble JE**, Pennington ZT, Perusini JN, Wascheck J, Fanselow MS. Amygdala AMPA receptor subunit specificity underlying fear sensitization following acute traumatic stress.

CHAPTER 1

General Introduction

FEAR AND ANXIETY

Fear and its associated behaviors are one of the most highly conserved emotional responses across animal phylogeny. Unlike many other emotional behaviors, making a successful and appropriate "fear response" can be the difference between life or death, giving it particular weight and evolutionary pressure. The animal that successfully avoids a dangerous situation will go on to procreate; the animal that succumbs to a dangerous situation will not. Taken together, fear can be operationally defined as the set of behaviors, neurobiological and neurochemical responses made to a proximal threat.

Due to its conserved nature, we can effectively model fear in animals in order to understand the neural mechanisms that underlie fear learning and memory in humans. We can use Pavlovian, or associative, conditioning to elicit conditioned fear responses in rodents. In Pavlovian conditioning, a conditional stimulus (CS; often a context or tone) is associated with an unconditional stimulus (US; often a footshock) such that the animal links the previously neutral CS with the aversive US. With adequate conditioning, the animal will exhibit a conditional response (CR; freezing, among others) to the CS presentation alone. The process of acquiring this CS-US relationship that allows a CR to occur is known as fear acquisition. This mechanism entails plasticity in different brain regions of the fear circuit. Specifically, plasticity in the basolateral amygdala (BLA) is required for an animal to learn a fear CS-US association (Maren et al., 1996). For a more complete description of the neural mechanisms underlying fear learning and memory, see the subsection "Neural Basis of Fear".

Although engaging in a fear response in a dangerous situation is adaptive, fear responses can become maladaptive if they are exhibited at either inappropriate times or at an inappropriate magnitude. Understanding the underlying mechanisms of adaptive and maladaptive fear responses becomes particularly important in clinical diagnoses of fear-related disorders such as anxiety.

However, a fundamental question that arises is how are fear and anxiety differentiated? It is thought that fear is a response to a specific situation or target while anxiety is a less specific response (Perusini and Fanselow, 2015). For example, in fear conditioning a “fear” response is often denoted by an animal’s response to a specific CS, such as a trained tone or context, while an ”anxiety” response can be measured by less specific responding, such as to a novel context where the animal has never experienced shock. When discussing fear- and anxiety-related disorders, such as Post-Traumatic Stress Disorder, differentiating between fear and anxiety is necessary for understanding symptoms and the neural mechanisms underlying these phenotypes.

One model that is particularly useful for differentiating fear and anxiety is the predatory imminence theory (Fanselow et al., 1988). In this model, there are three distinct stages of threat and associated behaviors that fall along a continuum: pre-encounter, post-encounter, and circa-strike. An animal is in a specific predatory imminence stage as determined by proximity (spatiotemporal distance) to the predator (Fanselow, 1989). In pre-encounter situations, which best characterize anxiety, there is a potential to encounter harm but this harm is either distant or has a low probability of occurring (Perusini and Fanselow, 2015). One would expect anxiety-like responses in pre-encounter situations. When harm becomes either more proximal or has a higher probability of occurring, animals transition into the post-encounter mode, which best characterizes fear (Perusini and Fanselow, 2015). This occurs when a threat in the environment

is detected or quickly approaching, and would lead to fear responses including freezing. The final stage, circa-strike, occurs when a predator makes contact with the animal and best characterizes panic behavior (Perusini and Fanselow, 2015). Responses generated during the circa-strike might include biting or vocalization. By linking behaviors driven by proximity to a threat to anxiety, fear and panic states, we can work toward deciphering how fear and anxiety interact at the level of brain circuits, but produce separable sets of responses in a clinical setting.

While there is overlap between the neural circuitry and some associated behaviors of fear and anxiety, differentiating these behaviors is critical to understand when these systems act adaptively and when these circuits go awry. In a disease such as PTSD, which sensitizes the fear and anxiety circuit to produce a number of maladaptive behaviors, using animal behavior as a model allows us fully comprehend the complexity of the disease.

NEURAL BASIS OF FEAR

The fear circuit, which consists of brain regions critical for learning and storing fear associations, has been well-defined and studied across decades of research. I will discuss the role of these brain regions – the amygdala, hippocampus, medial prefrontal cortex (mPFC) and periaqueductal gray (PAG).

Amygdala

The amygdala, named for its almond shape, is a crucial brain region in the fear circuit. Within the amygdala, the basolateral amygdala (BLA) and central amygdala (CeA) contribute greatly to an organism's ability to produce behaviors associated with fear and learn fear associations. The BLA receives sensory input from cortical and subcortical (e.g. thalamus and hippocampus) regions and integrates these sensory modalities into CS configurations, allowing

the animal to associate these CS's with an aversive US (footshock) (LeDoux et al., 1991; Orsini et al., 2011). Generally, afferents (presynaptic neurons) that innervate the amygdala code for and are activated by a particular CS. When those afferents are activated in concert with a footshock (US) delivery, which allows post-synaptic activation, this synapse is strengthened via *N*-methyl-*D*-aspartate (NMDA)-dependent long-term potentiation (LTP) (Fanselow and Kim, 1994). LTP, driven by NMDA-R activation, allows for a specific CS-US relationship to strengthen such that subsequent presentation of the CS without a US will cause the animal to exhibit a fear response. Critically, pharmacological inactivation of NMDA-R's in the BLA has been shown to block both contextual and cued fear conditioning (Miserendino et al., 1990; Fanselow and Kim, 1994; Rodrigues et al., 2001). The BLA is uniquely poised to serve as the site of CS-US convergence due to its ability to integrate CS-coding sensory information from cortical and subcortical regions with US-activated post-synaptic cellular responses.

There is a heterogeneous population of cells within the BLA that form complex networks of excitatory and inhibitory cells in this region. Different subregions of the BLA - basal (BA) and lateral (LA) subnuclei – can be differentiated both functionally and anatomically (Swanson and Petrovich, 1998; Fanselow and LeDoux, 1999). The LA is the major site of plasticity in auditory fear conditioning and receives input from both the medial geniculate nucleus (MGN) of the thalamus and auditory cortex (Li et al., 1996). Studies using a variety of techniques have implicated the LA as the initial site of cued CS and US convergence. For example, combining CS presentation and optogenetic activation of LA neurons in place of a footshock is sufficient to support cued fear conditioning (Johansen et al., 2010). Additionally, the immediate early gene (IEG) Arc has been shown to be upregulated in the LA after tone fear conditioning compared to controls, and blocking Arc translation through an antisense oligodeoxynucleotide (ODN) prevents the formation of long-term auditory fear memories (Ploski et al., 2008). Unlike afferents

carrying information about tone CS's, contextual CS's are thought to be represented by afferents from hippocampal and cortical regions innervate the BA, where they functionally activate neurons that are driven by the US. Specifically, the BA receives input from the ventral hippocampus (VH) and prelimbic cortex (PL) of the mPFC (Orsini et al., 2011). Regardless of the initial site of CS-US convergence (LA for cued conditioning and BA for contextual conditioning), this information is carried downstream out of the BLA to the CeA in order to generate the appropriate fear responses.

The CeA, comprised of both the lateral (CeL) and medial (CeM) subdivisions, is the major output region of the amygdala via downstream projections to brainstem regions to promote fear behavior such as freezing (Fanselow and LeDoux, 1999; Duvarci and Pare, 2014). The CeA is composed primarily of GABAergic medium-spiny neurons (striatum-like), which distinguishes the CeA from the nearby BLA which has substantially higher levels of excitatory neurons than inhibitory cells (McDonald, 1982). Information leaves the BLA to the CeA through one of two pathways – the BA projects to the CeL, and the LA projects to the CeM, which is the major output of the CeA to areas including the hypothalamus, midbrain and brainstem (LeDoux et al., 1988; Rizvi et al., 1991; Pitkänen et al., 1997; Paré et al., 2004). The connections from the BLA to CeA can either be direct or through a group of cells called the intercalated cells (mICCs) which modulate activity between the BLA and CeA (Pitkänen et al., 1997; Paré et al., 2004). The CeA also receives direct cortical input without connections through the BLA (McDonald, 1998). The CeA modulates fear behaviors through a variety of inhibitory and disinhibitory mechanisms, which ultimately lead to CeM excitatory output to other brain regions that modulate fear behaviors.

One crucial output of the CeM is to the PAG, a brain region important for driving freezing behavior (Rizvi et al., 1991). The PAG can be anatomically and functionally divided into dorsal

(dPAG) and ventral (vPAG) subdivisions with the vPAG primarily responsible for driving freezing behavior (Bandler and Shipley, 1994). Interestingly, the dPAG seems to be involved in expressing innate (unconditioned) behaviors (Kim et al., 1993; Kim et al., 2013). Taken together, the CeA and PAG are both critical brain regions in producing fear-related behaviors including freezing.

Hippocampus

When animals form contextual fear memories, the CS (context) is a complex combination of stimuli that must be integrated into a cohesive representation, the "context". Although the BLA serves as the primary site of CS-US convergence, the BLA is not responsible for forming the contextual representation; formation of the contextual representation occurs in the hippocampus (HPC) (Fanselow, 2000). The hippocampus can be functionally and anatomically subdivided into dorsal (dHPC) and ventral (vHPC) regions with distinct roles and connections (Fanselow and Dong, 2010). Broadly, decades of research on memory across both humans and non-human subjects have suggested that the HPC is important in forming and retaining new memories as hippocampal damage can produce both retrograde and anterograde amnesia.

The dHPC is critical for forming contextual representations, which is necessary for animals to then associate with a footshock. Evidence of the dHPC's role in forming contextual memories comes from studies that show disruption of the dHPC prevents animals from context fear conditioning (Maren et al., 1997; Zelikowsky et al., 2013). Although it seems as if the dHPC is the brain's preferred mechanism for forming contextual representations, there are compensatory mechanisms for forming contextual configurations in the absence of a dHPC (Zelikowsky et al., 2014). These compensatory mechanisms seem to be driven by mPFC

activity, specifically in the infralimbic (IL) and prelimbic (PL) regions (Zelikowsky et al., 2014). While the dHPC is important in effective context conditioning, it is not required for cued conditioning.

Unlike the dHPC, which is important for context but not cued fear conditioning, the vHPC plays a more general role in fear and anxiety behaviors. Importantly, the vHPC is poised to play a critical role in fear processes via direct connections to the prefrontal cortex, bed nucleus of the stria terminalis (BNST) and amygdala, making it poised to play a critical role in fear processes (Swanson and Cowan, 1977; Jay and Witter, 1991). Conditioning to both a context and cue is disrupted by damage to the vHPC, and selective lesions of the vHPC produce anxiolytic behavior on the elevated plus maze (Kjelstrup et al., 2002; Maren and Holt, 2004). Whereas the dHPC seems to be critical for forming contextual representations of environments, but not necessarily assigning emotional valence to the representation, the vHPC plays a more general role in emotional regulation, and hence of fear and anxiety (Fanselow and Dong, 2010).

Prefrontal Cortex

The medial prefrontal cortex's (mPFC) role in fear is primarily driven by two subdivisions: the infralimbic (IL) and prelimibic (PL) cortices. Within the fear circuit, the mPFC is bidirectionally connected to the BLA and hippocampus, making it an important regulator of fear behaviors (Marek 2013). The dorsal mPFC, PL, is involved in the expression of fear as sustained activity in the PL has been shown to augment fear expression (Fenton et al., 2014). Interestingly, lesioning the dorsal PL caused an increase in cued and context fear conditioning which indicates that dorsal PL lesions produce a non-specific increase in fear expression (Morgan and LeDoux, 1995). In the same study, lesions of the more ventral mPFC, IL, had no effects on acquiring cued or contextual fear but did reduce extinction learning selectively to the

cue (Morgan and LeDoux, 1995). Unlike the PL, the IL has been implicated primarily in extinction processes as activity in the IL has been shown to play a role in consolidating extinction memories (Quirk et al., 2000). There have been reports of increases, decreases, and no changes in fear learning after mPFC damage which questions the notion that the PL regulates fear expression while the IL is responsible for regulating fear suppression (extinction) (Giustino and Maren, 2015). Despite its potentially complex role, the mPFC has a critical part in fear regulation and expression.

There are many neural systems and circuits implicated in modulating fear and anxiety states. One such system of interest is the kappa opioid system, which consists of kappa opioid receptors (KORs) and its endogenous ligand, dynorphin. KORs and dynorphin are enriched in brain regions associated with fear and anxiety including the hippocampus (HPC), prefrontal cortex (PFC), basolateral amygdala (BLA), central amygdala (CeA), and bed nucleus of the stria terminalis (BNST) (Crowley and Kash, 2015). When activated, KORs lead to a cascade of intracellular events including activation of MEK, ERK, and MAPK pathways, which are important for cellular plasticity. There is some evidence that KORs are involved in regulating aspects of fear and anxiety, as delivery of a KOR antagonist directly into the BLA reduced fear potentiated startle (Knoll et al., 2011). Delivering a KOR antagonist has also been shown to produce anxiolytic effects as measured by increased time spent on the open arms of the elevated plus maze compared to saline-treated animals (Knoll et al., 2007).

Taken together, the amygdala, PAG, hippocampus and prefrontal cortex act as important players in the fear circuit and the activity and functional connectivity between these regions is critical for fear and anxiety regulation and expression.

POST-TRAUMATIC STRESS DISORDER (PTSD)

Post-Traumatic Stress Disorder (PTSD) is a debilitating disease that affects a number of individuals after experiencing a traumatic event. Like many other psychiatric diseases, symptoms of PTSD are complex and multi-faceted which makes the disease difficult to diagnose and treat. Additionally, not every individual who experiences a stressor will go on to develop PTSD; estimates vary, but generally 5 – 20% of individuals who undergo trauma will go on to develop PTSD (Perrin et al., 2014). Resilience and susceptibility to developing PTSD remains a complex and widely investigated area of study with likely involvement of a multitude of environmental and genetic components involved in this underlying susceptibility. PTSD susceptibility also seems to have a sex-dependent component as it is estimated to be twice as prevalent in women compared to men (Perrin et al., 2014).

PTSD was first described as a psychiatric disorder in 1980 within the American Psychiatric Association's (APA) *Diagnostic and Statistical Manual of Mental Disorders (DSM-III)* (Diagnostic and Statistical Manual of Mental Disorders, 1980). In the current DSM-V, the definitions and diagnoses of PTSD have changed considerably including re-classifying the disorder from an "Anxiety Disorder" into a new category called "Trauma and Stressor-related Disorders" (Pai et al., 2017). In DSM-V, there are 8 criteria listed as associated with PTSD: (A) exposure to a stressor, (B) intrusion symptoms, (C) avoidance symptoms, (D) negative affective symptoms (cognition and mood), (E) hyperarousal or hyperreactivity, (F) symptoms last for longer than 1 month, (G) symptoms negatively impact the individual with PTSD, and (H) symptoms are not due to medication, other illness or substance use (Diagnostic and Statistical Manual of Mental Disorders, 2013). Within each of these criteria there are a number of symptoms or circumstances that constitute fulfilling the criterion requirement. For example, Criterion A, exposure to a stressor, can be fulfilled by an individual suffering a stressor directly –

through combat, sexual assault, car accident, etc. – or through indirect exposure to a stressor such as witnessing a traumatic event . The wide array of criteria combined with the heterogeneity of fulfilling these criteria makes PTSD an extremely complex disease. Undeniably, the complexity of this disease has made diagnosing and treating the disease particularly difficult.

Understanding the genetic and environmental contributions to developing PTSD is a critical question in clinical psychology today. However, the complexity of the disease and its associated symptoms makes it unlikely that there are a small number of impactful genes that contribute to the disease; instead, it seems more plausible that there are a large number of genes with small contributions that together determine an individual's genetic susceptibility to developing PTSD. Many studies have presented a strong case for the heritability of PTSD (up to 30-50% heritability was found in some studies) through twin studies and parent-offspring transmission (Smoller, 2016). A variety of genetic components have been found to contribute to PTSD's heritability including but not limited to genetic variants in neurotransmitter, neuropeptide and receptor genes such as the opioid receptor-like 1 gene (*OPRL1*) and pituitary adenylate cyclase-activating polypeptide (PACAP) (Ressler et al., 2011; Andero et al., 2013; Smoller, 2016). The strongest of these associations has been seen both as a correlative measure in humans and proven for causation in an animal model; both *OPRL1* and PACAP fit these categories. Further investigation into the genetic basis of PTSD is necessary but difficult as these studies are often under powered and might miss critical genes with a small but significant contribution to PTSD heritability.

Alongside genetic components, environmental factors contribute greatly to an individual's susceptibility to developing PTSD following a traumatic event. These environmental factors include but are not limited to the type of trauma encountered and the individual's history

regarding stress and traumatic events. For example, PTSD risk among soldiers was found to increase in a dose-responsive manner with the length served or intensity of combat (Goldberg et al., 1990). Other environmental factors including low education, prior trauma exposure or psychological disorders, discharging a weapon, and witnessing trauma can serve as risk factors to developing PTSD after encountering a stressor (Xue et al., 2015). Generally, risk factors for PTSD can be divided into pre-trauma factors (prior stress or psychiatric disease, gender, socioeconomic status), peri-trauma factors (type and severity of trauma, initial reaction to trauma), and post-trauma factors (social support, treatment) (Kirkpatrick and Heller, 2014). Further investigation into these risk factors and how they contribute to an individual's susceptibility to developing PTSD could be hugely beneficial in diagnosing and understanding the disease.

Studying PTSD in the human population offers a unique insight into the genetic and environmental factors (G x E interactions), such as social vulnerabilities and stressors, that can contribute to developing the disease. Through large-scale analysis of genetic variants and careful environmental tracking, researchers have been able to disentangle some of the mystery as to why only a percentage of a population that encounters a traumatic event will go on to develop PTSD.

There are many theories, both biological and psychological, to explain how trauma can lead to the debilitating symptoms associated with PTSD. A common thread between these theories is that exposure to a traumatic stressor changes neural circuits and mechanisms to cause behavioral symptoms. Alterations in the hypothalamic-pituitary-adrenal (HPA) axis, which is intimately involved in regulating the stress response of an organism, have been suggested as a mechanism of PTSD due to hyperactivity following a traumatic event (de Kloet et al., 2006; Jones and Moller, 2011). Other lines of evidence point to changes in the limbic circuitry focusing

on the amygdala, hippocampus and medial prefrontal cortex. Human studies have indicated hyperresponsivity in the amygdala in PTSD patients when prompted with fear or stress stimuli including fearful facial expressions (Rauch et al., 2000; Shin et al., 2005). In the prefrontal cortex, studies have found decreased volumes of anterior cingulate cortex (ACC) and lower medial prefrontal cortex (mPFC) activation in humans with PTSD compared to healthy controls (Shin et al., 2004; Woodward et al., 2006; Gold et al., 2011). Finally, some studies indicate a reduction in hippocampal volume or activity in PTSD patients compared to healthy controls (Bremner et al., 1999; Gilbertson et al., 2002). In humans, PTSD symptoms have been tied to changes in limbic circuit brain regions including the amygdala, hippocampus and medial prefrontal cortex, as well as alterations in the HPA axis, which guide an individual's stress response.

PTSD is a complex and debilitating disease that manifests following experience with a traumatic event. Symptoms of PTSD include exaggerated fear responding, anxiety and depression, social isolation, increased risk of substance use disorder, difficulty sleeping, hyperarousal and flashbacks to the trauma. There are many genetic and environmental components underlying risk factors to developing PTSD, and studies within the human population have led to a variety of theories regarding PTSD's mechanism in the brain – primarily that brain regions in the limbic circuit become either hyperactive (amygdala) or hypoactive (hippocampus and prefrontal cortex) following trauma. However, we do not yet understand what specific changes in the brain lead to various PTSD symptoms, which is a major hurdle in effectively treating the disorder.

ANIMAL MODELS OF PTSD

Given the complexity and heterogeneity of PTSD symptoms, it is critical that animal models of the disease allows understanding the cellular, molecular, and neural circuit dynamics

involved in producing these phenotypes, which can allow effective diagnosis and treatment of the disease. For this reason, many laboratories have developed animal models of PTSD to dissect neural mechanisms, which is not feasible to do in the human population. I will discuss a variety of ways to model PTSD-like behaviors in animals, with an emphasis on how these models have bolstered our understanding of neural mechanisms engaged by stress that produce PTSD-like symptoms, including the Fanselow laboratory's PTSD model, Stress-Enhanced Fear Learning (SEFL).

In order to effectively model PTSD in animals, it is suggested that the chosen stressor must induce these 5 criteria: (1) mirror biological and behavioral PTSD-like symptoms, (2) generate these symptoms in an intensity-dependent manner, (3) the symptoms must persist across time, (4) symptoms must be bi-directional (increase in some phenotypes, decrease in others), and (5) must produce variability across subjects (Yehuda and Antelman, 1993). These 5 categories serve as a minimum qualification for any given animal model of PTSD to serve translational benefit such that these models could elucidate information that will go on to help diagnose or treat the disease in the human population. It is critical to note that any given animal model of PTSD cannot recapitulate *all* of the human symptoms but a powerful model of the disorder should mimic several of the human correlates (hyperarousal, increased fear responses, HPA stress modulations, depressive and anxiety-like behavior, enhanced substance or alcohol use disorder, etc).

PTSD develops following a variety of traumatic experiences, including physical and psychological stressors. Modeling PTSD in animals follows a similar pattern of heterogeneity as these models utilize physical stressors including footshock, which is used in the SEFL model, restraint stress, which involves immobilizing the animal for prolonged periods of time, or a mixture of different stressors, which might include footshock, periods of immobilization, forced

swim, and other stressors (Liberzon et al., 1997; Rau et al., 2005; Kohda et al., 2007; Zoladz et al., 2008). Inducing PTSD-like behaviors through physical stressors has been shown to produce symptoms resembling those seen in the human population (Takahashi et al., 2006; Vanderheyden et al., 2015; Perusini et al., 2016). In addition to physical stressors, some models of PTSD use either social stressors (social isolation or housing instability, social defeat and early life stress) or psychological stressors (predator or predator odor) to produce PTSD-like symptoms in animals (Marais et al., 2008; Zoladz et al., 2008; Mackenzie et al., 2010; Hammels et al., 2015; Rajbhandari et al., 2015; Skelly et al., 2015). When social or psychological stressors are used either in conjunction with physical stressors or independently, these types of stressors can also induce PTSD-like symptoms in animals.

Through our ability to manipulate molecular and cellular processes and alter circuitry in the rodent brain, researchers have been able to identify brain regions, mechanisms, and circuit changes associated with PTSD-like symptoms. Additionally, the variety of stressors that can be used to induce PTSD in animals provides a unique opportunity to validate and replicate neural mechanisms involved in PTSD across not only laboratories but also stressor manipulations. Neural mechanisms altered by drastically different types of stress provide parsimonious and potentially translationally useful insight into this complex disease.

However, animal models of PTSD also have their pitfalls. One critical complication is the fact that not all humans who undergo a stressor will go on to develop PTSD (there is a large amount of inter-individual variance in the human population) but by design, nearly all animals given a stressor develop PTSD-like symptoms. While this lack of inter-individual variance in animal models unarguably provides the power necessary to find effects with various manipulations, it disregards a critical question of developing PTSD in humans: why do some individuals go on to develop PTSD while others remain resilient to the disease? By utilizing

animal models of PTSD, we can characterize the neural mechanisms associated with the disease; however, these animal studies must be conducted in tandem with human studies to address aspects of PTSD that cannot be studied in animals.

NEURAL MECHANISMS OF STRESS

Organisms have developed a conserved mechanism to respond to stressful situations, known as the stress system. The stress system is primarily made up of the hypothalamic-pituitary-adrenal (HPA) axis and the locus coeruleus/norepinephrine-autonomic nervous system, both of which are activated by stress (Nicolaides et al., 2015). There are both central and peripheral nervous system components of the stress system, with many interactions between the two. Additionally, the stress system interacts with other central nervous systems including the dopaminergic reward system and the fear circuit through central amygdala activation (Wanat et al., 2013; Penzo et al., 2015; Farahimanesh et al., 2018). For the purposes of this dissertation, I will focus on reviewing the HPA axis and its regulatory role in the stress response as our model of PTSD involves behavioral changes characterized by neural mechanisms that are dependent on HPA axis activity. As this dissertation work focuses on exposure to an acute stressor, I will focus the neural mechanisms of acute stress.

Generally, stress triggers activation of the hypothalamic-pituitary-adrenal (HPA) axis, which leads to a variety of responses throughout the body. HPA axis activation begins in the paraventricular nucleus of the hypothalamus (PVN), where neurons release corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) into the pituitary gland (Shirazi et al., 2015). CRH release into the pituitary gland triggers the secretion of adrenocorticotropic hormone (ACTH), which acts on the adrenal glands to elicit the broad secretion of glucocorticoids (GCs) (Shirazi et al., 2015). In the case of an acute stressor, GC circulation peaks around 15 – 30 minutes, and GCs typically return to baseline approximately 1 hour after

the stressful event has ended (Sapolsky et al., 1984). The reduction of circulating GCs is due to negative feedback systems through the PVN, pituitary, medial prefrontal cortex (mPFC) and hippocampus (Shirazi et al., 2015).

GCs act on two classes of receptors: mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs). While MR expression is spatially restricted, with the largest expression pattern found in parts of the hippocampus, GRs are highly expressed throughout the brain (Reul and de Kloet, 1985). Areas with the highest expression of GRs include parts of the fear and anxiety circuit including the amygdala and hippocampus (Herman et al., 1989; Patel et al., 2000; Wang et al., 2014). Because MRs have a much higher affinity for binding GCs than that of GRs, most MRs are bound by GCs due to circadian-driven fluctuations in GC release (Shirazi et al., 2015). Thus, stress-driven spikes in GCs tend to drive GCs to bind to GRs.

Activation of GRs in the amygdala has been shown to modify learning and memory processes. For example, stress can lead to remodeling of synapses and dendritic branching, which has been linked to anxiogenic behavior and enhancements in fear conditioning (Mitra et al., 2005; Rau et al., 2005; Mitra and Sapolsky, 2008; Hoffman et al., 2015). In addition, GCs in the amygdala are thought to shift the excitation/inhibition balance toward more excitation by diminishing GABA transmission, which leads to increased firing of glutamatergic neurons (Duvarci and Paré, 2007; Liu et al., 2014b). Finally, stress-mediated increases in GCs have been linked to improved memory consolidation as indicated by enhanced recall of the stressful event (Roozendaal et al., 2008; Finsterwald and Alberini, 2014). Stress-induced GC release in the amygdala has been linked to a variety of modifications in learning and memory processes, including promoting anxiogenic behavior and augmenting consolidation of stressful memories.

Stress-induced releases of GCs also work on GRs in the hippocampus to mediate several responses. Generally, it is thought that acute or mild levels of stress lead to enhancements of long-term potentiation (LTP) and dendritic branching – behaviorally, an improvement in learning and memory – while chronic or high levels of stress reduce LTP and dendritic branching – behaviorally, a deficit in learning and memory (Sapolsky, 2003; Kim et al., 2006; Joëls and Krugers, 2007). This effect might be limited to the dorsal hippocampus, which proportionally has a much higher concentration of GRs than MRs (Robertson et al., 2005). GCs in the hippocampus also regulate adult neurogenesis, though there is disagreement as studies have indicated decreases, increases, and no change in the neural stem cell (NSC) population (Hanson et al., 2011; Kirby et al., 2013; Lucassen et al., 2015). Within the hippocampus, there are alternating effects of acute and chronic stress exposure, in which acute stress tends to augment learning and memory processes while chronic stress reduces learning and memory processes.

CRH release from the PVN acts on other systems beyond sparking the pituitary gland to release ACTH. These systems include regulating stress-mediated anxiety in the bed nucleus of the stria terminalis (BNST) and interacting with emotion, anxiety and learning processes in the amygdala and hippocampus (Kim et al., 2006; Roozendaal et al., 2009; Leuner and Shors, 2013; Roman et al., 2014; Gafford and Ressler, 2015; Gray et al., 2015). These non-pituitary gland processes induced by CRH release are critical in shaping the stress response following a stressor.

Encountering a stressor leads to a variety of neural and behavioral responses, together known as the stress response. This highly conserved response is critical for adaptation and an organism's safety but can also serve to produce maladaptive effects if either chronically active or overly active in certain circumstances. Activation of the stress response leading to

maladaptive behaviors can be seen in a variety of psychiatric disorders including PTSD, some anxiety disorders and depression.

STRESS-ENHANCED FEAR LEARNING (SEFL)

Our laboratory has developed a rodent model of PTSD called Stress-Enhanced Fear Learning (SEFL). In SEFL, animals are given an acute traumatic experience (a series of pseudorandomly delivered 1.0-mA, 1-sec footshocks; 10 for mice, 15 for rats) that induces a variety of PTSD-like phenotypes (Table 1.1). Behaviorally, it is critical that animals exhibit a variety of PTSD-like phenotypes across the fear and anxiety spectrum. For the purposes of this dissertation, I focused primarily on four assays of fear, anxiety and depression after the traumatic experience: (1) enhanced fear conditioning (fear, Chapters 2 - 4), (2) open field (anxiety, Chapters 2 - 3), (3) elevated plus maze (anxiety, Chapter 3), and (4) forced swim test (depression, Chapter 3).

The primary behavioral characteristic of SEFL is enhanced fear to a subsequent mild stressor (1 1.0-mA, 1-sec footshock) delivered in a novel context (Rau et al., 2005). This behavioral phenotype closely matches a symptom of PTSD in which patients exhibit hyper reactivity to a mild stressor (Table 1.1). Importantly, the stress-enhanced fear learning persists across a long duration of time, with rats showing the enhanced fear phenotype up to 3 months after the acute stressor (Rau and Fanselow, 2009). The DSM-V in part describes PTSD by its duration of trauma-induced symptoms, which aligns closely with SEFL's persistent phenotype. Although the commonly used SEFL protocol involves delivering 15 footshocks to rats (10 to mice), subsequent enhanced fear can be seen in animals that are given fewer footshocks as the traumatic experience. When rats are given 4 footshocks rather than 15, most animals still show a sensitized fear response to the subsequent mild stressor (Rau and Fanselow, 2009). Interestingly, the difference between delivering 4 or 15 footshocks as the acute stressor is not in

the magnitude of subsequent enhanced fear, but is instead the probability of an animal developing a PTSD-like phenotype. When 15 shocks are used as the acute stressor, nearly 100% of rats will develop PTSD-like phenotypes; when 4 shocks are used as the acute stressor, significantly fewer rats will develop PTSD-like phenotypes. Thus, the 15-shock protocol is used to ensure a robust development of SEFL.

The SEFL phenotype is thought to be mediated by a general, trauma-induced sensitization of the amygdala resulting in a nonassociative enhancement in subsequent fear conditioning. Previous work in the laboratory strengthens the hypothesized nonassociative nature of SEFL. Critically, SEFL does not depend on retention of the fear memory formed to the trauma context (Poulos et al., 2014). When rats are given the early life stress (ELS) procedure, which involves administration of the acute traumatic experience prior to an online and functional hippocampus (post-natal day 19, or P19), they exhibit amnesia to the trauma context while retaining the SEFL phenotype (Poulos et al., 2014). In addition, animals show enhanced fear to non-contextual CS's and non-shock US's. After trauma, rats will exaggerate fear learning to a tone CS, indicating that SEFL phenotypes are not necessarily due to alterations in associative learning (Rau et al., 2005). Exaggerated fear responses are also not limited to shock US's, as post-trauma rats will also show increased responses to a white noise startle (Perusini et al., 2016). Finally, animals will retain the SEFL phenotype even following extinction to the trauma context (Rau et al., 2005; Long and Fanselow, 2012). Regardless of the extinction procedure (spaced, immediate or massed), reduction of fear to the trauma context did not mitigate subsequent enhanced fear (Rau et al., 2005; Long and Fanselow, 2012). Taken together, these data make a strong argument for the nonassociative nature of SEFL.

Understanding the neural basis of SEFL is critical from both basic science and clinical perspectives. To understand SEFL's mechanism, it is important to differentiate between

induction of SEFL and *expression* of SEFL. SEFL induction refers to the neural changes and mechanisms *during* the 15-shock trauma, which induce a variety of PTSD-like phenotypes (Table 1.1). SEFL expression refers to the neural mechanisms associated with demonstrating the SEFL phenotypes, including exaggerated fear response and other anxiety measures. The temporal distinction between induction and expression of SEFL becomes critical for understanding what neural mechanisms guide aspects of developing and expressing PTSD-like symptoms. From a clinical perspective, this distinction is mandatory.

Unsurprisingly, SEFL induction is dependent on CORT as delivering metyrapone, a CORT synthesis blocker, prior to the acute trauma eliminated the SEFL phenotype (Perusini et al., 2016). Metyrapone was delivered systemically (i.p. injection) across a range of doses (0 mg/kg, 50 mg/kg, 100 mg/kg, and 150 mg/kg) 1 hour prior to the 15 shocks, and rats showed a dose-dependent reduction in SEFL as measured by retained context fear to the 1 shock context (Perusini et al., 2016). Importantly, rats also displayed a dose-dependent reduction in retained fear to the trauma context, indicating that metyrapone does not selectively eliminate SEFL while sparing the memory of the trauma (Perusini et al., 2016). When CORT was co-administered with metyrapone (10 mg/kg, i.p. injection) 1 hour prior to the trauma, the SEFL phenotype was rescued while the memory to the trauma context remained blocked (Perusini et al., 2016). Interestingly, administration of CORT (10 mg/kg, i.p. injection) without metyrapone (0 mg/kg, i.p. injection) and CORT (10 mg/kg, i.p. injection) in the absence of footshocks do not support the SEFL phenotype (Perusini et al., 2016). These findings indicate that CORT is necessary but not sufficient for SEFL.

Based on our understanding of the fear circuit, it seemed likely that CORT acts upon GRs in the BLA to induce SEFL. In one study, GR expression in the amygdala, dorsal hippocampus and medial prefrontal cortex was measured 2 months after ELS. The only brain

region with an increase in GRs (compared to non-traumatized controls) following the acute stressor was the amygdala (Poulos et al., 2014). To directly study the effect of CORT in the BLA during trauma, mifepristone (0.5 μg), a GR antagonist, was delivered via cannula directly into the BLA prior to the 15 shock trauma; mifepristone-treated rats did not exhibit SEFL (Perusini et al., 2016). Thus, prior work on SEFL has identified an upregulation in GRs in the amygdala following an acute stressor. Additionally, GRs are involved *during* the trauma as delivering a GR antagonist to the BLA during the acute stressor eliminates the SEFL phenotype. Further studies are needed to understand the contribution of non-amygdala brain regions, including the hippocampus and prefrontal cortex, to SEFL induction.

Parsing out the mechanisms of SEFL expression is a mandatory step toward developing intervention techniques for patients with PTSD. Given the variety of PTSD-like phenotypes seen in our model, it seems unlikely that there is a singular mechanism responsible for the gamut of subsequent phenotypic changes (Table 1.1). Thus far, most of the work to understand the neural mechanisms of SEFL expression are limited to the enhanced fear learning phenotype. Specifically, our laboratory has discovered that there is a selective increase in the GluA1 AMPA-R subunit in the BLA following the acute stressor (Perusini et al., 2016). There is no such increase in either the GluA2 AMPA-R subunit or the GluN1 NMDA-R subunit (Perusini et al., 2016). The GluA1 amplification in the BLA persists across multiple time points after trauma, and this increase is mitigated by pre-trauma administration of metyrapone (Perusini et al., 2016). Critically, GluA2-lacking AMPA-R's have a relatively high permeability for calcium relative to GluA2-containing AMPA-R's, and differential expression of AMPA-R subunits can drastically affect synaptic efficacy and neuronal survival (Hollmann et al., 1991; Liu and Zukin, 2007). It is important to note that fear conditioning generally results in a shift from GluA2-containing AMPA-R's to GluA2-lacking AMPA-R's; however, this transition is time-dependent, with the switch

peaking 24 hours after conditioning and subsiding by 1 week following conditioning (Jarome et al., 2012). Contrary to these findings, GluA1 remains upregulated in the amygdala beyond this 1 week time point (Perusini et al., 2016). Further studies will need to be done to understand what mechanisms lead to this persistent increase in GluA1 in the amygdala, and what cell types are primarily affected (excitatory or inhibitory).

There has always been an interest in using SEFL to understand how pharmacological manipulations, which could be used in the human population, can affect the observed PTSD-like phenotypes. However, prior studies in the laboratory have primarily failed to find pharmaceuticals that eliminate SEFL. One study tested the effect of three drugs on SEFL: midazolam (anxiolytic and amnestic compound, positive modulator at GABA-A receptors), propranolol (anxiolytic, beta-adrenergic antagonist), and allopregnanolone (anxiolytic and amnestic compound, positive allosteric modulator of GABA-A receptors) (Long et al., 2011). Each of these pharmaceuticals has been linked to stress and/or PTSD in animals and/or humans, making them worthwhile candidates to explore (Rodríguez Manzanares et al., 2005; Pibiri et al., 2008; Bali and Jaggi, 2014; Nagaya et al., 2015; Staff, 2015; Ronzoni et al., 2016; Brunet et al., 2018). Contrary to these findings, the tested compounds did not mitigate SEFL when administered 20 minutes prior to the 15-shock trauma (Long et al., 2011). It is possible that the pharmacological manipulations described above would have impacted other PTSD-like symptoms seen after an acute stressor (changes in open field, elevated plus maze, and forced swim test performance), however these studies only probed for a mitigation in the enhanced fear conditioning to a mild stressor.

SEFL is a robust model of PTSD that mimics the behavioral, biochemical, and neuroanatomical mechanisms observed in the human population. Using SEFL to characterize the behavioral phenotypes and their associated neural mechanisms could offer invaluable

insights to understanding and eventually treating those with PTSD. Additional investigations into the underlying mechanisms of SEFL, particularly to parse the role of non-amygdala brain regions in the fear circuit, are critical to furthering our understanding of this animal model of PTSD.

DISSERTATION OBJECTIVE

The objective of this dissertation is to characterize the behavioral and neural mechanisms underpinning SEFL. Through probing for PTSD-like phenotypes expanding past enhanced fear conditioning to include other measures of anxiety (open field, elevated plus maze) and depression (forced swim test), I comprehensively distinguished the phenotypes that may develop following an acute stressor. As PTSD is an extremely phenotypically complex disease, it is critical to incorporate multiple measures of PTSD-like phenotypes when using an animal model of the disease. Additionally, it is likely that these differing behavioral symptoms share some underlying neural mechanisms, but also has divergent, independent mechanisms of action.

In Chapter 2, I test for the role of housing condition in the development of SEFL. Prior to my work, all SEFL studies utilized isolation housing prior to and during the experiment. As isolation housing is a stressor in and of itself, I tested for whether isolation housing was necessary for the development of SEFL. In Chapter 3, I test for the role of the kappa-opioid receptor (KOR) in SEFL by using the KOR antagonist JDTic. KORs have been implicated in models of fear and anxiety, and have potential translational benefit as a clinical therapeutic (Knoll et al., 2007; Knoll et al., 2011; Helal et al., 2017). In Chapter 4, I use a variety of techniques to characterize the neural mechanisms underlying SEFL. The emergence of sophisticated techniques in behavioral neuroscience, including activity-dependent cell labeling and manipulation, has allowed for the advancement of understanding neural dynamics

associated with specific behaviors. This work will undoubtedly shed light on the functional circuitry and molecular mechanisms underpinning SEFL. Finally, in Chapter 5, I make general conclusions and offer further directions based on this dissertation.

PTSD Symptom	SEFL Parallel	Source
Hyper-reactivity to mild stress, lasting at least 90 days	Increased freezing to 1 shock or loud noise	*Rau et al, 2005*
Propensity to form new fears	Increased cued and contextual fear	*Rau et al, 2005*
Anxiety	Heightened anxiety, decreased locomotion in the open field, reduced open arm time in the elevated plus maze	*Perusini, 2015* *Tribble, 2018 (unpublished)*
Co-morbid alcohol and drug abuse	Increased voluntary alcohol consumption	*Meyer et al, 2013*
Symptoms present > 30 days post trauma	> 90 days	*Rau and Fanselow, 2009*
Increased startle reactivity	Hyper-reactivity to loud noise	*Perusini, 2015*
Co-morbid depression	Increased immobility time in the forced swim test	*Perusini, 2015*

Table 1.1 Parallels of PTSD symptoms and SEFL phenotypes.

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CHAPTER 2

Pair Housing Rats Does Not Protect From Behavioral Consequences Of An Acute Traumatic Experience

ABSTRACT

Post-Traumatic Stress Disorder (PTSD) is an extremely debilitating disease with a broad array of associated symptoms, making the disorder difficult to diagnose and treat. In humans, patients seem to benefit from group therapy or other means of promoting social behavior. To test these effects on our rodent model of PTSD, rats were housed in either single or pair conditions prior to and during an acute stressor to induce PTSD in these rats. Subsequently, rats were assessed for PTSD-like symptoms in order to determine the effect of social housing on stress-induced phenotypes. Post-trauma phenotypes, including enhanced fear conditioning and anxiolytic behavior, persisted regardless of the animal's housing condition. It is possible that any housing driven improvements to stress-induced phenotypes would require longer periods of pair housing than were used in these experiments. Although PTSD patients show improved health outcomes following social interaction or group therapy, the fear and anxiety phenotypes seen following an acute stressor in an animal model of the disease endured despite an animal's housing condition.

INTRODUCTION

Fear is a set of highly conserved neural and behavioral mechanisms that are critical for a living organism's survival. When kept at adaptive levels, fear allows an organism to respond appropriately to threats in their environment. However, in psychiatric diseases including Post-Traumatic Stress Disorder (PTSD), exposure to a traumatic event produces symptoms and behaviors in which the fear circuit sensitizes to generate maladaptive responses. These responses include exaggerated fear to mild threats, increased anxiety and depression, and other changes in affective mood (Fani et al., 2012; Spinhoven et al., 2014; Acheson et al., 2015; Powers et al., 2015).

Interestingly, PTSD and social anxiety disorder (SAD) can exhibit a high level of comorbidity, leading to social isolation among PTSD patients (McMillan et al., 2014; McMillan and Asmundson, 2016). It has also been suggested that co-morbidity of PTSD and SAD can heighten other PTSD symptoms, including exaggerated fear responses, depression and anxiety (McMillan et al., 2014). In fact, social isolation ranks highly among problems that PTSD patients hope to overcome when beginning treatment (Rosen et al., 2013). Including techniques to reduce social isolation or adding group therapy components could be particularly important for the efficacy of PTSD treatment (Echeburúa et al., 2014; Ellis et al., 2014; Resick et al., 2015).

In animal models, isolation housing can be used as a model of chronic stress. Social isolation in animals has been shown to produce PTSD-like phenotypes including enhanced contextual fear conditioning and impaired fear extinction (Pibiri et al., 2008; Zelikowsky et al., 2018). Many factors including the sex of the animal (male vs. female), the age at which the animal is housed in isolation (adolescent vs. adulthood) and the measure being analyzed (fear vs. depression vs. anxiety) all seem to play a critical role in determining the effect of isolation housing, often yielding results in disagreement (Pibiri et al., 2008; Arndt et al., 2009; Garrido et

al., 2013; McCormick et al., 2013; Skelly et al., 2015). Conversely, group housing animals after a stressful event has been shown to mitigate anxiety and depression phenotypes (Liu et al., 2013).

Our laboratory has developed a rodent model of PTSD called Stress Enhanced Fear Learning (SEFL), in which animals undergo an acute traumatic experience which leads to a variety of maladaptive behavioral consequences (Rau et al., 2005). These behaviors include an exaggerated fear response to a mild stressor and increased baseline anxiety (Poulos et al., 2015; Perusini et al., 2016). To date, all studies conducted to understand the behavioral consequences of SEFL have involved animals who are housed in isolation. As isolation housing can serve as a powerful stress inducer, it is critical to understand if isolated housing conditions are necessary for SEFL or in some way interacts with the SEFL procedure.

Here, we describe the behavioral portfolio of post-trauma SEFL rats in either single or pair housed conditions. We have previously shown the drastic effects of an acute traumatic experience in singly housed rats without demonstrating necessity of isolation housing in producing these maladaptive behaviors. The effect of housing condition on SEFL behaviors was tested through analyzing subsequent enhanced fear learning, changes in anxiety (open field), and extinction to the context in which animals received a mild stressor. We subsequently analyze the contribution of housing condition to generalization in a novel context following the acute stressor.

RESULTS

Experiment 1: Social housing does not alter the context memory of trauma, stress-enhanced fear learning, or generalization to a novel context.

Trauma Context

To test whether housing condition affected memory to the trauma context, animals were placed back in the trauma context for 8 minutes, one day following exposure to the acute traumatic event (15 or 0 unsignaled footshocks). Animals that experienced the 15 footshocks showed increased levels of fear compared to animals that were not shocked in the context (Stress: $F_{(1, 26)} = 209.16$, $p < 0.001$; Fig 2.1b), but there was no effect of housing condition on contextual fear (Housing: $F_{(1, 26)} = 0.24$, $p = 0.63$; Stress x Housing: $F_{(1, 26)} = 1.92$, $p = 0.18$; Fig 2.1b). Housing condition does not affect contextual fear of the trauma-associated context.

Stress-Enhanced Fear Learning

Previous findings indicate that traumatized animals show enhanced fear to a mild stressor in a novel context (Rau et al., 2005). However, it was previously unknown whether housing condition interacted with this enhanced fear learning as all prior studies were conducted in single housed animals. To understand whether housing condition interacted with the post-trauma sensitization phenotype, single and pair housed animals underwent a mild stressor in a novel environment (1 Shocks in Context B). One day later, animals were placed back in the 1 shocks context for 8 minutes to assess contextual fear. Stressed animals showed enhanced fear in this context (Stress: $F_{(1, 26)} = 98.70$, $p < 0.001$; Fig 2.1c), but there was no effect of housing condition (Housing: $F_{(1, 26)} = 2.89$, $p = 0.10$; Stress x Housing: $F_{(1, 26)} = 0.023$, $p = 0.88$; Fig 2.1c). Traumatized animals produce the stress-enhanced fear learning phenotype regardless of housing condition.

Generalization

Animals were placed in a third novel context (Context C) for 8 minutes the day after the 1 shocks context test to test for housing condition's effect on fear generalization. Traumatized animals showed increased generalization compared to non-traumatized animals (Stress: $F_{(1, 26)} = 137.72$, $p < 0.001$; Fig 2.1d), but housing condition did not alter generalization (Housing: $F_{(1, 26)} = 1.10$, $p = 0.30$; Stress x Housing: $F_{(1, 26)} = 0.31$, $p = 0.58$; Fig 2.1d). These findings indicate that housing condition does not affect generalization in traumatized animals.

Experiment 2: Social housing does not alter generalization to a novel context, and modestly augments extinction to the one shock context.

Pre-One Shock Baseline

Prior to the mild stressor (1 Shocks in Context B), animals can explore the context for 3 minutes, referred to as the pre-shock period or baseline. To understand whether housing condition altered animals' behavior during the pre-shock period, we analyzed freezing during this period across trauma and housing conditions. Stressed animals exhibited more freezing compared to non-traumatized animals (Stress: $F_{(1, 28)} = 22.23$, $p < 0.001$; Fig 2.2b), but housing condition did not alter freezing behavior during this period (Housing: $F_{(1, 28)} = 1.11$, $p = 0.30$; Stress x Housing: $F_{(1, 28)} = 1.07$, $p = 0.31$; Fig 2.2b). These findings indicate that group housing does not change freezing behavior during the pre-shock period in Context B.

Extinction of One Shock Context

To test whether extinction behavior to the 1 shocks context differs due to housing condition, animals underwent 20-minute extinction sessions for 4 consecutive days. Freezing behavior during the first 8 minutes of the extinction session was analyzed to determine

between-session extinction. There was a mild effect of housing across extinction days (Housing: $F_{(1, 120)} = 3.57$, $p = 0.061$; Fig 2.2c), and a large effect of both stress (Stress: $F_{(1, 120)} = 155.03$, $p < 0.001$; Fig 2.2c) and extinction day (Day: $F_{(1, 28)} = 162.62$, $p < 0.001$; Stress x Day: $F_{(1, 28)} = 78.17$, $p < 0.001$; Fig 2.2c). There was no interaction of housing with either stress or extinction day (Stress x Housing: $F_{(1, 120)} = 1.32$, $p = 0.25$; Housing x Day: $F_{(1, 120)} = 0.62$, $p = 0.43$; Stress x Housing x Day: $F_{(1, 120)} = 1.59$, $p = 0.21$; Fig 2.2c).

Two-way ANOVAs were performed to analyze effects of housing condition and trauma on freezing during the first 8 minutes of extinction day. On extinction day 1, stressed animals showed higher freezing levels compared to non-stressed animals (Stress: $F_{(1, 28)} = 160.67$, $p < 0.001$; Fig 2.2c), but there was no effect of housing (Housing: $F_{(1, 28)} = 0.57$, $p = 0.46$; Stress x Housing: $F_{(1, 28)} = 0.69$, $p = 0.41$; Fig 2.2c). On day 2, traumatized animals showed higher freezing levels compared to non-stressed animals (Stress: $F_{(1, 28)} = 82.57$, $p < 0.001$; Fig 2.2c), but there was no effect of housing (Housing: $F_{(1, 28)} = 0.041$, $p = 0.84$; Stress x Housing: $F_{(1, 28)} = 0.16$, $p = 0.69$; Fig 2.2c). On day 3, stressed animals showed higher freezing levels compared to non-stressed animals (Stress: $F_{(1, 28)} = 12.18$, $p = 0.002$; Fig 2.2c), and a modest effect of housing (Housing: $F_{(1, 28)} = 5.90$, $p = 0.022$; Stress x Housing: $F_{(1, 28)} = 3.34$, $p = 0.078$; Fig 2.2c). Single housed stressed animals were freezing significantly more than pair housed stressed animals during the first 8 minutes of extinction on day 3 ($t(10) = 2.30$, $p = 0.043$; Fig 2.2c). By day 4, all animals were fully extinguished and there were no longer effects of stress (Stress: $F_{(1, 28)} = 0.0003$, $p = 0.99$; Fig 2.2c) or housing (Housing: $F_{(1, 28)} = 1.37$, $p = 0.25$; Stress x Housing: $F_{(1, 28)} = 0.80$, $p = 0.38$; Fig 2.2c). Housing had a modest effect on extinction behavior to the 1 shocks context, where single housed traumatized animals extinguished modestly slower than pair housed traumatized animals.

Experiment 3: Social housing does not change behavior in the open field following an acute traumatic experience.

Total Open Field Crossings

Previous findings indicate that stressed animals show decreased mobility in an open field task (Perusini et al., 2016). To test whether housing condition interacts with behavior in an open field, animals were placed in an open field for 8 minutes on the day following trauma (15 or 0 unsignaled footshocks). Mobility was measured by the number of times animals crossed gridlines on the open field. Across the full 8 minute session, stressed animals were significantly less mobile than non-stressed animals (Stress: $F_{(1, 28)} = 20.29$, $p < 0.001$; Fig 2.3b), but there was no effect of housing on mobility in the open field (Housing: $F_{(1, 28)} = 0.16$, $p = 0.70$; Stress x Housing: $F_{(1, 28)} = 0.26$, $p = 0.62$; Fig 2.3b). Animals show decreased mobility in the open field following an acute traumatic experience, but housing condition does not interact with this anxious phenotype.

Open Field Crossings by Minute

To test whether mobility was differentially expressed across the 8-minute session, data were analyzed across 1 minute bins (Fig 2.3c). Across the 1 minute bins, stressed animals were significantly less mobile (Stress: $F_{(1, 244)} = 66.92$, $p < 0.001$; Fig2.3c), and animals were significantly less mobile across the session (Minute: $F_{(1, 244)} = 14.48$, $p < 0.001$; Fig 2.3c), but there were no effects of housing on mobility (Housing: $F_{(1, 244)} = 0.14$, $p = 0.71$; Stress x Housing: $F_{(1, 244)} = 1.47$, $p = 0.23$; Housing x Minute: $F_{(1, 244)} = 0.38$, $p = 0.54$; Stress x Housing x Minute: $F_{(1, 244)} = 0.092$, $p = 0.76$; Fig 2.3c). These findings indicate that housing condition does not interact with the anxious phenotype shown by stressed animals in the open field task.

Experiment 4: Housing condition modestly impacts generalization to a novel context.

Pre-One Shock Baseline

In past SEFL studies, rats do not generalize fear to the novel context as measured by low pre-shock baseline freezing (Rau et al., 2005). However, animals in Experiments 1 – 3 froze significantly higher than typically observed in SEFL studies, indicating a generalization of fear (Fig 2.2b). To understand why rats were generalizing fear, I systematically compared the housing conditions used in Experiments 1 – 3 (single and pair housed in large, plastic tubs) with the housing conditions typically used in our laboratory (single housed in metal cages). There was a significant effect of housing condition, indicating that housing condition mediates fear generalization to a novel context (Housing: $F_{(2, 42)} = 3.65$, $p = 0.035$; Fig 2.4b). *Post-hoc* analysis indicate that there is a significant decrease in freezing between animals single housed in metal cages compared to rats pair housed in plastic tubs ($t(29) = 2.44$, $p = 0.010$; Fig 2.4b). There were modest differences in generalization between animals single housed in metal cages and those single housed in plastic tubs ($t(29) = 1.09$, $p = 0.14$; Fig 2.4b), and animals housed singly in plastic tubs and paired housed in plastic tubs ($t(29) = 1.35$, $p = 0.094$; Fig 2.4b). These data indicate that housing condition affects generalization to a novel context.

One Shock Context

To verify that housing condition does not affect subsequent expression of fear to the one shock context, animals were given an 8-minute context test in the one shock context the day after conditioning. There was no significant effect of housing condition on this fear expression (Housing: $F_{(2, 42)} = 2.73$, $p = 0.077$; Fig 2.4c). These data indicate that housing condition does not affect expression of fear to the one shock context as measured during a subsequent context test.

DISCUSSION

The above studies indicate that PTSD-like phenotypes observed following an acute stressor in our animal model of the disease persist regardless of housing conditions before, during, or following experience with the acute stressor. Stressed animals continue to show post-trauma increases in fear and anxiety independent of housing condition. However, there were modest effects between groups with varying housing conditions during extinction to the context where animals were given a mild (1 shock) stressor following trauma. In this measure, stressed pair-housed animals showed slightly enhanced extinction compared to stressed single-housed animals across days of extinction. Given that all other measures of fear and anxiety endured across single and pair housed animals, it seems unlikely that housing condition contributes substantially to developing PTSD-like phenotypes after a stressor. In our model, this indicates that the potential mild, chronic stress of single housing does not impact the behaviors associated with the acute, severe stressor (15 shocks) which produces enhanced fear conditioning and heightened anxiety behaviors.

Given the evidence in both animal models and humans for the role of isolation housing in exacerbating effects of stress, it is somewhat surprising that housing does not contribute to enhanced fear and anxiety changes following an acute stressor. Data from humans indicates that there can be marked benefits to individuals who suffer from PTSD when given social support through activities including group therapy (Ellis et al., 2014; Resick et al., 2015). Likewise, some animal models of stress indicate a relationship between housing condition and the effects of stress on subsequent phenotypes (Pibiri et al., 2008; Liu et al., 2013; Ravenelle et al., 2014; Murínová et al., 2017). However, in our model of PTSD we fail to find an effect of housing condition on an array of subsequent fear and anxiety related phenotypes, including enhanced fear conditioning and decreased activity in the open field task.

There are a few reasons as to why we might have failed to find an effect of housing condition on the PTSD-like symptoms seen in stressed animals. First, animals undergoing the 15 shock stressor show an extreme phenotype, particularly in subsequent fear conditioning where the animals are freezing at a near maximum rate (typically, 90-100%) across the context test. While we might have expected to see a reduction in this freezing for animals that were group-housed, there is not much room for a more severe phenotype in isolated animals. Additionally, this ceiling effect could have prevented the ability to disentangle a mitigating effect from pair housing animals. Future studies could address this ceiling effect by reducing the severity of the stress in order to tease apart a potential mitigation in fear and anxiety symptoms following an acute stressor.

Animals were housed in their respective condition (isolated or pair) for approximately one week prior to stress, and immediately following stress through the remainder of the experimental timeline. Some animal studies indicate a necessity of chronicity of isolation or pair housing to yield effects of this housing condition on behavioral measures, so it is possible that the present studies failed to produce effects of housing condition on PTSD-like measures because of the shortened timeline of housing condition (Baker and Bielajew, 2007; Garrido et al., 2013). For example, one study indicating a stress-reducing effect of social housing in rats involved housing rats either in isolation or in pairs for a period of three weeks, across which time the rats were subjected to a chronic stress procedure (Westenbroek et al., 2005). Another critical difference between the positive results of this study and the experiments presented here is that our PTSD model involves an acute stressor to initiate PTSD-like symptoms while studies indicating an effect of social housing on mitigating fear, anxiety, and depression phenotypes often involve a chronic stressor (Westenbroek et al., 2003b; Babygirija et al., 2010).

Finally, all of the experiments presented here were done in male Long-Evans rats. Our rationale for using male rats was to match most of the previous work done with SEFL, as we were primarily interested in understanding whether isolation housing was necessary to produce PTSD-like symptoms seen after an acute stressor. A variety of studies have indicated a sex-dependent nature of housing effect on susceptibility to stress with female rats showing a heightened response to pair housing compared to male rats (Westenbroek et al., 2003a; Westenbroek et al., 2004; Westenbroek et al., 2005; Baker and Bielajew, 2007; Leasure and Decker, 2009). Therefore, it is possible that female rats undergoing the same acute stress protocol presented here would show benefits of social housing following the acute stressor while few to no advantages were seen in male rats. Critically, the neuropeptide oxytocin has been shown to reduce CORT following a stressor and administration of oxytocin following stress can protect hippocampal function, which is normally impaired following a stressor (Windle et al., 1997; Lee et al., 2015; Park et al., 2017). Additionally, oxytocin-deficient female mice exhibit increased anxiety-like behavior on the Elevated Plus Maze, and elevated levels of CORT alongside increased *fos* expression in the medial amygdala following stress (Amico et al., 2004; Mantella et al., 2004). Thus, if oxytocin is mediating subsequent development of PTSD-like symptoms after an acute stressor, it is possible these effects would be seen selectively in a female population of rats compared to the male population used in these experiments. Future studies to disentangle the contribution of housing condition to the development of PTSD-like symptoms in our animal model should probe for these effects in female rats to get a more holistic understanding of the potential sex differences.

Prior work with SEFL indicates that the enhanced fear learning is due to nonassociative sensitization. Therefore, animals tend to exhibit minimal generalization when placed in a novel context for the one shock conditioning as seen by a low level of freezing during the pre-shock

baseline. In these experiments, the rats demonstrated higher than expected levels of fear during this pre-shock baseline which indicates an increase in generalization. This increase in generalization could be due to one, or both, of the major differences in housing compared to prior work with SEFL: that animals were housed in large, plastic tubs rather than hanging metal cages, or that animals were pair housed. To investigate the contribution of these housing manipulations to post-trauma generalization, these factors were operationally manipulated and animals were tested for their fear generalization to a novel context. Single-housed animals in the metal cages froze significantly less than pair-housed animals in the plastic tubs, with single-housed animals in the plastic tubs exhibiting an intermediate level of freezing. Thus, these data indicate that both the type of cage and the presence of a cage-mate contribute to fear generalization.

Given that the fear conditioning boxes closely resemble the metal cages that animals are housed in, there is a larger contextual overlap for the single-housed animals in the metal cages than those living in the plastic tubs, which do not resemble the fear conditioning boxes. Thus, it could be the degree of similarity between housing condition and the fear conditioning boxes that drives the magnitude of generalization due to either high (single-housed metal cages) or low (pair-housed plastic tubs) contextual overlap. Animals with high contextual overlap might generalize less because their home cage, a safe environment, closely resembles the fear conditioning boxes, an unsafe environment. Understanding how housing condition contributes to measures of fear, including generalization, has important implications in designing experiments where these effects might unintentionally muddle data.

In the present set of studies, we sought to understand the role of pair housing animals on PTSD-like symptoms following an acute stressor. As housing rats in isolation can be a stressor independently, it was important for us to understand whether isolation housing rats

prior to, during, and following an acute stressor was necessary for developing PTSD-like symptoms including exaggerated subsequent fear conditioning and increases in anxiety-like behavior. These studies indicate that PTSD-like symptoms arising from an acute stressor persist regardless of the animal's housing condition. Future studies to understand the contribution of housing condition to development of PTSD-like symptoms could include longer periods of respective housing condition, a chronic stress protocol rather than the acute stressor used here, or inclusion of female rats as animal studies indicate a sex-specific effect of housing condition on the development of stress-dependent changes in fear, anxiety and depression.

MATERIALS AND METHODS

Animals

A total of 144 adult (PND 60-90), male Long Evans rats (Experiment 1: $n = 32$, Experiment 2: $n = 32$, Experiment 3: $n = 32$, Experiment 4: $n = 48$) were used. All rats were housed in a vivarium at UCLA with food and water given ad libitum except during experimental periods. Animals were housed on a 12-h on/off light cycle. These rats were assigned to their specific housing condition for at least one week prior to experiments and were handled for approximately 1 minute per day. All procedures were approved by the Chancellor's Animal Research Committee at UCLA.

Behavioral Testing

Apparatus

All fear conditioning took place in Med Associates conditioning chambers (28 x 21 x 21 cm; Lafayette Instrument Co.; Lafayette, IN) that were controlled through Med Associates Video Freeze software, as described previously (citation here). The conditioning chambers were configured into 3 distinct contexts: Context A, Context B, and Context C. Footshocks were

delivered to the animals through Med Associates shock scramblers in each conditioning chamber (ENV 414-S). Conditioning sessions were recorded by near infrared cameras. Freezing behavior was analyzed via Med Associates Video Freeze software (citation here).

Context A consisted of a flat grid floor, windex odor and white light illumination. Animals were transported to Context A in their homecages, which were mounted on a hanging rack. Context B consisted of an alternating thick and thin grid floor, acetic acid odor, red light illumination, and an A-frame insert. Animals were transported to Context B in a novel, large plastic box. Context C consisted of a white plastic floor, simple green odor, red light illumination, and a white plastic insert. Animals were transported to Context C in a plastic cage with fresh bedding.

Trauma (Experiments 1, 2, 3 and 4)

On Day 1, animals were subjected to an acute traumatic experience consisting of 15 pseudorandom, unsignaled footshocks (1-sec, 1.0-mA) across a 90-minute session in Context A. In all experiments, SEFL designates exposure to the acute traumatic experience while NS designates control animals exposed to the context for 90 minutes without any footshock presentations.

Trauma Context Test (Experiment 1)

One day later, animals were placed back in Context A, the trauma context, for 8 minutes to assess contextual fear. Transport and all contextual configurations were identical to the trauma session.

One Shock Conditioning (Experiments 1, 2 and 4)

Following trauma, animals were placed in a novel context, Context B, which was distinct from Context A in contextual configuration (distinct grid flooring, odor, illumination, and contextual cues) and animal transportation method. Animals could explore the context for 3 minutes (pre-shock baseline) before receiving a single 1-sec, 1.0-mA footshock. 30 seconds following the footshock, animals were removed from the conditioning chamber.

One Shock Context Test (Experiments 1 and 4)

One day later, animals were placed back in Context B, the one shock context, for 8 minutes to assess contextual fear. Transport and all contextual configurations were identical to the one shock context. Greater freezing in the SEFL rats compared to the NS rats during this test indicates the presence of stress-enhanced fear learning.

Generalization Test (Experiment 1)

Animals were placed in a novel context, Context C, that was distinct from both Contexts A and B in contextual configuration (distinct grid flooring, odor, illumination, and contextual cues) and animal transportation method. Animals could explore the context for 8 minutes, and freezing was measured to assess generalization of contextual fear to the novel chamber.

One Shock Extinction (Experiment 2)

Animals underwent 20-minute extinction sessions to the one shock context (Context B) across 4 days following the one shock conditioning. Freezing was assessed during the first 8 minutes of the session to analyze contextual fear. Transport and all contextual configurations were identical to the one shock context.

Open Field (Experiment 3)

One day after trauma, animals were placed in an open field arena for 8 minutes. The open field arena measured 91.4 cm x 91.4 cm with 30.5 cm high walls. All behavior was digitally recorded and subsequently blindly hand scored to determine mobility of the animals as measured by number of times an animal crossed a gridline on the open field floor (see Figure 3b). Animal transportation method and the contextual configuration (odor and illumination) were distinct from those used during the trauma.

Data Analyses

All data were exported from Med Associates software and analyzed using R (RStudio, v1.0.136). Data were analyzed by between-subjects ANOVAs or repeated-measures ANOVAs where appropriate. If there were significant effects found with the ANOVA, appropriate *post-hoc* tests were performed. Data are plotted as group means ± SEM.

ACKNOWLEDGEMENTS

Thank you to members of the Fanselow laboratory for their intellectual assistance to this project.

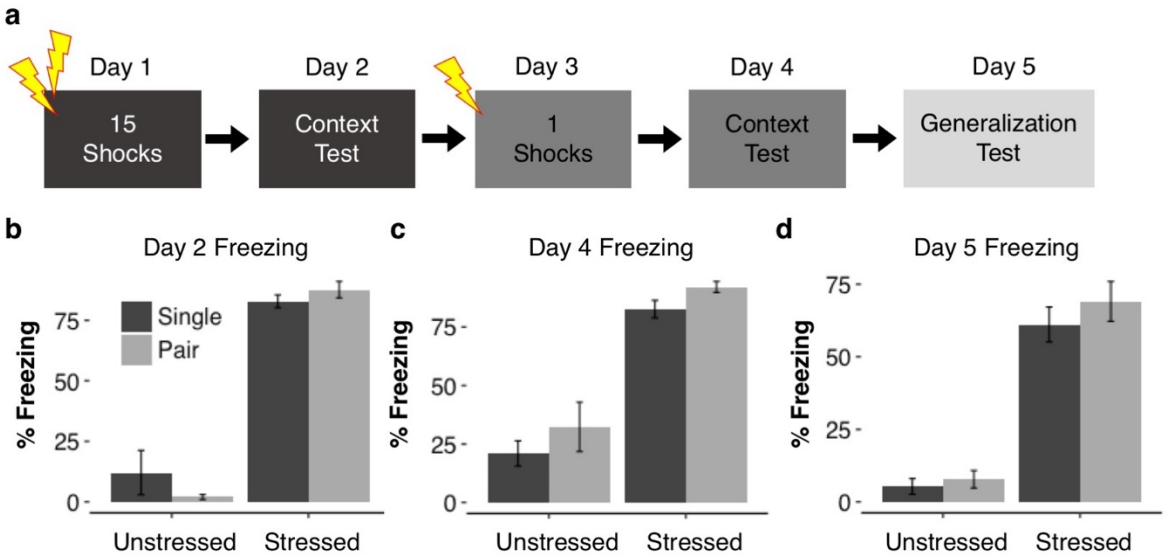

Figure 2.1: Social housing animals does not alter the context memory of trauma, stress-enhanced fear learning, or generalization to a novel context. (a) Experimental design. (b) Freezing across an 8 minute context test in the 15 shock (trauma) context. Stressed animals froze significantly more than unstressed animals, but there was no effect of housing on this measure. (c) Freezing across an 8 minute context test in the 1 shock context. Stressed animals froze significantly more than unstressed animals, but there was no effect of housing on this measure. (d) Freezing across an 8 minute context test in a novel context to probe for fear generalization. Stressed animals froze significantly more than unstressed animals, but there was no effect of housing on this measure.

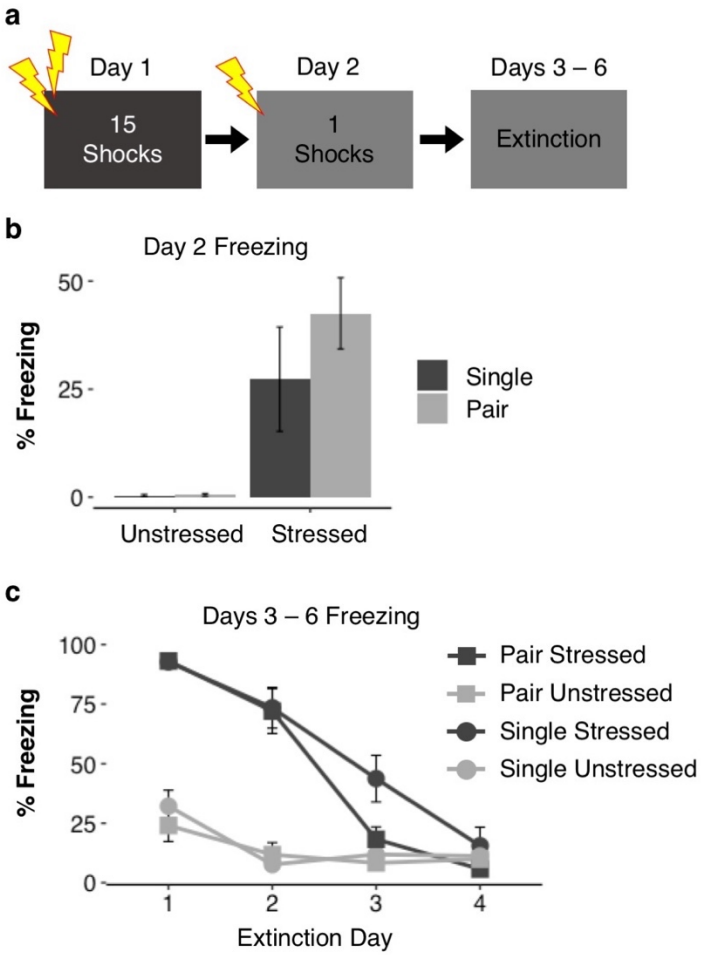

Figure 2.2: Social housing animals modesty augments extinction to the one shock context. (a) Experimental design. (b) Freezing during the 3 minute baseline period prior to the one shock. Stressed animals froze significantly more than unstressed animals, but there was no effect of housing on this measure. (c) Freezing across the first 8 minutes of each extinction day for a four day extinction period. On Day 3, pair housed stressed animals exhibited less freezing (more extinction) than single housed stressed animals, indicating a slight enhancement of extinction due to pair housing. Housing did not have an effect at any other time point.

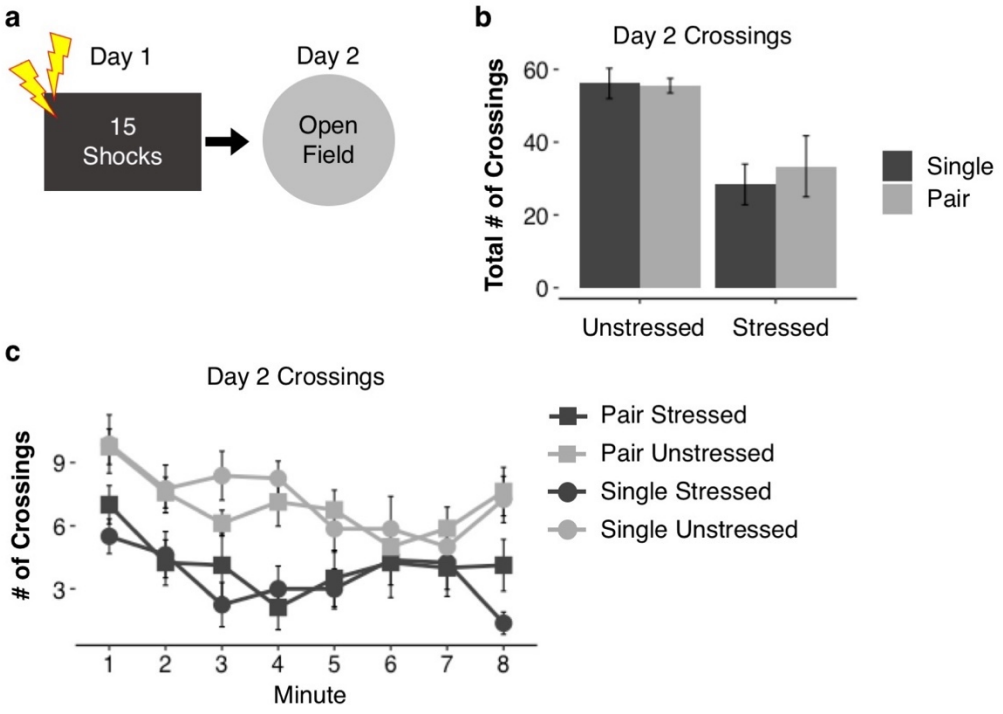

Figure 2.3: Social housing animals does not change behavior in the open field test following an acute traumatic experience. (a) Experimental design. (b) Total number of crossings across an 8-minute test in the open field. Stressed animals exhibited less locomotion (indicated by a reduced number of crossings) than unstressed animals, but there was no effect of housing on this measure. (c) Minute by minute number of crossings across an 8 minute test in the open field. Stressed animals exhibited less movement in the open field, but there was no effect of housing on this measure.

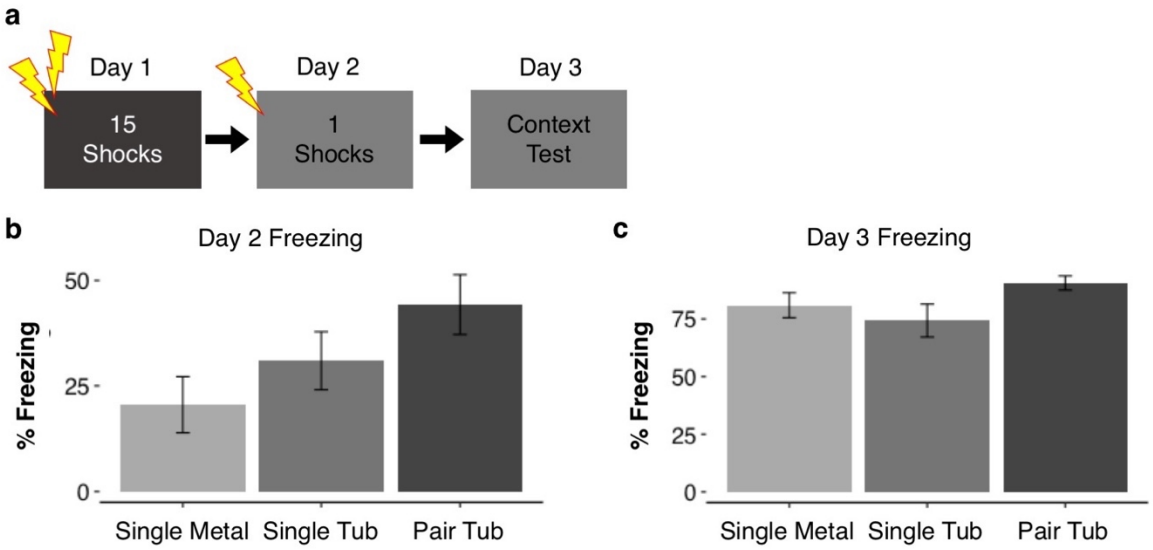

Figure 2.4: Housing condition modestly impacts generalization to a novel context. (a) Experimental design. (b) Freezing during the 3 minute baseline period prior to the one shock. Pair housed animals living in plastic tubs froze significantly more than single housed animals living in metal cages, and single housed animals living in plastic tubs exhibited an intermediate level of freezing. (c) Freezing during the 8 minute context test of the 1 shock context. There were no significant differences in the amount of freezing exhibited based on housing condition.

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CHAPTER 3

JDTic, a Kappa-Opioid Antagonist, Mitigates Anxiety Behavior on the Elevated Plus Maze following an Acute Stressor

ABSTRACT

Stress-Enhanced Fear Learning (SEFL) is a rodent model of Post-Traumatic Stress Disorder (PTSD) in which exposure to an acute traumatic experience produces a variety of PTSD-like phenotypes including increased measures of fear, anxiety and depression. Kappa Opioid Receptors (KORs) are known to play a role in many of these phenotypes, and are found abundantly in the fear and anxiety circuit. In this study, we administered the KOR antagonist JDTic immediately after an acute stressor to characterize the role of KORs in the expression of post-trauma PTSD-like phenotypes. Post-trauma administration of JDTic altered behavior on the elevated plus maze (EPM) by rescuing the stress-induced reduction in open arm exploration. However, we found no effect of JDTic treatment on the primary index of SEFL, exaggerated fear conditioning in a novel context. Across an 8-minute test session on the EPM, stressed animals treated with saline reduced the amount of time spent in open arms. In contrast, stressed animals treated with JDTic increased open arm exploration across the test session. Thus, administration of JDTic rescues stress-induced anxiety behavior on the EPM. JDTic, acting through KOR antagonism, mediates some aspects of post-trauma alterations in fear and anxiety. Characterizing behavioral consequences of stress and understanding their neural mechanisms is a critical step toward understanding how stress modifies the fear and anxiety circuit.

INTRODUCTION

Kappa-opioid receptors (KORs) are a class of G-protein coupled receptors (GCPRs) in the brain that respond to the endogenous ligand dynorphin (Knoll and Carlezon, 2010). Activation of KORs produces a variety of cellular effects, including decreasing calcium currents, mitigating cyclic AMP (cAMP) and activating MEK, ERK, and MAPK pathways (Knoll and Carlezon, 2010; Crowley and Kash, 2015). Importantly, KORs and dynorphin are highly expressed in regions of the fear and anxiety circuit including the hippocampus (HPC), prefrontal cortex (PFC), basolateral amygdala (BLA), central amygdala (CeA), and bed nucleus of the stria terminalis (BNST) (Crowley and Kash, 2015). The rich expression patterns of KORs and dynorphin-releasing neurons throughout the fear and anxiety circuit have made this system a prime candidate in the stress, anxiety, fear and depression literature. KOR activation leads to both rapid (altering cell excitability and neurotransmitter release) and prolonged (changes in gene expression) cellular changes. Taken together, some believe that these rapid and prolonged changes might contribute differentially to acute and chronic stress, respectively (Knoll and Carlezon, 2010).

Likewise, KORs interact closely with other modulators of the stress system, including corticotropin-releasing factor (CRF). Both dynorphin and CRF are co-expressed in regions including the periventricular nucleus of the hypothalamus, the hypothalamic supraoptic nucleus, and in axon terminals of the locus coerulues (Crowley and Kash, 2015). Behaviorally, effects of either KORs or CRF seem to depend on each other. For example, anxiogenic behaviors produced by CRF depend upon the KOR system (Bruchas et al., 2009). Relatedly, KOR-mediated reinstatement of drug seeking behavior requires CRF activity, and can be blocked by administration of a CRF-1R antagonist (Zhao et al., 2007). KORs are also thought to mediate

activation of the hypothalamic-pituitary-adrenal (HPA) axis, as administration of KOR antagonists in mice has been found to reduce CORT levels.

Behaviorally, the KOR system has been linked to a variety of effects mediating fear and anxiety behaviors. Broadly, antagonizing or genetically restricting expression of KORs has been shown to produce anxiolytic effects while KOR agonism produce anxiogenic or depressive-like states (Mague et al., 2003; Carlezon et al., 2006; Knoll et al., 2007). More specifically, the KOR system is thought to be engaged in response to stress, and can mediate future behavioral consequences of stress including stress-induced reinstatement and conditioned place aversion following stress. These KOR-mediated stress effects have also been shown in learning and memory tasks, as animals that undergo stress (repeated forced swim) or are treated with a systemic KOR agonist show a deficit in subsequent tests of novel object recognition. Interestingly, this deficit can be mitigated by co-occurring treatment of a KOR antagonist, indicating a causal role of KORs in producing this stress-induced deficit in learning and memory (Carey et al., 2009; Paris et al., 2011).

Changes in the KOR system have been seen following fear conditioning. When probing for changes in KOR and pro-dynorphin (PDyn, the precursor of dynorphin) in the fear circuit, there is evidence for an upregulation of KOR mRNA in the BLA and a downregulation of KOR mRNA in the striatum following fear conditioning, while no such changes were observed in the CeA or HPC (Knoll et al., 2011). To indicate a causal contribution of KORs in the BLA to fear conditioning, microinfusions of JDTic, a KOR antagonist, into various brain regions prior to testing for fear potentiated startle indicated that only the BLA microinfusions blocked fear potentiated startle, while JDTic microinfusions into the striatum had no effect on this phenotype (Knoll et al., 2011). Taken together, the KOR system has been shown to be involved in mediating effects of stress, fear, anxiety and depression in both animal models.

Interestingly, KORs have not been widely studied as pertaining to Post-Traumatic Stress Disorder (PTSD), which involves exposure to a traumatic event leading to stress-induced changes in fear, anxiety, and depression. Given KOR's role in mediating effects of stress, this system seems like a likely candidate to be involved in contributing to aspects of PTSD including anxiogenic and depressive phenotypes often seen following a stressor. Although some investigators have suggested a potential therapeutic benefit of KOR antagonists in the treatment of PTSD, the suitable pre-clinical and clinical work to justify KOR antagonism as a treatment for PTSD patients remains uninvestigated. Despite this lack of causal understanding of the contribution of KORs to PTSD, a variety of studies have demonstrated that CRF, which is tightly connected to the KOR system, is upregulated in human and animal models of PTSD. Understanding how the KOR system contributes to PTSD symptoms is a necessary step toward potentially using KOR antagonists as a therapeutic for PTSD patients.

Interest surrounding the role of KORs and dynorphin in regulating stress and its effects have led to excitement around KOR antagonists as a pharmaceutical intervention post-stress to mitigate some of stress's most deleterious consequences, including an increased risk of drug addiction or relapse and depression (Chavkin and Martinez, 2015). JDTic is a KOR antagonist that has been considered for such clinical trials, and in 2014 there was a Phase I clinical trial conducted to assess JDTic's safety, tolerability, and pharmacokinetics in a double-blind, placebo controlled, randomized trial in healthy adults (Buda et al., 2015). Unfortunately, this trial was halted when patients began experiencing nonsustained ventricular tachycardia (NVST), suggesting potentially selective JDTic toxicity in humans (Buda et al., 2015). The large number of pre-clinical studies indicating that KOR antagonists have a role in preventing drug or alcohol relapse, curbing stress-induced depression, and broadly alleviating symptoms of stress have caused discussion regarding KOR antagonists as a clinical tool to continue despite these failed

clinical trials (Newton et al., 2002; Mague et al., 2003; Beardsley et al., 2005; Schank et al., 2012; Lalanne et al., 2014). Further work is required to understand how KOR antagonists such as JDTic could be used clinically, including a deeper understanding of the physical and psychological side effects of KOR antagonist administration in both stressed and healthy control populations.

Using the SEFL model, we can understand the neural mechanisms responsible for certain PTSD-like phenotypes. In this study, we examined the role of KORs in SEFL through administering the KOR antagonist JDTic immediately following an acute stressor. Then, PTSD-like phenotypes including exaggerated subsequent fear conditioning, anxiety and depression measures were assessed to understand the selective contribution of KORs. By administering JDTic systemically following an acute stressor, then testing rats on a variety of behavioral tasks including fear conditioning, the elevated plus maze (EPM), open field (OF), and forced swim test (FST), we can specifically understand how KOR's in the fear and anxiety system mediate distinct behavioral consequences following an acute stressor. Previously, our laboratory has shown that an acute stressor leads to these changes in fear, anxiety and depression including stress-enhanced fear learning, anxiogenic behavior in the OF and depressive behavior in the FST (Rau et al., 2005; Perusini et al., 2016). Further, we could test the efficacy of JDTic as a potential clinical treatment following an acute stressor to mitigate aspects of PTSD including enhanced fear, anxiety, and depression measures.

RESULTS

Experiment 1: An acute stressor enhances subsequent fear conditioning that is not disrupted by administration of JDTic.

Trauma Context

During an 8-minute context test of the trauma context to assess fear retention, animals receiving 15 shocks in the trauma context showed higher levels of fear than non-shocked animals (SEFL: $F_{(1, 28)} = 4762.56$, $p < 0.001$; Fig 3.1b). JDTic administration had no effect on fear retention (Drug: $F_{(1, 28)} = 0.036$, $p = 0.85$; Drug x SEFL: $F_{(1, 28)} = 0.024$, $p = 0.88$; Fig 3.1b). These findings indicate that post-trauma administration of JDTic does not affect the memory of the trauma context when fear retention was analyzed in these animals.

One Shock Baseline

To test fear generalization to a novel context, freezing was measured during the first 3-minutes in a novel context (Context B), one day after the trauma context test. Stressed animals showed significantly higher levels of freezing during this 3-minute baseline (SEFL: $F_{(1, 28)} = 30.48$, $p < 0.001$; Fig 3.1c). JDTic administration did not have an effect on freezing during this time (Drug: $F_{(1, 28)} = 2.84$, $p = 0.10$; Drug x SEFL: $F_{(1, 28)} = 2.95$, $p = 0.097$; Fig 3.1c). Post-trauma administration of JDTic does not mitigate fear generalization to a novel context.

One Shock Context Test

Fear retention to the one shock context, the primary SEFL phenotype, was tested to determine the effect of JDTic on post-stress fear learning. Traumatized animals showed highly elevated freezing levels when placed back in the one shock context test for 8 minutes compared to non-traumatized animals (SEFL: $F_{(1, 28)} = 255.18$, $p < 0.001$; Fig 3.1d), but JDTic did not

mitigate this phenotype (Drug: $F_{(1, 28)} = 0.32$, $p = 0.57$; Drug x SEFL: $F_{(1, 28)} = 0.040$, $p = 0.84$; Fig 3.1d). Overall, JDTic does not mitigate aspects of stress enhanced fear learning.

Experiment 2: Post-trauma administration of JDTic changes behavior on the Elevated Plus Maze task, but does not affect anxiety or depression behaviors in the Open Field or Forced Swim Test tasks.

Elevated Plus Maze

Animals were placed on the elevated plus maze and allowed to explore freely for 8 minutes to assess anxiety behavior. Across the full 8 minute session, there was no effect of stress on the percent of time animals spent in the open arms, a common indicator of an anxious phenotype (SEFL $F_{(1, 37)} = 1.47$, $p = 0.23$; Fig 3.2b). There was also no effect of JDTic on the percent time animals spent in the open arms across the full 8-minute session (Drug: $F_{(1, 37)} = 0.005$, $p = 0.94$; Drug x SEFL: $F_{(1, 37)} = 2.10$, $p = 0.16$; Fig 3.2b). Interestingly, JDTic and stress did affect the animal's behavior on the elevated plus maze when analyzing the first half of the session (Minutes 1-4) compared to the last half of the session (Minutes 5-8). Stress alone did not affect behavior on the EPM when binned by first and last half of the session (SEFL: $F_{(1, 82)} = 2.59$, $p = 0.11$; Fig 3.2b). JDTic administration alone did not affect the binned percent time spent in the open arms (Drug: $F_{(1, 82)} = 0.0086$, $p = 0.93$; Fig 3.2b). Interestingly, there was a significant interaction of JDTic and stress on this measure (Drug x SEFL: $F_{(1, 82)} = 4.20$, $p = 0.044$; Fig 3.2b). While both unstressed saline-treated animals and stressed JDTic-treated animals showed increased time spent in open arms across the test session, both unstressed JDTic-treated animals and stressed saline-treated animals were either stagnant or decreased in their time spent in the open arms. There were no significant effects of bin on this measure (Bin: $F_{(1, 82)} =$

0.17, $p = 0.68$; Drug x Bin: $F_{(1, 82)} = 0.090$, $p = 0.76$; SEFL x Bin: $F_{(1, 82)} = 0.29$, $p = 0.59$; Drug x SEFL x Bin: $F_{(1, 82)} = 1.86$, $p = 0.18$; Fig 3.2b).

The difference in percent time spent exploring the open arm between Minutes 5-8 and 1-4 was taken to quantify this JDTic x SEFL interaction. There was no main effect of stress on this measure (SEFL: $F_{(1, 41)} = 1.01$, $p = 0.32$; Fig 3.2f). There was no main effect of JDTic on this measure either (Drug: $F_{(1, 41)} = 0.32$, $p = 0.58$; Fig 3.2f). However, there was a significant interaction of JDTic and stress (Drug x SEFL: $F_{(1, 41)} = 6.52$, $p = 0.014$; Fig 3.2f). Post-hoc analysis indicates that the stressed saline-treated animals were significantly different than stressed JDTic-treated animals ($t(15) = 1.88$, $p = 0.04$) and unstressed saline-treated animals ($t(20) = 3.03$, $p = 0.0032$), but were not significantly different than unstressed JDTic-treated animals ($t(20) = 1.25$, $p = 0.11$). Stressed JDTic-treated animals were not significantly different than unstressed saline-treated animals ($t(16) = 0.28$, $p = 0.39$). Unstressed JDTic-treated animals, however, were significantly different than unstressed saline-treated animals ($t(20) = 1.73$, $p = 0.05$).

Animals were measured based on the percent of open arm entries taken across the 8-minute session. There were no significant effects of JDTic or stress on this measure (Drug: $F_{(1, 35)} = 0.15$, $p = 0.70$; SEFL: $F_{(1, 35)} = 0.13$, $p = 0.72$; Drug x SEFL: $F_{(1, 35)} = 1.77$, $p = 0.19$; Fig 3.2e). When looking across 4-minute bins (Minutes 1-4, Minutes 5-8), there were likewise no significant effects of JDTic or stress on this measure (Drug: $F_{(1, 78)} = 0.21$, $p = 0.65$; SEFL: $F_{(1, 78)} = 0.17$, $p = 0.68$; Bin: $F_{(1, 78)} = 0.066$, $p = 0.80$; Drug x SEFL: $F_{(1, 78)} = 2.13$, $p = 0.15$; Drug x Bin: $F_{(1, 78)} = 0.14$, $p = 0.71$; SEFL x Bin: $F_{(1, 78)} = 0.052$, $p = 0.82$; Drug x SEFL x Bin: $F_{(1, 78)} = 0.19$, $p = 0.67$; Fig 3.2d).

To assess overall mobility, the total number of arm entries (open and closed) was assessed across 8 minutes. There were no significant effects of JDTic or stress on the total number of arm entries (Drug: $F_{(1, 35)} = 0.035$, $p = 0.85$; SEFL: $F_{(1, 35)} = 2.70$, $p = 0.11$; Drug x SEFL: $F_{(1, 35)} = 2.08$, $p = 0.16$; Fig 3.2g).

These results indicate that there is a tendency for animals to habituate to the elevated plus maze during the session, as shown through an anxiolytic phenotype across the session in the unstressed, saline-treated controls. However, untreated stress (stressed, saline-treated animals) or unstressed animals given JDTic treatment eliminate this phenotype and fail to habituate across the 8-minute test session. This reduction in open arm exploration cannot be explained by mobility on the elevated plus maze.

Open Field

Animals were tested for 8 minutes in the open field to assess locomotor activity. Across the full 8-minute session, stressed animals moved significantly less than unstressed animals, as has been previously reported in the SEFL model (SEFL: $F_{(1, 44)} = 11.90$, $p = 0.0013$; Fig 3.3b). There was no effect of JDTic on locomotion in the open field (Drug: $F_{(1, 44)} = 0.45$, $p = 0.50$; Fig 3.3b). There was no significant interaction of JDTic and stress on this measure (Drug x SEFL: $F_{(1, 44)} = 0.021$, $p = 0.89$; Fig 3.3b).

When analyzing mobility across bins (Minutes 1-4, Minutes 5-8), there was still a significant decrease in mobility among the stressed animals (SEFL: $F_{(1, 88)} = 19.1$, $p < 0.0001$; Fig 3.3c). Again, there was no significant effect of JDTic (Drug: $F_{(1, 88)} = 0.73$, $p = 0.40$; Fig 3.3c). There was no effect of bin nor were there any significant interactions on this measure (Bin: $F_{(1, 88)} = 3.83$, $p = 0.053$; Drug x SEFL: $F_{(1, 88)} = 0.033$, $p = 0.86$; SEFL x Bin: $F_{(1, 88)} = 1.35$, $p = 0.25$; Drug x Bin: $F_{(1, 88)} = 0.090$, $p = 0.76$; Drug x SEFL x Bin: $F_{(1, 88)} = 0.011$, $p = 0.92$; Fig 3.3c).

These data indicate that exposure to an acute stressor reduces overall mobility in the open field, and JDTic does not mitigate this reduction in mobility.

Forced Swim Test

One day after a 15-minute exposure in the forced swim test, animals were tested during a 5-minute session and assessed for the number of immobility bouts exhibited and the latency to the first immobility bout as measures of depression. There was no significant effect of JDTic or stress on the number of immobility bursts during this 5-minute session (Drug: $F_{(1, 27)} = 0.94$, $p = 0.34$; SEFL: $F_{(1, 27)} = 0.017$, $p = 0.90$; Drug x SEFL: $F_{(1, 27)} = 3.22$, $p = 0.084$; Fig 3.4b). Likewise, there was no significant effect of JDTic or stress on the latency to the first immobility bout (Drug: $F_{(1, 27)} = 0.056$, $p = 0.81$; SEFL: $F_{(1, 27)} = 0.21$, $p = 0.65$; Drug x SEFL: $F_{(1, 27)} = 0.12$, $p = 0.74$; Fig 3.4c). These results indicate that there was no effect of JDTic treatment or stress on behavior in the forced swim test.

DISCUSSION

In these experiments, we sought to understand the contribution of KOR's following stress to development of PTSD-like phenotypes including heightened measures of fear, anxiety and depression as measured by stress-enhanced fear learning, and behavior in the OF, EPM, and FST. Surprisingly, post-trauma administration of JDTic, a KOR antagonist, did not alter subsequent fear behavior as indicated by the perseverance of the stress-enhanced fear learning phenotype. This result was unexpected, as previous studies have implicated a role of KOR's in fear learning – however, JDTic did not affect fear behavior in either stressed or unstressed animals in these experiments (Knoll et al., 2007; Knoll et al., 2011). Likewise, ICV administration of nor-binaltorphimine (10 μg/rat), a KOR antagonist, prior to fear conditioning reduced freezing to the trained context when animals were placed back in the context for a fear retention test the

following day (Fanselow et al., 1991). Despite the lack of effect on fear measures, the most robust and seemingly resistant phenotype following experience with an acute stressor, we continued to investigate the potential contribution of post-trauma KOR's to other PTSD-like phenotypes that arise following an acute stressor including heightened anxiety and depression.

To test for the role of KORs in developing anxiogenic and depressive behaviors, a systemic post-trauma injection of JDTic was given prior to testing behavior on the OF, EPM and FST. On the EPM, stressed animals given JDTic trended toward increased time spent in the open arms compared to stressed animals given saline. However, a more interesting trend arises when analyzing behavior on the EPM temporally, comparing the first half of the test session (Minutes 1-4) to the second half of the test session (Minutes 5-8). Dissecting the EPM session temporally gives further insight into how behavior during this anxiety-provoking test is affected by various experimental conditions (Carobrez and Bertoglio, 2005). Control (unstressed saline) animals exhibited habituation across the EPM test session, as indicated by an increase in time spent in the open arms in the second half of the session compared to the first half, indicating that anxiety diminished with increased exposure to the EPM. Stressed animals given JDTic also habituated across the test session. However, stressed animals given saline did not habituate to the EPM, instead showing a decrease in the time spent in the open arms across the test session. This key difference between stressed animals – JDTic treated habituating to the anxiogenic EPM test and saline treated not habituating – indicates that KORs could be involved in stress-induced anxiogenic behavior as this phenotype is rescued in JDTic treated animals. It is important to note that unstressed animals given JDTic also failed to habituate to the EPM, and closely resembled stressed animals given saline in that time spent in the open arms decreased across the test session. This result was unexpected, as prior studies have indicated a general anxiolytic effect of JDTic administration (Knoll et al., 2007). However, these data

indicate that JDTic's ability to mitigate anxiogenic behavior is specific to animals that have undergone an acute stressor, and actually produces anxiogenic behavior in unstressed animals. Understanding how JDTic affects stressed and unstressed animals differentially is critical as the drug is considered for therapeutic purposes. If JDTic's benefits are not only restricted to individuals whose neural circuitry has been affected by stress but also produce negative effects in control individuals, extreme caution should be taken when contemplating JDTic as a therapeutic intervention.

Interestingly, post-trauma administration of JDTic had no effect on behavior in the OF. Stressed animals showed reduced locomotor activity compared to unstressed animals, an effect seen previously in our laboratory, but giving JDTic following stress did not mitigate this phenotype (Perusini et al., 2016). Likewise, there was no effect of stress or JDTic on behavior in the FST. Previously, our laboratory has demonstrated an increase in depressive behavior in the FST following an acute stressor, which failed to replicate in this study (Perusini et al., 2016). One critical difference between previous FST experiments and the one presented here is the amount of time left between the initial experience (15 minutes of forced swim) and the probe trial (5 minutes of forced swim, where data are taken). Previously, one week separated these two experiences; in this study, there was only one day of separation between the initial forced swim and probe test. It is possible that the depressive changes observed previously require an incubation period, and that one day of separation is not sufficient to unearth the depressive phenotype. Regardless, administration of JDTic does not appear to mitigate either anxiogenic behavior in the OF or depressive behavior in the FST, as demonstrated by a lack of effect in these experiments.

KORs and its endogenous ligand dynorphin are highly expressed throughout the fear and anxiety circuit, including in the HPC, BLA, CeA, PFC, and BNST (Crowley and Kash, 2015).

Although a functional circuit outlining how KORs and dynorphin in these brain regions contribute to anxiety states is currently unknown, previous studies implicate a role of KORs in reducing glutamatergic activation of the BNST via BLA inputs (Crowley et al., 2016). Additionally, KORs have been demonstrated to contribute to GABAergic connectivity between the CeA and BNST, another critical pathway in the fear and anxiety circuit (Li et al., 2012). Given the role of the BNST in stress and anxiety, and accumulating evidence of the role of KORs and dynorphin with the BNST, this brain region is poised to play a critical role in the stress-mediated anxiolytic behavior on the EPM observed in these experiments and rescued by post-trauma administration of JDTic (Adhikari, 2014; Gungor and Pare, 2014; Taugher et al., 2014; Gungor et al., 2015; Avery et al., 2016). Previously, KORs in the BNST have been shown to be involved in mediating stress-induced alcohol seeking, as delivery of nor-BNI, a KOR antagonist, into the BNST blocked reinstatement of alcohol seeking induced by a systemic injection of U50,488, a KOR agonist (Lê et al., 2018). The BNST's involvement in regulating anxiety states in combination with its demonstrated role in mediating aspects of stress and rich expression of KORs and dynorphin make it an excellent contender for playing a role in the stress-induced anxiety changes observed in these studies. However, further studies are necessary to validate the causal role of KORs in the BNST for developing stress-induced anxiogenic states.

PTSD is an extremely complex, multi-faceted disease with a variety of symptoms. Although many of these symptoms have been successfully mimicked in our animal model of the disease (heightened startle reaction, emotional distress, negative affect, and subsequent enhanced fear responses), many symptoms remain unexplored (Diagnostic and Statistical Manual of Mental Disorders, 2013; Perusini et al., 2016). One PTSD-like phenotype that has yet to be explored in our animal model of the disease is difficulty sleeping (alterations in circadian rhythm). Importantly, other stress models have demonstrated alterations in circadian rhythm

following stress which can interact with other stress-induced phenotypes including changes in fear, anxiety and depression (Loh et al., 2010; Miyazaki et al., 2013; Landgraf et al., 2014). Pituitary adenylate cyclase-activating peptide (PACAP) has been implicated in both PTSD and circadian rhythm, demonstrating a connection between stress and regulators of sleep and circadian rhythm (Colwell et al., 2004; Ressler et al., 2011; Dias and Ressler, 2013). Additionally, there is evidence that KORs play a role in these stress-induced alterations in circadian rhythm, as administration of JDTic prior to stress reduced circadian rhythm and sleep disruptions (Wells et al., 2017). Exploring other PTSD-like symptoms including alterations in circadian rhythm could provide a more holistic understanding of the neural mechanisms contributing to selective PTSD-like phenotypes. In order to wholly comprehend the neural changes that lead to stress-induced behavioral changes, we should incorporate as many PTSD-like phenotypes as possible as evidence thus far indicates that there might be distinct neural mechanisms contributing to the array of PTSD-like changes induced by exposure to an acute stressor.

Collectively, these experiments demonstrate a selective role of KOR's in mediating certain anxiogenic behaviors following stress without altering other enhanced fear and depressive measured produced by experience with an acute stressor. Through administering JDTic systemically and post-trauma, these experiments gave us the unique ability to discern effects that could be translated directly into a clinical intervention. In these studies, post-trauma administration of JDTic selectively rescued anxiogenic behavior on the EPM in stressed rats without producing anxiolytic, anti-depressive, or fear-reducing effects in any other measures following a stressor. Additionally, although JDTic rescued open arm habituation on the EPM in stressed animals, administering JDTic to unstressed animals caused those animals to reduce their open arm exploration across the test session and closely resemble stressed, saline-treated

animals. Taken together, these experiments signify a potential role of KOR's in mediating PTSD-like phenotypes following an acute stressor. However, given the narrow scope of JDTic's effects and the consequential effects on unstressed animals, additional pre-clinical work is needed prior to considering KOR antagonists such as JDTic as a potential therapeutic for PTSD.

MATERIALS AND METHODS

Animals

96 male, Long Evans rats with an age range of 60 to 90 days were utilized for these experiments (Experiment 1: $n = 32$, Experiment 2: $n = 64$). Rats were housed in isolation for at least one week prior to experimentation, and were handled ~1 minute per day by the experimenter. All rats were given unrestricted access to food and water except when briefly removed from the vivarium for behavioral testing. Animals were housed on a 12-hour on/off reverse light cycle. The Chancellor's Animal Research Committee at UCLA approved all behavioral protocols involving animals.

Behavioral Testing

Apparatus

All fear conditioning experiments were conducted in Med Associates conditioning chambers (28 x 21 x 21 cm; Lafayette Instrument Co.; Lafayette, IN). These chambers were controlled through Med Associates Freeze software. Two distinct contexts were used: Context A and Context B. Context A consisted of white light, flat grid flooring, Windex cleaner, and no additional inserts. Context B consisted of red light, thick/thin grid flooring, acetic acid cleaner, and an A-frame insert. Footshocks were delivered through Med Associates shock scramblers

(ENV 414-S). Near infrared cameras were used to record conditioning sessions, and freezing behavior was analyzed using Med Associates Video Freeze software.

Stress-Enhanced Fear Learning (Experiment 1)

Animals received an acute traumatic experience consisting of 15 pseudorandom, unsignaled footshocks (1-sec, 1.0-mA) across 90 minutes in Context A. Unstressed animals received equal context exposure without footshock delivery. Immediately after trauma, animals were given an i.p. injection of JDTic (10 mg/kg) or saline. 10 days later, animals are given an additional i.p. injection of JDTic (10 mg/kg) or saline. An additional 2 days later, animals received a 8-minute context test of the trauma context (Context A) to assess retention of the trauma fear memory. The next day, animals were fear conditioned with a single (1-sec, 1.0-mA) shock delivered in a novel context (Context B). This shock was delivered after a 3-minute baseline period, and animals were removed from Context B 30 seconds after the footshock. 24 hours later, animals were placed back in Context B for an 8-minute context test to assess fear to the one shock context.

Anxiety and Depression Assays (Experiment 2)

Animals received a stressor consisting of 15 pseudorandom, unsignaled footshocks (1-sec, 1.0-mA) across 90 minutes in Context A. Unstressed animals received equal context exposure without footshocks. Immediately after trauma, animals were given an i.p. injection of JDTic (10 mg/kg) or saline. 3 to 6 days later, animals received a battery of behavioral tests to assess changes in anxiety and depression including the Elevated Plus Maze (Day 1), Open Field (Day 2), and Forced Swim Test (Days 3 and 4).

Elevated Plus Maze

Animals were transported to a novel testing room with a distinct transport method from that used for the acute traumatic experience. Then, animals were placed in the center of the elevated plus maze and allowed to explore for 8 minutes under dim red lighting. Activity on the elevated plus maze was recorded with a mounted video camera. Data was hand scored after completion of the task by a blind observer. The animal had to completely enter the open arm to qualify for open arm entries, and had to have at least half of its body in the open arm to qualify as time spent in the open arm. Each arm of the elevated plus maze measured 92 cm in length. The apparatus was 60 cm off the ground with 19 cm high walls enclosing the closed arms.

Open Field

The same transport and testing room was used for Open Field as for Elevated Plus Maze. Animals were transported to the room then placed in the center of an open field apparatus and allowed to explore freely for 8 minutes under dim red lighting. Behavior in the open field was recorded by a video camera mounted above the open field. Data was hand scored after completion of the task by a blind observer. Crossings were counted if the animal fully crossed one of the marked black gridlines in the open field. The open field arena measured 91.4 cm x 91.4 cm with 30.5 cm high walls.

Forced Swim Test

The same transport was used for Forced Swim Test as for Open Field and Elevated Plus Maze, but a distinct testing room was used. This testing room employed bright white light. Animals were brought to the testing room, then placed in a large cylinder filled with water (temp) for 15 minutes (Day 3). No data was recorded on this day. 24 hours later, animals were brought back to the testing room and placed back in the large cylinder filled with water for 5 minutes.

Data was recorded by a video camera and was hand scored after completion of the task by a blind observer. The animal had to cease all movement to qualify for an immobility bout.

Data Analyses

All data were analyzed using R. ANOVAs were used to evaluate the effects of treatment between groups, and when appropriate, followed by post hoc *t*-tests.

ACKNOWLEDGEMENTS

Thank you to Eric Harvey and Pamela Kennedy for their intellectual contribution and assistance with these experiments. Thank you to Dickson Chen for assistance with handling animals and behavioral testing. Thank you to the Fanselow laboratory for contributions to these experiments.

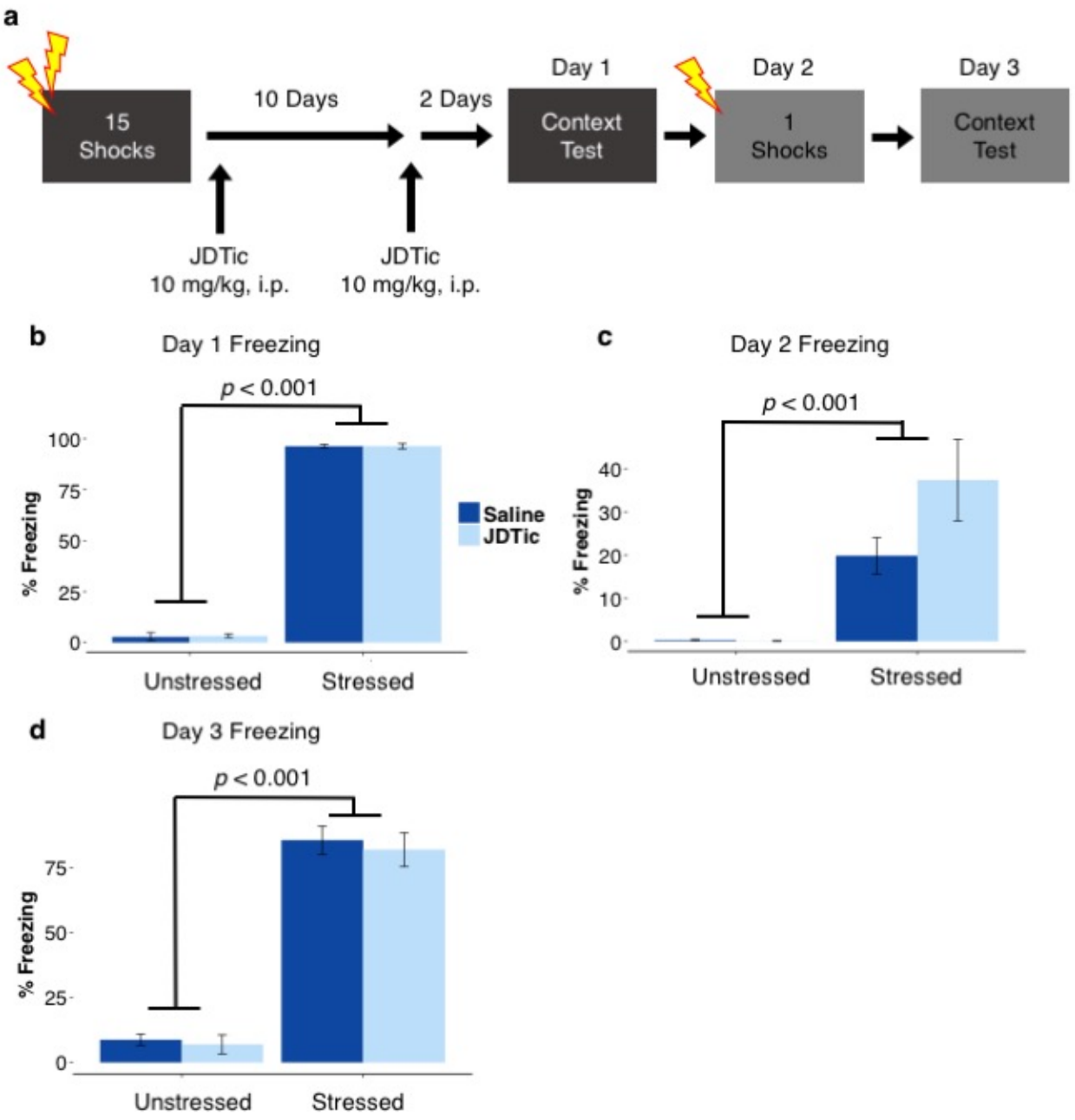

Figure 3.1: Administration of JDTic immediately after an acute stressor does not mitigate subsequent enhanced fear conditioning. (a) Experimental design. (b) Freezing across an 8 minute context test in the 15 shocks (trauma) context. Stressed animals froze significantly more than unstressed animals but there was no effect of JDTic on this measure. (c) Freezing across the 3 minute baseline prior to the one shock mild stressor. Stressed animals froze significantly more than unstressed animals but there was no effect of JDTic on this measure. (d) Freezing

across an 8 minute context test to the one shock context. Stressed animals froze significantly more than unstressed animals during this test but there was no effect of JDTic.

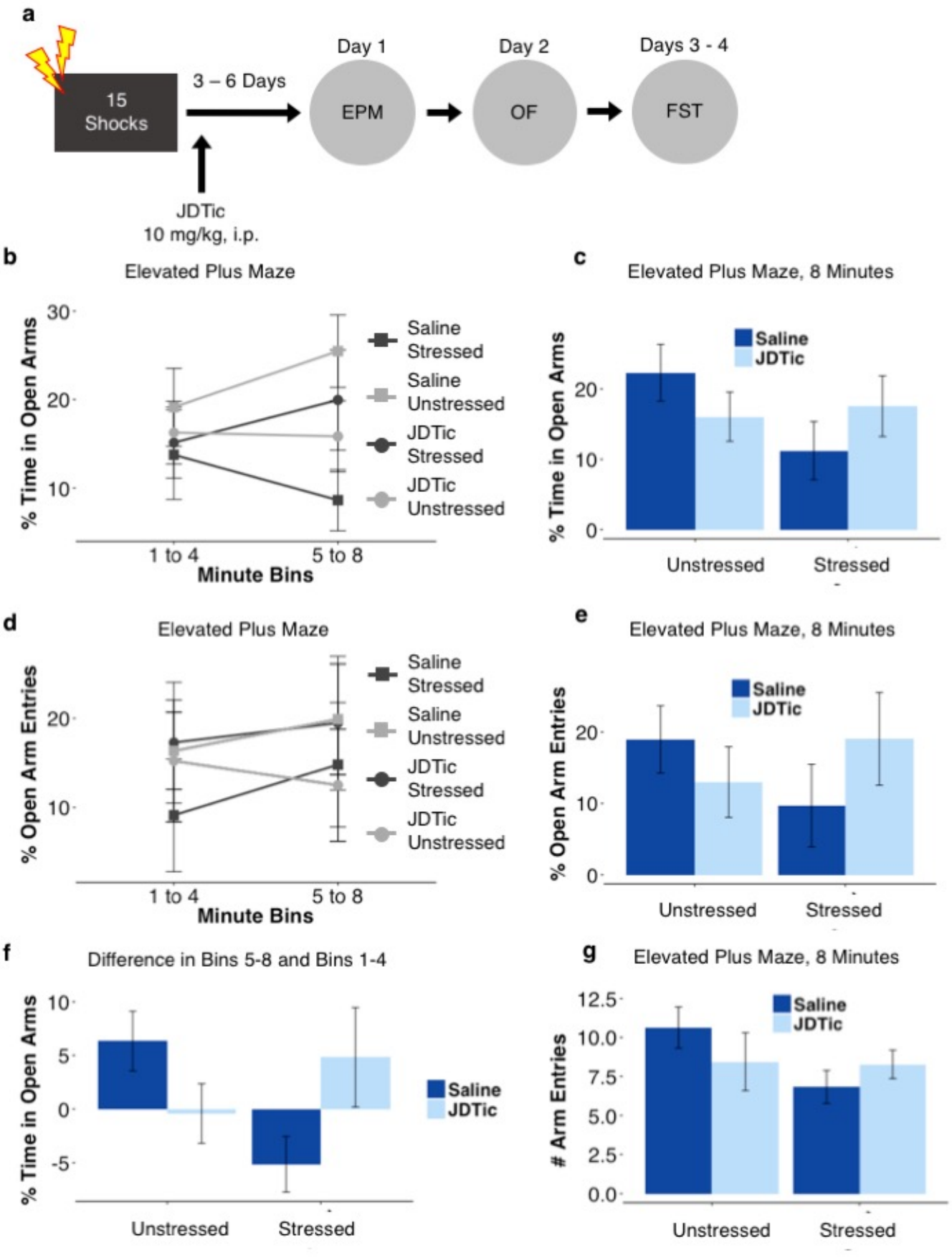
a
15 Shocks
3 – 6 Days
Day 1
EPM
Day 2
OF
Days 3 - 4
FST
JDTic
10 mg/kg, i.p.
b
Elevated Plus Maze
% Time in Open Arms
Saline Stressed
Saline Unstressed
JDTic Stressed
JDTic Unstressed
1 to 4
5 to 8
Minute Bins
c
Elevated Plus Maze, 8 Minutes
% Time in Open Arms
Saline
JDTic
Unstressed
Stressed
d
Elevated Plus Maze
% Open Arm Entries
Saline Stressed
Saline Unstressed
JDTic Stressed
JDTic Unstressed
1 to 4
5 to 8
Minute Bins
e
Elevated Plus Maze, 8 Minutes
% Open Arm Entries
Saline
JDTic
Unstressed
Stressed
f
Difference in Bins 5-8 and Bins 1-4
% Time in Open Arms
Saline
JDTic
Unstressed
Stressed
g
Elevated Plus Maze, 8 Minutes
Arm Entries
Saline
JDTic
Unstressed
Stressed

Figure 3.2: Post-trauma administration of JDTic alters behavior on the elevated plus maze in both stressed and unstressed animals. (a) Experimental design. (b) Percent time that animals spent in the open arm during the first half (minutes 1-4) and second half (minutes 5-8) on the elevated plus maze. Both saline unstressed and JDTic stressed groups showed habituation across the test session as indicated by an increase in the time spent in the open arms in the latter half of the session. Both saline stressed and JDTic unstressed groups failed to show this habituation. (c) Across the full 8 minute session, there was no effect of stress or JDTic on the total percent of time spent in the open arms. (d) Percent of all arm entries that were open arm entries comparing between the first half (minutes 1-4) and second half (minutes 5-8) of the test session. There was no effect of stress or JDTic on this measure. (e) Percent of all arm entries that were open arm entries across the full 8 minute session. There was no effect of stress or JDTic on this measure. (f) Calculated difference scores between the first half and second half of the test session in percent time spent in open arms (percent time spent in minutes 5-8 minus percent time spent in open arms in minutes 1-4). Positive values indicate more time spent in the open arms in the latter half of the session. Saline unstressed and JDTic stressed animals show an increase in percent time spent in the open arms across the test session, while saline stressed and JDTic unstressed animals do not. (g) Total number of arm entries across the 8 minute test session. There was no effect of stress or JDTic on this measure.

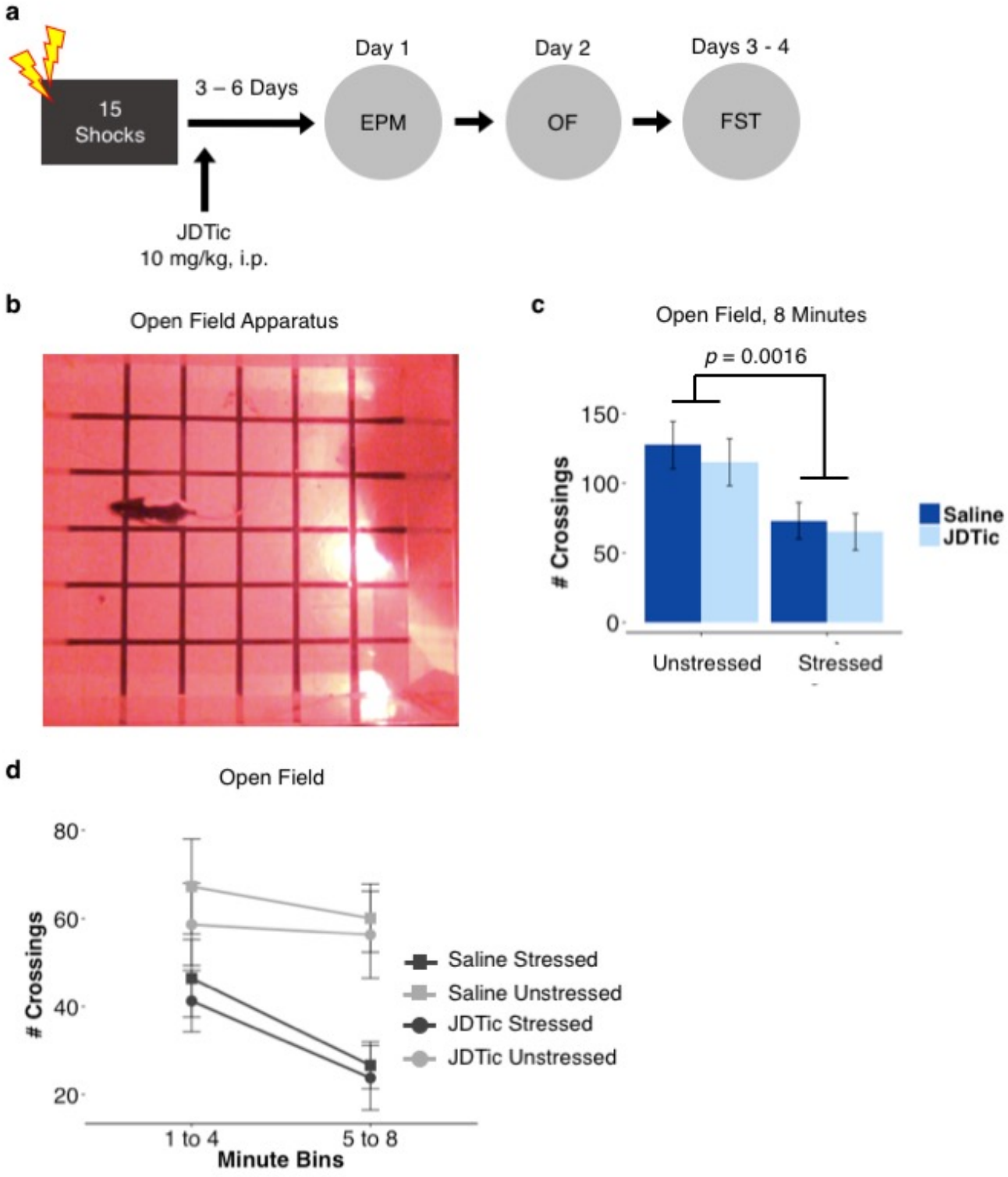

Figure 3.3: Post-trauma administration of JDTic does not affect anxiety-like behavior in the open field test. (a) Experimental design. (b) Open field apparatus. (c) Total number of

crossings that rats make in the open field (a measure of locomotion) across an 8 minute session. Stressed animals move significantly less than unstressed animals but there is no effect of JDTic on this measure. (d) Number of crossings that rats make in the open field binned by the first half (minutes 1-4) and second half (minutes 5-8) of the test session. Stressed animals move significantly less than unstressed animals but there is no effect of JDTic on this measure.

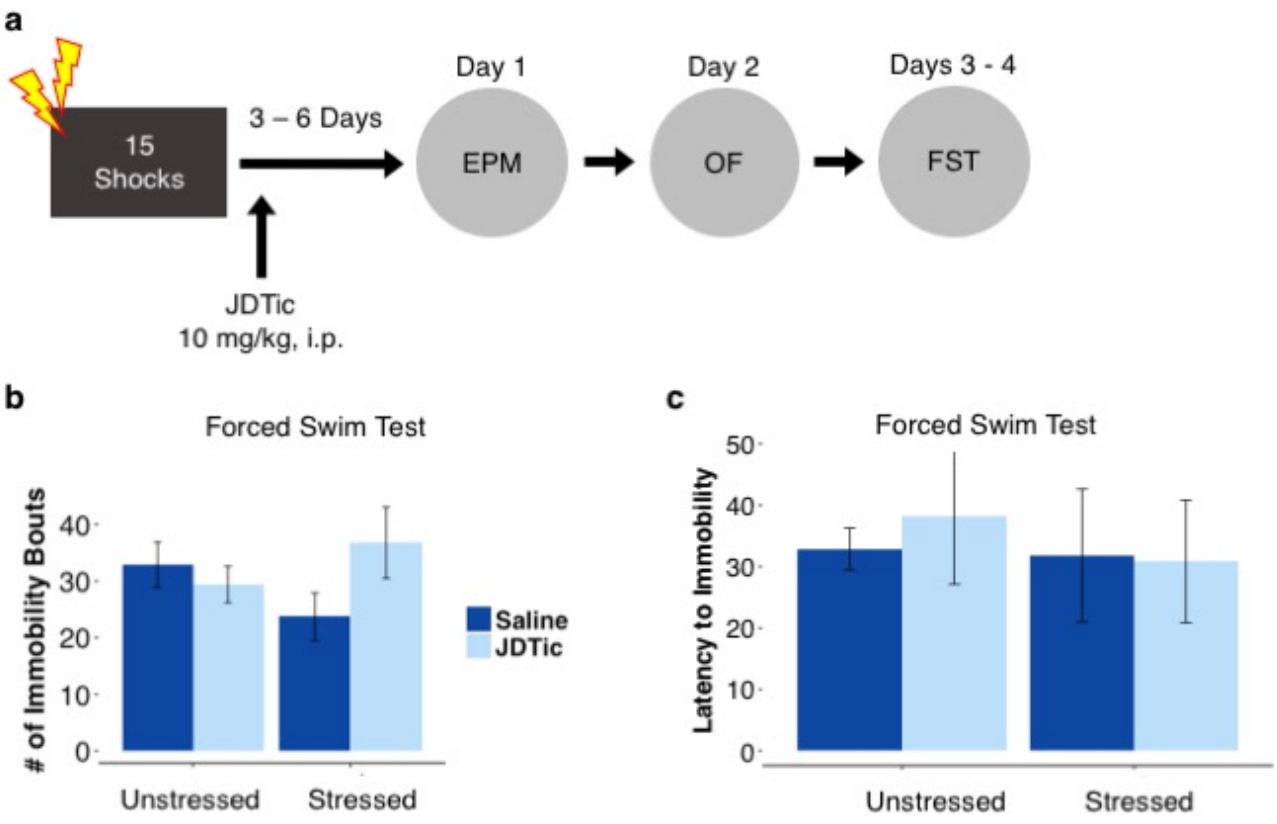

Figure 3.4: Post-trauma administration of JDTic does not alter behavior in the forced swim test. (a) Experimental design. (b) Number of immobility bouts across a 5 minute session, as measured by a rat ceasing all motor activity. There was no effect of stress or JDTic on this measure. (c) Latency to the first immobility bout that each rat displayed, in seconds. There was no effect of stress or JDTic on this measure.

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CHAPTER 4

Neural Mechanisms Underlying Stress-Enhanced Fear Learning

ABSTRACT

Behavioral consequences of experience with an acute stressor are driven by molecular and circuit neural changes. In our rodent model of Post-Traumatic Stress Disorder, animals that undergo an acute stressor develop a variety of PTSD-like symptoms including heightened fear responses, anxiety-like behavior depressive-like behavior. To fully understand how these PTSD-like phenotypes arise, and therefore successfully diagnose and treat these symptoms in the human population, we must understand the associated underlying neural mechanisms. The following experiments begin to address this by utilizing a variety of molecular manipulations and analyses to understand how the brain changes following stress to cause one PTSD-like symptom observed in our model, stress-enhanced fear learning (SEFL). Specifically, I analyze how changes in glutamate transmission contribute to SEFL by first examining RNA and protein changes of GluA1, an AMPA-R subunit implicated in fear learning, throughout the fear circuit following an acute stressor. Interestingly, there is a distinct RNA *Gria1* upregulation in the ventral hippocampus, and a separate protein GluA1 upregulation in the basolateral amygdala, indicating potentially contrasting mechanisms of AMPA-R changes in these two brain regions. Next, NMDA-R's were deleted throughout the amygdala following stress to understand their selective contribution to SEFL. Deleting amygdala NMDA-R's following stress interfered with consolidation of the trauma memory itself, and mitigated the SEFL phenotype as seen by reduced fear learning in the animals with depleted NMDA-R's. Finally, I studied the population dynamics in the amygdala using the immediate early gene *fos* to parse how cells in the

amygdala contribute to the SEFL phenotype. Each of these experiments sheds light on the underlying molecular mechanisms contributing to SEFL, and provides a framework by which subsequent experiments can continue to inform us of the pertinent neural changes that underlie specific stress-induced behavioral changes. In the future, these techniques and approaches can be used to investigate the neural mechanisms underlying other stress-induced behavioral changes seen in our model, including selective changes in anxiety and depression.

INTRODUCTION

Generally, stress can lead to a multitude of behavioral and molecular changes in an individual ranging from suppressed immune response, increases in glucocorticoid levels, heightened anxiety and depression, and the facilitation of insulin resistance (Black, 2006; Musazzi et al., 2011; McEwen et al., 2012; Carlsson et al., 2014; Huybrechts et al., 2014; Nicolaides et al., 2015). Due to the multitude and diversity of responses stress causes in an individual, I will focus on how stress alters the fear and anxiety circuit, as is most relevant to this dissertation. Critical to understanding how stress alters cellular and circuit function in the brain is paying particular attention to the type, severity, and longevity of the stress in question. For example, there is evidence that mild stress can facilitate forms of learning and memory while severe stress highly impairs performance on these same behavioral measures (Adlard et al., 2011; Maras et al., 2014). Given the complexity of stress-induced molecular and behavioral changes, and the sensitivity of the type, severity, and longevity of the given stress protocol on these measures, it is critical that researchers consider how, when, and at what strength animals are stressed for any subsequent comparisons of molecular and behavioral changes.

Acute stress is known to affect cellular function in key brain regions involved in fear and anxiety including the hippocampus (HPC), the amygdala (both basolateral amygdala, BLA, and central amygdala, CeA) and the prefrontal cortex (PFC). One such change in these brain regions is a noted increase in excitatory cellular transmission, driven primarily by an increase in glutamate release and transmission in the fear and anxiety circuit (Musazzi et al., 2011). These increases in glutamate transmission have been linked to consequences of stress including the induction of major depressive disorder (Tordera et al., 2011; Wang et al., 2015). Stress has also been shown to alter dendritic integrity, though with opposing effects in differing brain regions; in the PFC and HPC, stress has been shown to cause dendritic retraction and atrophy while in the

amygdala, stress often enhanced dendritic integrity through increasing dendritic length and augmenting spine density (Vyas et al., 2002; Morales-Medina et al., 2009). Several lines of evidence have implicated *N*-Methyl-*D*-Aspartate Receptors (NMDA-R's) as playing a critical role in these stress-induced changes in dendritic integrity, as blocking NMDA-R's mitigates these effects (Martin and Wellman, 2011; Andres et al., 2013). Taken together, stress, through release of stress factors including corticosteroids (CORT) and corticotropin-releasing hormone (CRH) is known to mediate cellular and circuit dynamics of the fear and anxiety circuit by altering critical aspects of cellular transmission including glutamate release and transmission, and either reducing or enhancing dendritic integrity in these brain regions. These cellular changes undoubtedly underlie some stress-induced changes in behavior, including heightened anxiety, depression, and fear responses.

Recent trends in neuroscience have highlighted how powerful experiments that causally manipulate cellular, molecular, or circuit-level function in the brain to observe a specific behavioral effect can be. To fully understand a phenotype and how it arises given certain genetic and environmental conditions, it is imperative that causal manipulations leading to changes in behavior can be linked. Therefore, the following experiments will build upon our behavioral characterization of stress-induced phenotypes by introducing molecular analyses and manipulations such that the observed behavioral changes can be causally linked to its associated neural mechanisms.

Previous experiments in our laboratory have demonstrated that stress-enhanced fear learning (SEFL) induction is dependent upon CORT's mechanisms in the BLA, as administering mifepristone, a glucocorticoid receptor (GR) antagonist, or metyrapone, a CORT synthesis inhibitor, into the BLA eliminates the SEFL phenotype (Perusini et al., 2016). Likewise, we have shown that SEFL expression is mediated by an upregulation of the GluA1 subunit of the AMPA

receptor in the BLA (Perusini et al., 2016). However, what is currently unknown is how neuromodulators of stress, including CORT, act upon cells in the fear and anxiety circuit to precipitate molecular changes that ultimately produce behavioral effects of stress. Understanding how modulators of stress can alter neural pathways involved in fear learning and memory, anxiety, depression, and other measures of PTSD is a critical step in understanding how stress interacts with these systems to produce maladaptive behaviors.

The following experiments seek to address what neural changes following an acute stressor lead to PTSD-like symptoms seen in our rodent model of the disease. Critically, advances in cellular and molecular techniques to probe at neural mechanisms have allowed the field to pinpoint specific neural changes occurring in particular brain regions at a given time. This type of specificity is crucial for beginning to understand how a system as complex as the fear and anxiety circuit becomes sensitized following stress. First, we seek to further characterize how stress leads to protein and RNA changes in the AMPA-R subunit GluA1, a calcium-permeable subunit that can contribute greatly to cellular plasticity. Next, we investigate the role of NMDA-R's in the expression of SEFL through selective NMDA-R depletion in the BLA following the acute stressor. Finally, we investigate the circuit dynamics of SEFL by tagging cells that are active at certain timepoints during and following stress to understand how network activity in the BLA contributes to enhanced fear conditioning following stress. Taken together, these studies provide a platform for which to build subsequent experiments intended to address how neural and molecular changes within the fear and anxiety circuit guide PTSD-like symptoms observed following an acute stressor.

RESULTS

Experiment 1: Post-trauma molecular analysis of GluA1 protein and RNA indicates an upregulation of GluA1 RNA in the VH but not in either the BLA or DH, while there is a protein upregulation of GluA1 in the BLA but not in the VH or the DH.

RNA Analysis

Immediately after the acute stressor, one hemisphere of each brain region (BLA, VH and DH) was taken from each animal to analyze *Gria1* RNA levels via RT-PCR. Two different primers (Gria1-1 and Gria1-2) were used for amplifying and analyzing *Gria1* RNA levels.

The Gria1-1 primer produced no overall effect of stress on RNA levels (Stress: $F_{(1, 48)}$ = 0.70, p = 0.79; Fig 4.1c). However, there was a main effect of RNA levels across brain region without an interaction between stress and brain region (Region: $F_{(2, 48)}$ = 4.30, p = 0.019; Stress x Region: $F_{(2, 48)}$ = 0.044, p = 0.96; Fig 4.1c; Fig 4.1c). Within the BLA, there was no effect of stress or hemisphere on *Gria1* RNA levels (Stress: $F_{(1, 16)}$ = 0.011, p = 0.92; Hemisphere: $F_{(1, 16)}$ = 1.25, p = 0.28; Stress x Hemisphere: $F_{(1, 16)}$ = 0.13, p = 0.72; Fig 4.1b). Within the VH, there was no effect of stress or hemisphere on *Gria1* RNA levels (Stress: $F_{(1, 12)}$ = 0.057, p = 0.82; Hemisphere: $F_{(1, 12)}$ = 1.24, p = 0.29; Stress x Hemisphere: $F_{(1, 12)}$ = 0.017, p = 0.90; Fig 4.1b). Within the DH, there was also no effect of stress or hemisphere on *Gria1* levels (Stress: $F_{(1, 14)}$ = 0.051, p = 0.82; Hemisphere: $F_{(1, 14)}$ = 1.04, p = 0.75; Stress x Hemisphere: $F_{(1, 14)}$ = 0.11, p = 0.75; Fig 4.1c).

The other primer, Gria1-2, did not yield an overall effect of stress on RNA levels (Stress: $F_{(1, 48)}$ = 0.14, p = 0.71; Fig 4.1e). There was, however, a main effect of brain region on RNA levels without an interaction between stress and brain region (Region: $F_{(2, 48)}$ = 7.84, p = 0.0011; Stress x Region: $F_{(2, 48)}$ = 2.33, p = 0.11; Fig 4.1e). Within the BLA, there was also no effect of

stress or hemisphere on *Gria1* RNA levels (Stress: $F_{(1, 16)} = 0.16$, $p = 0.70$; Hemisphere: $F_{(1, 16)} = 0.0036$, $p = 0.95$; Stress x Hemisphere: $F_{(1, 16)} = 1.00$, $p = 0.33$; Fig 4.1d). In the VH, there was no significant effect of stress or hemisphere on *Gria1* RNA levels but there was a trending interaction such that the left hemisphere of stressed rats showed enhanced levels of *Gria1* RNA levels compared to the left hemisphere of unstressed animals (Stress: $F_{(1, 12)} = 2.53$, $p = 0.14$; Hemisphere: $F_{(1, 12)} = 1.32$, $p = 0.27$; Stress x Hemisphere: $F_{(1, 12)} = 03.38$, $p = 0.09$; Fig 4.1d). In the DH, there was also no effect of stress or hemisphere on *Gria1* RNA levels (Stress: $F_{(1, 14)} = 1.91$, $p = 0.19$; Hemisphere: $F_{(1, 14)} = 0.74$, $p = 0.40$; Stress x Hemisphere: $F_{(1, 14)} = 0.30$, $p = 0.59$; Fig 4.1d). Taken together, these data indicate that *Gria1* RNA levels are differentially regulated by stress throughout the fear and anxiety circuit (BLA, DH and VH). There were no significant effects of stress on *Gria1* RNA expression in the BLA or DH. However, there was an enhancement in *Gria1* RNA levels in the left hemisphere of stressed animals compared to unstressed animals, indicating a potential lateralization of *Gria1* modulation.

Protein Analysis

Immediately following trauma, the other hemisphere of each brain region (BLA, VH and DH) was taken to analyze GluA1 protein levels via Western Blotting. There was a significant main effect of stress on GluA1 protein levels (Stress: $F_{(1, 50)} = 0.4.26$, $p = 0.044$; Fig 4.2b). There was a significant difference in protein quantification (GluA1/GAPDH) between each of these brain regions, but no interaction of stress and brain region (Region: $F_{(2, 50)} = 9.42$, $p < 0.001$; Stress x Region: $F_{(2, 50)} = 1.73$, $p = 0.19$; Fig 4.2b). Within the BLA, there was a trend toward an increase in GluA1 protein in stressed animals but no effect of hemisphere on this measure (Stress: $F_{(1, 18)} = 3.67$, $p = 0.071$; Hemisphere: $F_{(1, 18)} = 0.79$, $p = 0.39$; Stress x Hemisphere: $F_{(1, 18)} = 0.41$, $p = 0.53$; Fig 4.2c). Within the VH, there was a trend toward more GluA1 protein in the left hemisphere but no effect of stress on this measure (Stress: $F_{(1, 14)} = 0.067$, $p = 0.80$;

Hemisphere: $F_{(1, 14)} = 3.37$, $p = 0.088$; Stress x Hemisphere: $F_{(1, 14)} = 0.26$, $p = 0.62$; Fig 4.2c). Within the DH, there was no effect of stress or hemisphere on GluA1 protein levels (Stress: $F_{(1, 12)} = 1.95$, $p = 0.19$; Hemisphere: $F_{(1, 12)} = 0.0021$, $p = 0.96$; Stress x Hemisphere: $F_{(1, 12)} = 0.0015$, $p = 0.97$; Fig 4.2c). These data indicate that GluA1 protein levels are differentially modulated by stress in brain regions associated with fear and anxiety (BLA, DH and VH). There were no changes in GluA1 levels due to stress in the DH or VH, but there was a trend toward enhanced GluA1 protein expression in the BLA of stressed animals compared to unstressed animals, as has been previously reported (Perusini et al., 2016).

Experiment 2: NMDA-R's in the BLA and CeA are necessary for consolidation of the trauma memory and stress-enhanced fear learning.

Trauma Context Test

Animals underwent the acute stressor protocol (10 footshocks), then received surgery to infuse either Cre-recombinase, to deplete NMDA-R's from the amygdala, or GFP, a control, the following day. Ten days after surgery, animals were placed back into the stressor context for 8 minutes to measure fear retention. Stressed animals froze significantly more than unstressed animals during this test, and animals receiving an infusion of Cre-recombinase froze significantly less than animals receiving GFP (Stress: $F_{(1, 26)} = 178.36$, $p < 0.001$; Virus: $F_{(1, 26)} = 4.72$, $p = 0.039$; Fig 4.3b). There was also a significant interaction of stress and virus infused (Stress x Virus: $F_{(1, 26)} = 4.30$, $p = 0.048$; Fig 4.3b). Stressed animals receiving Cre-recombinase froze significantly less than stressed animals that received GFP ($t(12) = 2.12$, $p = 0.055$; Fig 4.3b). Unstressed animals receiving Cre-recombinase did not freeze significantly less than unstressed animals that received GFP ($t(10) = 0.60$, $p = 0.56$, Fig 4.3b). These data indicate that stressed animals freeze significantly more than unstressed animals in the stress

conditioning context, and that depleting NMDA-R's in the amygdala reduces freezing across both stressed and unstressed animals.

One Shock Context Test

The primary PTSD-like measure of enhanced fear was measured by placing animals back in the context in which they received a single footshock, one day following conditioning. Across the 8-minute context test, stressed animals froze significantly more than unstressed animals (Stress: $F_{(1, 26)} = 9.32$, $p = 0.0052$; Fig 4.3c). Additionally, animals receiving Cre-recombinase, with depleted NMDA-R's in the amygdala, froze significantly less than animals with GFP (Virus: $F_{(1, 26)} = 4.23$, $p = 0.050$; Fig 4.3c). There was no significant interaction of stress and virus infused (Stress x Virus: $F_{(1, 26)} = 2.51$, $p = 0.12$; Fig 4.3c). Interestingly, stressed Cre-recombinase treated animals did not significantly differ in their freezing from unstressed Cre-recombinase animals ($t(7) = 1.80$, $p = 0.11$; Fig 4.3c) or from unstressed GFP animals ($t(8) = 1.11$, $p = 0.30$; Fig 4.3c). However, stressed GFP treated animals did freeze significantly more than unstressed Cre-recombinase animals ($t(7) = 2.86$, $p = 0.024$; Fig 4.3c) and unstressed GFP animals ($t(7) = 2.57$, $p = 0.035$; Fig 4.3c). These data indicate that depleting NMDA-R's in stressed animals eliminates the SEFL phenotype by reducing their conditioning to a single footshock to levels comparable to previously unstressed animals. Depleting NMDA-R's in the amygdala in the unstressed animals also slightly reduces conditioning to a single shock.

Experiment 3: Stress-Enhanced Fear Learning results in increased *fos* expression in the BLA during the one shock context test, aligning with increased freezing by stressed animals during this test.

Trauma Context Test

One day following the acute stressor, animals were placed back into the context for 8 minutes to measure fear retention to the trauma context. Stressed animals (received the 15 shocks) froze significantly more than unstressed animals (no previous shocks) when placed back into the trauma context (Stress: $F_{(1, 8)} = 93.77$, $p < 0.001$; Fig 4.4b).

One Shock Context Test

Two weeks after the acute stressor, animals were given one footshock to test for stress-enhanced fear learning. Subsequently, animals were placed back in the one shock context for 8 minutes to assess fear retention to the one shock context. Previously stressed animals froze significantly more than unstressed animals during this fear retention test (Stress: $F_{(1, 8)} = 16.12$, $p = 0.003$; Fig 4.4c).

c-fos Immunohistochemistry

Animals were sacrificed 90-minutes following the one shock context test and their brains stained for *fos* expression. Across all planes in the BLA, there was a modest increase in *fos* staining in the stressed animals compared to unstressed animals (Stress: $F_{(1, 11)} = 3.05$, $p = 0.11$; Fig 4.4e-g) but there was no effect of Anterior/Posterior plane on this increased expression (Plane: $F_{(2, 11)} = 0.77$, $p = 0.49$; Stress x Plane: $F_{(2, 11)} = 0.58$, $p = 0.58$; Fig 4.4e-g). In the anterior plane of the BLA, there was no difference between stressed and unstressed animals in *fos* activity ($t(2) = 0.095$; $p = 0.47$; Fig 4.4e). In the middle plane of the BLA, there

was more *fos* activity in stressed animals compared to unstressed animals ($t(4) = 2.65$; $p = 0.028$; Fig 4.4f). Given there was only one data point collected for the unstressed posterior plane of the BLA, no *post-hoc* t-test was run. Overall, stressed animals had more *fos* activity in the BLA compared to unstressed animals following the one shock context test. This finding might have differential expression patterns based on the plane (anterior, middle, posterior) of the BLA being analyzed.

DISCUSSION AND FUTURE DIRECTIONS

The studies presented here seek to elucidate the neural mechanisms underlying SEFL, or maladaptive conditioning to a single footshock following an acute stressor. Previously, most SEFL studies utilized pharmacological or behavioral interventions with the intention to identify novel therapeutics that could translate into the human population. However, these experiments utilize sophisticated molecular and genetic analyses to instead identify the neural changes associated with brain regions in the fear and anxiety circuit that could mediate SEFL. Using a combination of post-behavior molecular analysis and interventions, I outlined preliminary evidence for the role of plasticity changes and cell population dynamics in mediating SEFL. Specifically, I found that, immediately after trauma, there is an upregulation of *Gria1* RNA in the VH, but not in the DH or the BLA. Critically, we utilized two different *Gria1* primers, and found significant effects with only one of the two. Further studies to replicate these effects and parse why the two primers yielded inconsistent results is necessary to fully understand how *Gria1* RNA is modulated by stress in the fear and anxiety circuit. Interestingly, there was an upregulation of *Gria1*'s protein, GluA1 (an AMPA-R subunit), in the BLA but not in the VH or the DH immediately following trauma. Next, I found that deleting NMDA-R's in the amygdala after the acute stressor interferes with expression of the trauma memory, and mitigates subsequent fear learning. Finally, I examined the population dynamics in the BLA by measuring *fos* activity

following the one shock context test. These data indicated that, compared to unstressed animals, stressed animals exhibited higher levels of *fos* activity after the one shock context test, which matches the increased freezing observed in these animals. These data indicate that there are an increased number of cells in the BLA that are recruited during one shock conditioning following an acute stressor compared to control animals. Collectively, these experiments begin to examine molecular contributions to SEFL, and provide rationale for subsequent experiments to continue identifying the neural basis of SEFL.

These experiments indicate there is a dissociation between RNA and protein changes of the GluA1 AMPA-R subunit in different brain regions associated with fear and anxiety; in the VH, there is a selective upregulation of *Gria1* RNA and in the BLA there is a selective upregulation of GluA1 protein. The GluA1 AMPA-R subunit, when forming homomers to produce a full AMPA-R, creates calcium permeability and has been shown to be necessary for forms of learning and memory (Benke, 2013; Freudenberg et al., 2013; Zhou et al., 2015). These data indicate that there is a role of GluA1 upregulation contributing to PTSD-like phenotypes seen following experience with an acute stressor, although the mechanism underlying this upregulation could differ between the VH and the BLA. CREB and CaMKII have been shown to play a role in mediating an observed increase in GluA1 following fear conditioning, and are potential players in contributing to the GluA1 protein increase seen following the acute stressor (Middei et al., 2013; Tran and Keele, 2016). Potential mechanisms underlying the selective *Gria1* RNA increase are less clear, but could be driven by epigenetic modifications or post-transcriptional modifications, as one study indicated that *Gria1* levels were reduced in animals on methyl-donor-deficient diet, which leads to DNA hypermethylation in the brain, while other AMPA-R subunits, including *Gria2* and *Gria3* were not affected by the manipulation (Tomizawa et al., 2015). This *Gria1* reduction was linked to impairments in

consolidating a fear extinction memory, indicating a causal role of sufficient *Gria1* expression levels in driving learning and memory processes (Tomizawa et al., 2015). Subsequent work should focus on further characterizing the molecular changes that lead to *Gria1* RNA or GluA1 protein upregulation in these brain regions. Additionally, further analyses regarding GluA1 receptor availability and phosphorylation patterns would contribute to our understanding of how these AMPA-R's contribute to SEFL.

GluA1-containing AMPA-R's represent one avenue for calcium to enter the cell; another critical pathway for calcium to enter the cell and mediate plasticity is through NMDA-R's. Previously, the role of amygdala NMDA-R's in SEFL was unknown. More broadly, there is evidence that NMDA-R's in the amygdala are necessary for both acquisition and expression of fear memories, as opposed to the hippocampal dogma that describes NMDA-R's as necessary for only acquisition, not expression, of these memories (Kim et al., 1991; Maren et al., 1996). These experiments indicate that NMDA-R's in the BLA and CeA are required for either consolidation or expression of the trauma memory, as animals with depleted NMDA-R's in these regions showed freezing deficits in the trauma context fear memory recall session (Maren et al., 1996; Schmidt et al., 2015). Interestingly, this deletion of NMDA-R's also blocked the one shock conditioning in both unstressed controls and stressed animals, indicating that SEFL is NMDA-R dependent in the amygdala just as standard fear conditioning is. These data contradict previous studies that demonstrate animals are able to develop a fear memory to a second environment (Context B) with a NMDA-R antagonist if previously trained in a different environment (Context A) (Sanders and Fanselow, 2003; Tayler et al., 2011). However that study, and many others focused on forms of cellular plasticity, analyzes the hippocampus rather than the amygdala. These data alongside other evidence of a difference in NMDA-R contribution to learning in the amygdala compared to the hippocampus suggest that NMDA-R's role in fear learning could

differ greatly between these two brain regions. For example, while plasticity at thalamo-amygdala synapses is calcium-mediated, it is not dependent on NMDA-R's (Weisskopf et al., 1999). In addition, the role of NMDA-R's in the CeA in forming and expressing fear memories remains unknown, though there is evidence of a selective role in acquiring fear memories (Zimmerman and Maren, 2010). Critically, this study suggests that SEFL is not solely driven by NMDA-R independent forms of plasticity, such as GluA1-mediated calcium influx. However, this study fails to address whether NMDA-R's are required for acquisition, expression, consolidation, or some combination of these, for the one shock fear memory. This study begins to unearth the plasticity mechanisms that contribute to SEFL by suggesting that NMDA-R's in the amygdala are necessary for components of the SEFL phenotype.

Finally, the BLA population dynamics contributing to SEFL are analyzed in Experiment 3. These data indicate that a larger population of BLA neurons are recruited to the memory trace associated with the one shock conditioning in stressed animals compared to unstressed animals, despite the two groups receiving identical conditioning procedures. Given SEFL's phenotype of increased freezing to the one shock context compared to unstressed controls, it is unsurprising that this increased freezing corresponds to higher levels of BLA activity, as measured by *fos*. Previous studies have mixed findings regarding *fos* expression in the amygdala and its correspondence to freezing behavior, with some studies reporting increased *fos* expression mapping onto increased freezing while other studies fail to find such a pattern (Holahan and White, 2004; Hoffman et al., 2014; Skórzewska et al., 2015). The data presented here do show heightened *fos* expression in the BLA corresponding to increased freezing in stressed animals compared to unstressed animals. These data are in agreement with the notion that acute stress causes sensitization in the amygdala, leading to hyperexcitability and the formation of maladaptive fear responses to a mild stressor.

These experiments, while limited in animal sizes and therefore power, introduce a framework by which advances in molecular and genetic tools can allow us to identify specific molecular and pathway changes that underlie PTSD-like phenotypes seen in our acute stress model. In these experiments, which utilize both rats and mice, post-behavior analysis including RT-PCR and Western Blotting, and manipulations to alter behavior such as HSV-driven reductions in NMDA-R's within a specific brain region of the fear and anxiety circuit, we can begin to understand how neural pathways in distinct brain regions contribute to the variety of fear, anxiety, and depression phenotypes observed following experience with an acute stressor.

Utilizing these sophisticated methods of measuring RNA and protein levels and manipulating specific pathways can be used in combination with behavioral (Chapter 2) or pharmacological (Chapter 3) manipulations that alter the PTSD-like phenotypes observed after an acute stressor. Together, this provides an extremely powerful framework for identifying interventions that can be translated into the human population (behavioral or pharmacological interventions) and allows for a basic science understanding of how the brain changes after stress to cause PTSD-like phenotypes.

In Experiment 1, preliminary data indicates that there is a double dissociation between a *Gria1* RNA upregulation in the VH and a GluA1 protein upregulation in the BLA. Both the VH and BLA are critical components of the fear and anxiety circuit, and could mediate various aspects of post-stress phenotypes observed in our PTSD model. The dissociation between RNA and protein upregulation suggests that there might be different underlying mechanisms driving molecular consequences of stress in these different brain regions. There is a variety of evidence suggesting an interacting role of stress and GluA1 throughout the fear and anxiety circuit. For instance, one study indicated that propranolol, a beta-adrenoceptor antagonist, delivered systemically or directly into the BLA reduced fear reactivation driven increases in GluA1 in the

LA by interfering with the PKA and CaMKII pathways (Zhou et al., 2015). There is additional evidence for a contribution of the BDNF/MEK/MAPK signaling cascade contributing to stress-induced phosphorylation of AMPA receptors in the PFC and hippocampus (Yang et al., 2004; Mailliet et al., 2008; Qi et al., 2009). Critical next steps in understanding how stress modulates glutamatergic transmission within the fear and anxiety circuit include comprehensive analysis of various signaling pathways that contribute to phosphorylation or availability of AMPA receptors on the cell surface (PKA, CaMKII, MEK, MAPK), dissecting phosphorylation and surface availability patterns of GluA1 in these brain regions, and identifying pharmacological or genetic manipulations that alter behavior by interfering with these recognized pathways. Establishing the molecular pathways contributing to changes in glutamatergic transmission within the fear and anxiety circuit in our model of PTSD will contribute to the growing literature seeking to understand how stress interacts with changes in cellular transmission and plasticity (Yang et al., 2005; Kim et al., 2006; Caudal et al., 2010; Liu et al., 2015; Bonini et al., 2016; Caudal et al., 2016).

NR1-floxed mice were used to understand how stress changes plasticity within the fear and anxiety circuit, and how these changes contribute to SEFL. These mice allow us to delete NMDA-R's from specific brain regions by infusing a virus carrying Cre-recombinase, which will excise the *Grin1* gene, coding for the obligatory NR1 subunit of the NMDA-R, from the genome, leading to permanent NMDA-R deletion. This strategy allows for more precise and targeted manipulations of NMDA-R's as compared to pharmacology. In the experiment presented here, depletion of NMDA-R's from the amygdala (both BLA and CeA, in this study) reduces expression of fear to the trauma context, and interferes with conditioning to the subsequent one shock in a novel context. This preliminary study indicates that NMDA-R's in the amygdala are needed for either the acquisition or expression of the one shock context fear memory, though

there is no way to determine which is interfered with using this protocol. Going forward, it is imperative that these amygdala sub-nuclei (BLA and CeA) are studied in isolation, as NMDA-R's in these regions can have drastically different effects on fear behavior, as discussed above. Additionally, the contribution of NMDA-R's in other regions of the fear and anxiety circuit, such as the hippocampus, should be studied to understand their contribution to the SEFL phenotype. Finally, it is worth noting that the permanence of this manipulation remains a critical confound when studying amygdala plasticity. Although typically understood that NMDA-R's in the dorsal hippocampus are required for acquisition but not expression of a fear memory, NMDA-R's in the BLA are required for both acquisition and expression (Kim et al., 1991; Maren et al., 1996; Quinn et al., 2005). Therefore, it becomes difficult to disentangle acquisition effects from expression effects. Future studies could seek to address this by altering the timepoint that animals receive surgery (for example, deleting NMDA-R's between learning and the fear retention test), although the suspected contribution of NMDA-R's to consolidation further complicates the ability to discern NMDA-R's specific effect on SEFL (Liu et al., 2014a; Schmidt et al., 2015). Collectively, the NR1 mouseline offers the ability to study how NMDA-R's in the fear and anxiety circuit contribute to SEFL, although many subsequent experiments are necessary to parse the specific role of NMDA-R's in this phenotype.

The fos-cre transgenic mouse line, utilized in Experiment 3, can be used in future experiments to further understand neural circuit dynamics and how populations of cells throughout the fear and anxiety circuit contribute to various phenotypes that arise from experience with an acute stressor. In Experiment 3, population activity in the BLA, a known site of molecular changes induced by stress, was monitored to observe whether a larger population of cells was recruited to the one shock enhanced conditioning seen in stressed animals compared to unstressed animals. However, this pilot experiment barely scratches the surface of

possibilities for experimentally parsing population contributions to PTSD-like phenotypes. Currently, experiments are ongoing to double label cells in the BLA to compare network activity during the stressor compared to the one shock. That is, cells will be labeled using the fos-driven cre-recombinase activity permitted by 4OHT injection during a trauma-associated behavior, then animals will be sacrificed 90-minutes following another behavior (in this case, one shock conditioning) and stained for *fos* to monitor whether the same group of cells associated with the stressor are selectively recruited for the one shock conditioning. This mouse line could also be used to functionally tag cells active during the stressor with an optogenetic or chemogenetic channel that would allow for turning on or turning off this stress-induced population of cells in subsequent behaviors including one shock fear conditioning, open field, elevated plus maze or forced swim test. For additional specificity, the cre-dependent virus carrying an optogenetic channel could be infused in one brain region while the fiber optic implanted in another, downstream region in order to manipulate only those cells that project from region A to region B. Finally, future experiments with this mouse line could involve electrophysiology to ascertain how stress causes changes in cellular transmission properties including AMPA-R and NMDA-R currents. The advantage of utilizing this mouse line for such work is that cells active at certain time points (for example, during trauma) can be labeled using a cre-dependent fluorophore, then those specific cells can be patched to measure electrophysiological properties. This technique provides additional power and specificity compared to blindly patching onto cells in the brain that may or may not have been associated with stress-induced changes. Together, the study presented in Experiment 3 combined with other pilot studies not presented in this dissertation lay the groundwork for follow-up experiments that have the potential to further our understanding of the neural circuit dynamics associated with stress-induced changes in fear, anxiety and depression.

In Chapters 2 and 3 of this dissertation, interventions that could be directly translated into a human population (behavioral or pharmacological, administered post-trauma) were utilized to understand the complexity of the PTSD-like phenotypes that arise from experience with an acute stressor, and how distinct neural pathways might contribute differentially to the multitude of symptoms. In this chapter, I begin to outline how molecular and genetic techniques can be utilized to specifically parse the cellular changes that drive the previously described behaviors. Incorporating these molecular analyses is a critical component of understanding how the brain changes after stress to yield behavioral phenotypes including heightened fear reactions, and increases in anxiety and depression.

MATERIALS AND METHODS

Animals

20 adult, male Long-Evans rats were used for Experiment 1. 32 adult, mixed male and female floxed-NR1 C57/Bl6 mice were used for Experiment 2. 10 adult, mixed male and female fos-cre C57/Bl6 mice were used for Experiment 3. All animals were housed in a vivarium at UCLA with food and water given ad libitum except during experimental periods. Animals were housed on a 12-h on/off light cycle. All animals were single housed for at least one week prior to experiments and were handled for approximately 1 minute per day. All procedures were approved by the Chancellor's Animal Research Committee at UCLA.

Behavioral Testing

Apparatus

All fear conditioning experiments were conducted in Med Associates conditioning chambers (28 x 21 x 21 cm; Lafayette Instrument Co.; Lafayette, IN). These chambers were controlled through Med Associates Freeze software, as previously described. Two distinct

contexts were used: Context A and Context B. Context A consisted of white light, flat grid flooring, Windex cleaner, and no additional inserts. Context B consisted of red light, thick/thin grid flooring, acetic acid cleaner, and an A-frame or white insert. Footshocks were delivered through Med Associates shock scramblers (ENV 414-S). Near infrared cameras were used to record conditioning sessions, and freezing behavior was analyzed using Med Associates Video Freeze software.

Rat Stress-Enhanced Fear Learning (Experiment 1)

Rats received an acute stressor consisting of 15 pseudorandom, unsignaled footshocks (1-sec, 1.0-mA) across 90 minutes in Context A. Unstressed rats received equivalent context exposure. Approximately 5 minutes after the acute stressor, rats were sacrificed by rapid decapitation, and their brains were submerged into chilled saline. Punches were taken to extract the BLA, VH and DH from each of the brains. Appropriate tissue from one hemisphere was placed in a 1.5-mL Eppendorf tube for RNA analysis and tissue from the other hemisphere was placed in a 1.5-mL Eppendorf tube for protein analysis. Both tubes were rapidly frozen in dry ice, then stored at -80 C for subsequent molecular analysis.

NR1 Mouse Stress-Enhanced Fear Learning (Experiment 2)

Mice received an acute stressor consisting of 10 pseudorandom, unsignaled footshocks (1-sec, 1.0-mA) across 60 minutes in Context A. The day following trauma, animals were given surgery (for details, see “Stereotaxic Surgery” below). 10 days following surgery, animals were placed back in Context A for 8 minutes to assess fear retention. The following day, animals were given 1 shock (1-sec, 1.0-mA) in a novel context, Context B, with a 3 minute baseline pre-shock and 30 seconds post-shock. 24 hours following the one shock, animals were placed back in Context B for 8 minutes to assess fear retention. Animals were sacrificed on the same day as

the Context B fear test, and the brain was post-fixed in 4% paraformaldehyde for subsequent validation of viral expression.

Fos-cre Mouse Stress-Enhanced Fear Learning (Experiment 3)

Mice underwent surgery to infuse a cre-dependent AAV prior to behavior. One month after surgery, mice received an acute stressor consisting of 10 pseudorandom, unsignaled footshocks (1-sec, 1.0-mA) across 60 minutes in Context A. The next day, animals received an i.p. injection of 4OHT to activate fos-dependent synthesis of cre-recombinase, and 5 minutes after the injection were placed back in Context A for 8 minutes to assess fear retention. Active cells during this 8-minute test were labeled because of the 4OHT administration. Two weeks later, mice were given one shock conditioning (2-sec, 1.0-mA) with a 3 minute pre-shock baseline and 30 seconds post-shock in Context B. 24 hours later, animals were placed back in Context B for 8 minutes to test for fear retention. 90 minutes after the fear retention test, animals were sacrificed and the brain was post-fixed in 4% paraformaldehyde for subsequent immunohistochemistry.

Stereotaxic Surgery

All animals were weighed, and their health monitored and recorded pre-operatively. Animals were anesthetized using 3% isoflurane, then transferred to the stereotaxic set-up and maintained on 1-2% isoflurane depending on breathing pattern. All animals toes were pinched at least every 15 minutes to ensure complete anesthesia. Mice were given a s.c. injection of Rimadyl (5 mg/kg) prior to any incision. After placing in earbars, trimming hair and administering artificial tears, the top of the head was wiped with alternating alcohol and betadine 3 times each. A scalpel was used to make an incision down the midline of the mouse's head. Bregma was measured and the skull was appropriately flattened. Then, small holes were drilled through the

skull at the appropriate coordinates to target the BLA (Experiment 2: Males: -1.600 A/P, ± 3.100 M/L, -4.750 D/V, Females: -1.350 A/P, ± 3.000 M/L, -4.750 D/V; Experiment 3: -1.580 A/P, ± 2.900 M/L, -4.580 D/V). Then, the appropriate virus was infused (Experiment 2: HSV-cre or HSV-GFP, 0.5 μL at 0.1 μL/min then 10 minutes of diffusion per infusion site; Experiment 3: AAV5-flex-ArchT-GFP, 0.4 μL at 0.05 μL/min then 5 minutes of diffusion per infusion site). The incision was then sutured and wiped with antibiotic cream. Animals were monitored with appropriate post-operative care (additional Rimadyl s.c. injection (5 mg/kg) the following day, treated with antibiotics (Enroflox, 0.25 mg/mL) in drinking water for 5 days post-surgery, weighed and health monitored daily).

Molecular Analyses

Western Blotting (Experiment 1)

Tissue samples were homogenized with 50 μL of lysis buffer (LB: 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate, 1 mM Na3VO4 and 1 mM NaF) + 1% protease inhibitor (P.I.) cocktail for 30 seconds, or until uniform. Then, samples were incubated on ice for 30 – 45 minutes and subsequently centrifuged for 10 minutes at 14,000 RPM (max speed). The total protein concentration was determined using a Pierce BCA kit and BSA standards. Then, aliquots of 30 μg protein in 15 μL aliquots were prepared using protein samples. loading sample buffer (LSB), and lysis buffer (LB). Samples were boiled for 5 minutes at 90 F, then stored at -80 C.

Acrylamide gels were assembled into the gel apparatus and filled with 1X TGS, then checked to ensure that the apparatus was not leaking. 5 μL of ladder was loaded into the first lane, then 15 μL of each sample was loaded into subsequent lanes. The gel was allowed to run at 75 V for 30 minutes, then at 130 V until the ladder ran past the black line at the end of the gel

(approximately 1 hour). The protein from the gels were transferred to PVDF membranes using a transfer sandwich and TG buffer. Following transfer to the membrane, we immunoblotted by first blocking in 5% milk diluted in TBST (20 mM Tris-HCl, 137 mM NaCl and 0.1% Tween-20; pH 7.4) for 1 hour at room temperature. Then, the membrane was incubated in primary antibody solution (1:1000 rabbit polyclonal anti-GluA1(abcam) and 1:2000 mouse monoclonal anti-GAPDH (abcam) in 2.5% milk-TBST) overnight at 4 C on a shaker. The following morning, each blot was washed 3 x 10 minutes each in TBST. The blot was then incubated in secondary antibody (1:5000 HRP-conjugated anti-rabbit (Cell Signaling Technology) secondary antibody and 1:5000 HRP-conjugated anti-mouse (Cell Signaling Technology) secondary antibody in 2.5% milk-TBST) for 2 hours at room temperature. Blots were subsequently washed 3 x 10 minutes in TBST. Blots were developed using ECL2 (Thermo Fisher Scientific, Rockford, IL), then imaged on a BioRad ChemiDoc Touch Imaging System. Analysis was conducted using an ImageJ 1.47 software package (NIH, Bethesda, MD).

RT-PCR (Experiment 1)

The appropriate volume of Trizol (350 – 1000 μL, depending on size of tissue sample) was added to each tissue sample. Samples were then homogenized until uniform. Following homogenization, 100 μL of chloroform (200 μL for larger samples) was added. Samples were centrifuged at 13,000 RPM at 4 C for 5 minutes. Then, the clear top layer was pipetted off and added to a new labeled tube. In this tube, isopropanol was added (1/2 of the Trizol volume used), and 1 μL of GlycoBlue was added to stain the RNA. The samples were shaken and then incubated for 10 minutes prior to centrifuging for 10 minutes at 13,000 RPM. The resulting liquid was poured out while retaining the RNA pellet (stained blue). The pellet was dislodged and washed with 75% ethanol, then centrifuged for 10 minutes at 13,000 RPM. This step was repeated to wash the RNA pellet again. Then, a vacuum pipet was used to remove all ethanol

from the tube, ensuring the RNA pellet was left in the tube. Tubes were left open to dry on the bench for 10 minutes. 20 μL of nuclease free water was added (50 μL for larger samples). The RNA concentration was determined by adding 1 μL of sample onto a NanoDrop for analysis.

RNA was converted to cDNA using a cDNA SuperMix and the appropriate protocol was used for cDNA conversion (Lid at 105 C, 10 minutes at 25 C, 45 minutes at 42 C, 5 minutes at 85 C). cDNA samples were utilized for PCR amplification using a SybrGreen assay. GAPDH and 36b4 were amplified as housekeeping genes in order to normalize *Gria1* values. The sequences for the primers are: Gria1-1 – FWD: AACCACCGAGGAAGGATACC, RVS: ACCACCAGCCTCTCCTTTTT; Gria1-2 – FWD: CAATTTGTCCTTCAGCTACGC, RVS: CGGTGGTTGTCAGAATGTTG.

HSV Viral Validation (Experiment 2)

Brains were cryo-sections into 40 μm sections and slices containing the BLA were retained in PBS for analysis. Approximately 12 slices spanning the anterior/posterior plane of the BLA were chosen from each animal for subsequent viral validation. Sections were washed in PBS briefly, then mounted onto a slide and coverslipped following Vectashield with DAPI administration. Images were taken on the microscope (Keyence BZ-X710) and subsequently analyzed. All GFP controls were included for analysis. Only Cre-recombinase animals with viral expression in either the BLA or CeA were included for analysis (1 unstressed animal was excluded due to a lack of viral expression).

c-fos immunohistochemistry (Experiment 3)

Brains were cryo-sectioned into 40 μm sections and slices containing the BLA were retained in phospho-buffered saline (PBS) for analysis. Approximately 12 slices spanning the anterior/posterior plane of the BLA were chosen from each animal for subsequent

immunohistochemistry. Slices were washed in PBS, then incubated in primary antibody (1:10,000; Millipore ABE 457 anti-c-fos) dissolved in blocking solution (0.3% Triton X-100, 3% NGS in PBS) overnight at 4 C. The following morning, slices were washed 3 x 10 minutes in PBS. Then, slices were incubated in secondary antibody (1:500 goat anti-rabbit Alexa Fluor 594, 1:1000 DAPI) dissolved in blocking solution (0.3% Triton X-100, 3% NGS in PBS) for 2 hours at room temperature. Then, slices were washed 3 x 10 minutes in PBS, mounted onto a slide and coverslipped following administration of Vectashield. Images were taken on the microscope (Keyence BZ-X710) and subsequently analyzed.

Data Analyses

All data were analyzed using R. ANOVAs were used to evaluate the effects of treatment between groups, and when appropriate, followed by post hoc *t*-tests.

ACKNOWLEDGEMENTS

Thank you to Abha Rajbhandari and Sarah Gonzalez for their help and intellectual contribution to these experiments and analyses. Thank you to Allison Furterer, Prashant Rajbhandari, the Coppola laboratory and the Tontonoz laboratory for guidance and assistance with the RT-PCR. Thank you to the Fanselow laboratory for their guidance and intellectual contribution to these experiments.

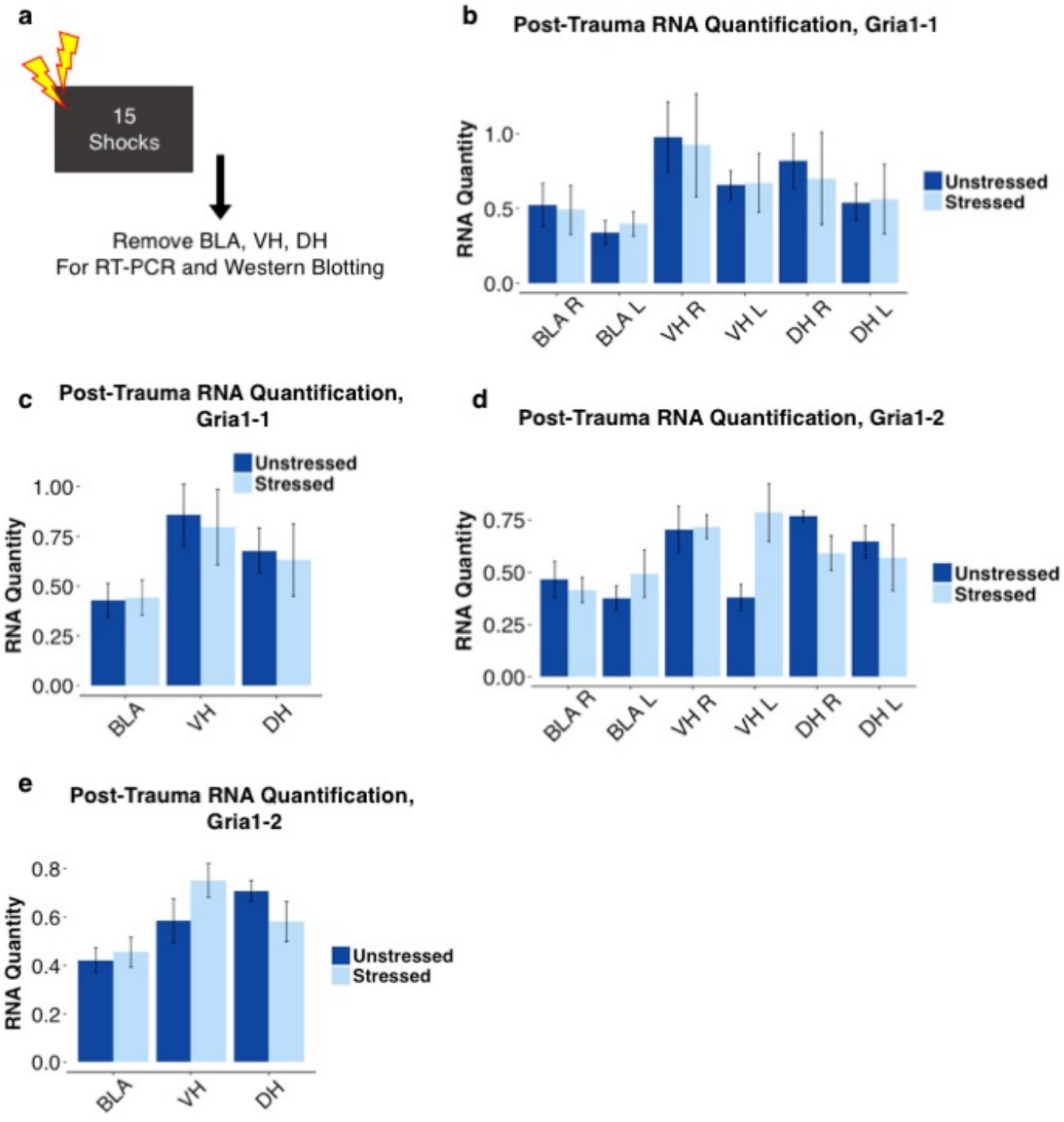

Figure 4.1: Following an acute stressor, there is an immediate upregulation of *Gria1* RNA in the VH but not in the DH or the BLA. (a) Experimental design. (b) *Gria1* RNA levels immediately following an acute stressor by brain region and by hemisphere, using the first

primer (Gria1-1) for amplification. *Gria1* values are normalized by two housekeeping genes, GAPDH and 36b4. (c) *Gria1* RNA levels by brain region (hemispheres collapsed) using the first primer (Gria1-1) for amplification. *Gria1* values are normalized by two housekeeping genes, GAPDH and 36b4. (b) *Gria1* RNA levels immediately following an acute stressor by brain region and by hemisphere, using the second primer (Gria1-2) for amplification. *Gria1* values are normalized by two housekeeping genes, GAPDH and 36b4. (c) *Gria1* RNA levels by brain region (hemispheres collapsed) using the second primer (Gria1-2) for amplification. *Gria1* values are normalized by two housekeeping genes, GAPDH and 36b4.

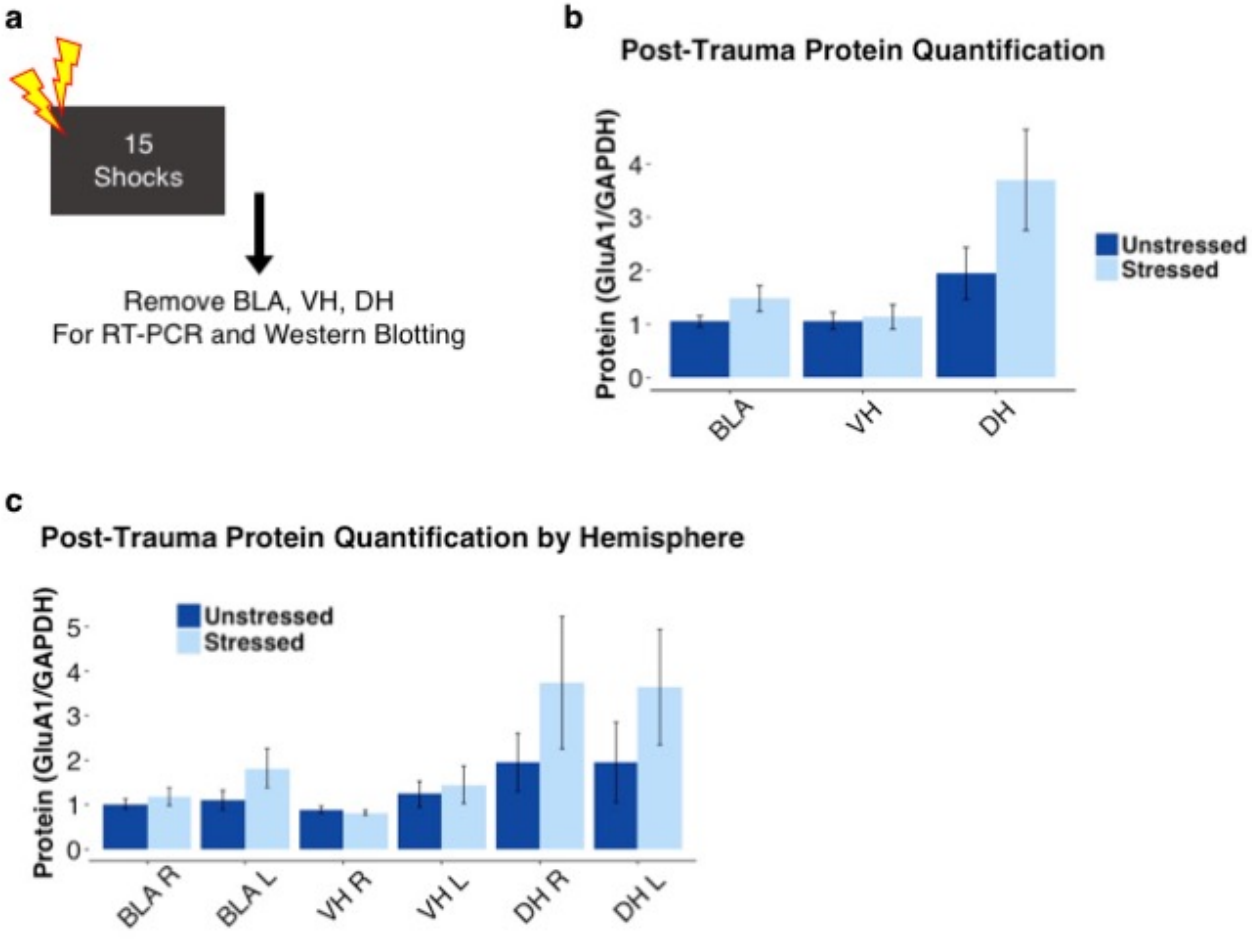

Figure 4.2: Following an acute stressor, GluA1 protein is upregulated in the BLA but not in the VH or the DH. (a) Experimental design. (b) GluA1 protein levels, normalized to GAPDH protein, by brain region after Western Blot analysis. There was a trend toward more GluA1 protein in the BLA following stress, as seen previously in our laboratory. (c) GluA1 protein levels, normalized to GAPDH protein, separated by brain region and hemisphere.

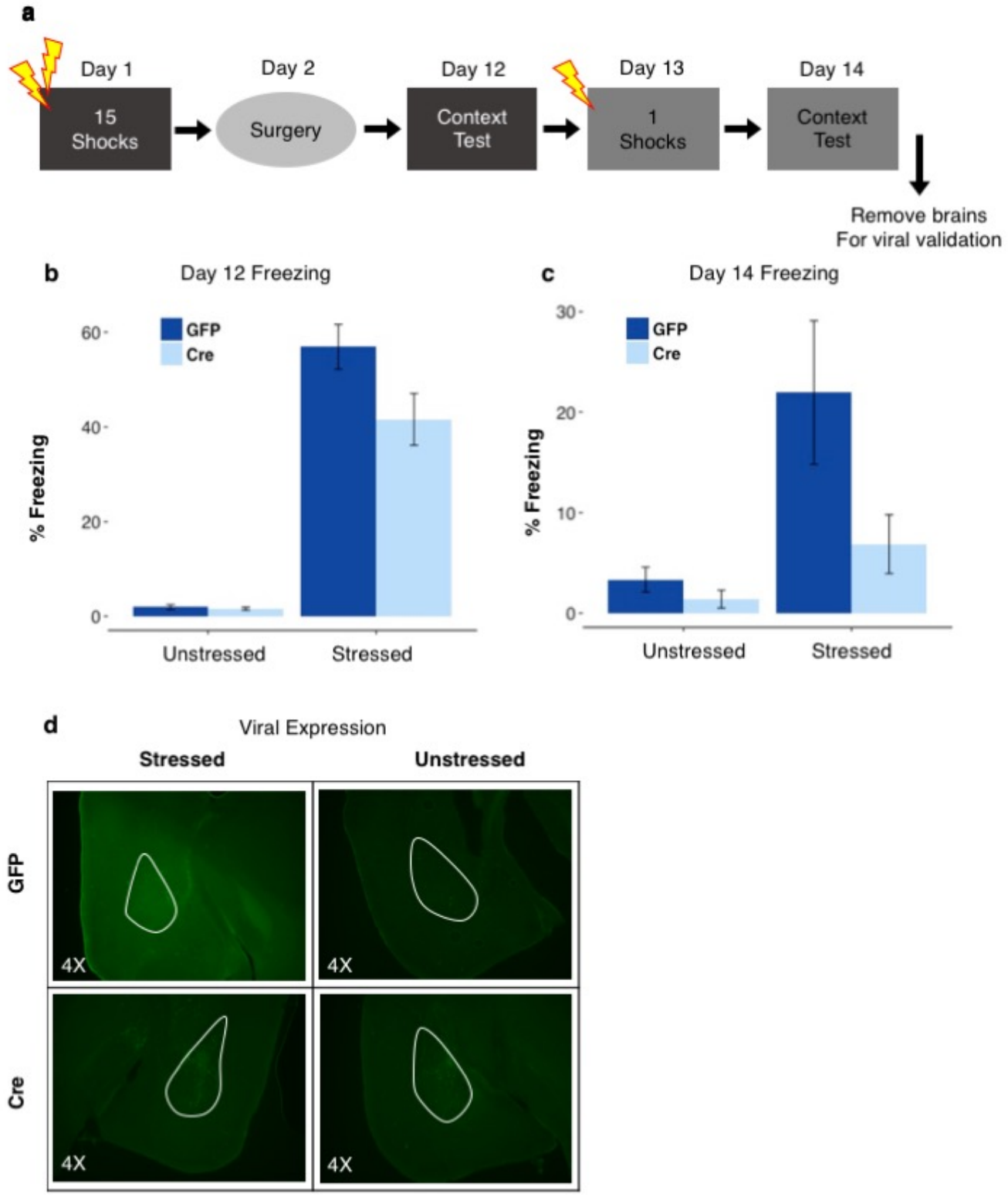

Figure 4.3: HSV-driven deletion of NMDA-R's from the amygdala, including both BLA and CeA, reduces fear retention to the trauma context and impairs SEFL. (a) Experimental

design. (b) Freezing across the 8-minute trauma context recall, 11 days following trauma and 10 days following surgery. Animals with NMDA-R depletion (Cre) froze less than control (GFP) animals during this fear recall session. (c) Freezing across the 8-minute one shock context recall session, the day following one shock conditioning. NMDA-R depletion in the amygdala overall reduced freezing during this test session, in both stressed and unstressed conditions. (d) Images to validate placements and HSV expression. Signal was either detected due to the HSV carrying GFP (control) or Cre-GFP (NMDA-R deletion).

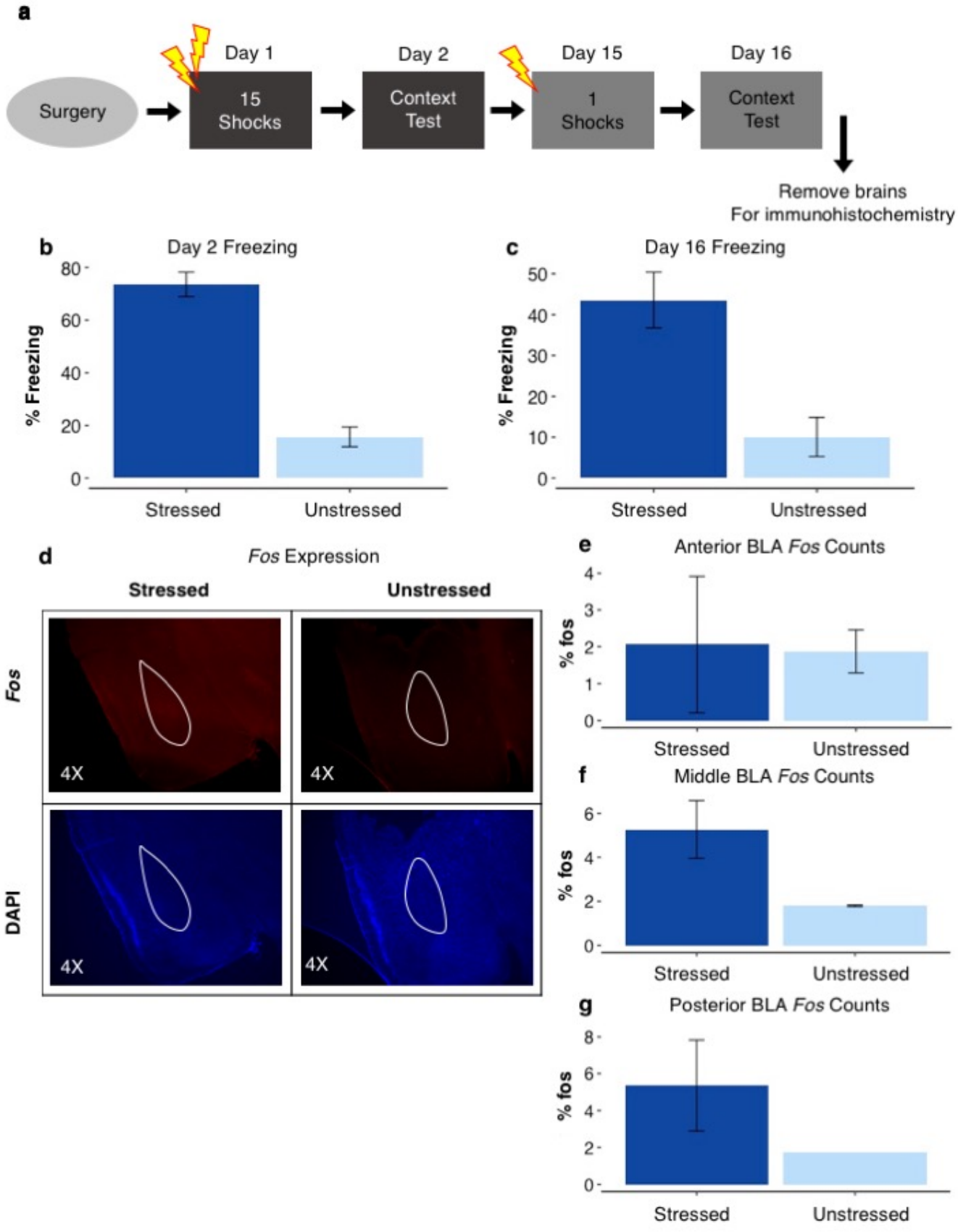
a
Day 1
Day 2
Day 15
Day 16
Surgery
15 Shocks
Context Test
1 Shocks
Context Test
Remove brains
For immunohistochemistry
b
Day 2 Freezing
% Freezing
80
60
40
20
0
Stressed
Unstressed
c
Day 16 Freezing
% Freezing
50
40
30
20
10
0
Stressed
Unstressed
d
Fos Expression
Stressed
Unstressed
Fos
DAPI
4X
e
Anterior BLA Fos Counts
% fos
4
3
2
1
0
Stressed
Unstressed
f
Middle BLA Fos Counts
% fos
6
4
2
0
Stressed
Unstressed
g
Posterior BLA Fos Counts
% fos
8
6
4
2
0
Stressed
Unstressed

Figure 4.4: A larger population of BLA neurons are recruited during the one shock conditioning in stressed animals compared to unstressed animals. (a) Experimental design. (b) Freezing across the 8-minute trauma context recall session. Stressed animals froze significantly more than unstressed animals during this test session. (c) Freezing across the 8-minute one shock context recall session. Stressed animals froze significantly more than unstressed animals during this test session, displaying the SEFL phenotype, although all animals received the same one shock conditioning. (d) Representative images of *fos* immunohistochemistry expression in stressed and unstressed animals. DAPI was used for normalizing cell counts. (e) Normalized *fos* expression (to DAPI) of the anterior portion of the BLA in stressed and unstressed animals. There was no significant difference in %fos activated cells across these groups. (f) Normalized *fos* expression (to DAPI) of the middle portion of the BLA in stressed and unstressed animals. There was more *fos* activity in the stressed animals compared to unstressed animals. (g) Normalized *fos* expression (to DAPI) of the posterior portion of the BLA in stressed and unstressed animals. There was more *fos* expression in stressed animals compared to unstressed animals.

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CHAPTER 5

General Conclusions

Post-Traumatic Stress Disorder (PTSD) is a psychiatric disease that affects a subset of individuals, generally 5 – 20%, who undergo trauma (Perrin et al., 2014). Although the symptoms of PTSD are vast and multi-faceted, generally they involve changes in the fear and anxiety circuit that might lead to heightened fear responses, increased anxiety and depression, and negative affective symptoms such as compromised cognition and mood (Diagnostic and Statistical Manual of Mental Disorders, 2013). To effectively diagnose and treat PTSD, it is critical that we understand how the brain changes following stress to lead to the symptoms observed in individuals with the disease. As fear and its underlying mechanisms are a widely conserved set of emotional behaviors, we can study maladaptive forms of fear and anxiety in animal models to more fully comprehend the underlying neural mechanisms that lead to fear-related diseases such as PTSD in humans.

In this dissertation, I have demonstrated how using an animal model of PTSD, which involves animals receiving an acute, traumatic experience, leads to a variety of PTSD-like phenotypes that can be studied to further our holistic understanding of the disease. As a complex and multi-faceted disease with a variety of symptoms, PTSD remains difficult both to diagnose and treat in the human population. Alongside genetic or behavioral human experiments and clinical trials, studying animal models of the disease offers the unique ability to discern information regarding the neural mechanisms underlying stress-induced changes in behavior, and to strive to develop novel interventions that could translate into treatments for humans with PTSD. To fully understand the disease and its underlying mechanisms, it is critical

to combine knowledge and experimental evidence across species, techniques used to induce PTSD in animal models, and treatments that have successfully mitigated some or all of the PTSD symptoms that arise following experience with an acute stressor. Collectively, this dissertation offers insight into the behavioral and neural characterization of one such model of PTSD, in which animals develop heightened fear responses and increases in anxiety and depression after an acute traumatic experience.

Modeling diseases in an animal model is useful for a variety of reasons. Importantly, causal mechanisms underlying certain changes in behavior can be studied in animal models while no such work is permissible in human subjects. That is, manipulations including molecular and genetic alterations of cells and neural circuits that give critical information about what brain systems are involved in the disease can be used in animal models but not in human subjects. Without these animal models, it would be next to impossible for researchers to confidently understand and verify hypotheses regarding what neural mechanisms contribute to the disease. Alongside the ability to discern causal mechanisms of the disease, animal models are important for testing potential translational interventions for the diseases modeled. In our model of PTSD, we often target these potentially translational interventions to occur after the stress but before phenotypes emerge to best match when human subjects could be treated. The most directly translational types of interventions are either (1) behavioral or (2) systemic pharmacological manipulations. Clearly, these two types of interventions could be translated to treat human subjects directly as compared to other manipulations that involve central nervous system intervention or use of newer techniques such as viruses to manipulate populations of cells. Together, modeling diseases in an animal is a critical part of understanding these diseases for the unique ability to map causal underlying neural mechanisms, and for the potential to identify directly translational disease interventions.

In Chapters 2 and 3 of this dissertation, I utilize our animal model of PTSD to test for therapeutic interventions that could be employed in human subjects. In Chapter 2, I use a behavioral manipulation, group housing animals, to test for its effects on stress-induced alterations in the fear and anxiety circuit. Unfortunately, this behavioral manipulation had no effects on subsequent stress-induced behavioral changes, which calls into question its efficacy for use with human subjects. In Chapter 3, I administered a systemic post-trauma injection of a kappa opioid receptor (KOR) antagonist, JDTic, to test its effects on stress-induced enhanced fear learning and anxiogenic behavior. While JDTic did not mitigate stress-enhanced fear learning, it did change behavior on the elevated plus maze (EPM), indicating a potential anxiolytic effect in stressed animals. However, JDTic administration in unstressed animals led to anxiogenic effects on the EPM, indicating a potential confound in translating to human subjects if there are adverse consequences when administering to controls. Both of these chapters attempt to use an animal model of PTSD to test potential clinical translations to treat individuals who suffer from PTSD. Characterizing these pre-clinical manipulations is an important step in understanding how we can begin to treat the complexity of symptoms that arise in human subjects with PTSD.

In Chapter 4, molecular and genetic manipulations and analyses were conducted to characterize the neural mechanisms underlying our animal model of PTSD. While a distinct strategy from the pre-clinical manipulations utilized in Chapters 2 and 3, the ability to directly manipulate cells and circuits or subsequently analyze brain tissue to quantify changes in neural markers is a unique and powerful benefit to studying diseases in animal models. Through using these techniques, I discovered a dissociation between protein and RNA upregulations in two different brain regions, the basolateral amygdala (BLA) and ventral hippocampus (VH), respectively, of the AMPA-R subunit GluA1, which we believe contributes to some of the stress-

induced changes in our model. Directly labeling, and in the future manipulating, populations of cells that are active during stress, or during subsequent stress-induced changes in behavior, can also provide novel insight into the neural circuitry involved in producing PTSD-like symptoms. This type of work is simply not doable in the human population, and requires an animal model of the disease to progress our understanding of these neural dynamics.

The experiments presented in this dissertation also offer insight into the complexity and variety of symptoms that can arise following experience with an acute stressor. The DSM outlines many different categories of symptoms that can arise and contribute to a PTSD diagnosis, including but not limited to changes in fear, anxiety and depression (Association, 2013). Our ability to model these different PTSD-like symptoms is an important tool to more fully comprehend the disease as a whole rather than as a set of individual components. Additionally, not all patients with PTSD develop an identical set of symptoms which makes understanding the symptoms both independently and as a part of the disease as a whole incredibly important. Our model of PTSD, which mirrors a number of these symptoms, can and should be used to parse distinct neural changes that map onto subsequent behavioral symptoms induced by stress. That is, our model can be utilized to identify the specific changes in the brain that contribute to development of distinct symptoms. In this dissertation, one such system was discovered as antagonizing KOR's following stress did not perturb the enhanced fear phenotype but did mitigate post-stress changes in anxiety. This is one example of how our model can be harnessed to learn how distinct neural pathways, systems, circuits, and changes following stress guide behavioral phenotypes. A more comprehensive understanding of both the neural changes responsible for and PTSD-like symptoms that arise from our model could potentially unearth novel means of targeting PTSD treatments in patients who present with their own individual array of symptoms.

Most prior work on SEFL focused on behavioral characterization of the model, or pharmacological interventions that could be translated into the human population (Rau et al., 2005; Rau and Fanselow, 2009; Long et al., 2011; Perusini et al., 2016). Critically, previous studies have demonstrated that CORT's effects in the amygdala are necessary for SEFL, and have indicated that the acute stressor leads to an upregulation of GluA1 protein in the amygdala, which alters glutamatergic synaptic transmission to make the amygdala hyper-excitable (Perusini et al., 2016). However, what is fundamentally missing in this line of work is characterizing the molecular pathways and changes that mediate the enhanced fear phenotype, and expanding beyond SEFL to exploring the neural mechanisms underlying other PTSD-like symptoms that arise following an acute stressor, such as alterations in anxiety and depression. Due to the complexity and variety of PTSD-like symptoms that arise after an acute stressor, it seems fundamentally unlikely that changes in cellular plasticity or glutamatergic transmission alone mediate all of these behaviors. In this dissertation, I demonstrated that KOR's are involved in developing the increased anxiety phenotype following SEFL, as post-trauma administration of a KOR antagonist mitigates anxiogenic behavior on the EPM. This is one such example of how different neural systems and circuits could be differentially involved in various PTSD-like symptoms, which collectively define the induction of PTSD in our animal model. Going forward, work on this animal model should prioritize characterizing the neural processes responsible for different PTSD-like phenotypes, such as decreased locomotion on the OF or changes in depression as measured through the FST, such that neural pathways that are either conserved or differentially regulate these phenotypes can be assessed. By expanding past the enhanced fear phenotype to studying these other measures of PTSD, we will gain a better understanding of the disease holistically.

Alongside the neural systems described in this chapter, there are other candidate mechanisms shown to be mediated by stress and involved in producing fear and anxiety states. One such system is corticotropin-releasing factor (CRF), a neuromodulator released primarily by the paraventricular nucleus (PVN) of the hypothalamus, mediates many responses to stress, including behavioral responses. Interestingly, the amygdala contains both CRF neurons and a high level of CRF receptors, making it a brain region poised for stress-induced mediation of behavior through the CRF system (Koob and Bloom, 1985; Shekhar et al., 2005). Already, there is evidence that CRF is expressed following an acute stressor, as one study indicated that there was increased *fos* activity in the CRF-containing neurons of the PVN following an acute restraint stress (de Andrade et al., 2014). Likewise, mRNA of CRF-binding protein (CRF-BP), which binds CRF and mitigates its ability to bind CRF receptors, was found to be selectively upregulated in the BLA following stress without an observed upregulation in the CeA (Lombardo et al., 2001; Herringa et al., 2004). This same study failed to find any mRNA expression differences of CRF or CRF receptors in either the BLA or CeA, indicating that there was a selective disruption in CRF-BP mRNA (Herringa et al., 2004). This dynamic regulation of CRF-BP seems to be specific to acute stress, as chronic stress procedures did not affect levels of CRF-BP in either the BLA or the DH (Lombardo et al., 2001). Further studies elucidated that the CRF-BP upregulation is due to CRF's actions, rather than mediated by CORT, as only injections of CRF yielded an increase in CRF-BP mRNA in the BLA (Herringa et al., 2006). Additional evidence indicates that CRF mRNA is upregulated in the hypothalamus following acute, but not repeated, stress, and that chronic stress actually eliminates the acute stress-induced upregulation (Hsu et al., 2001). Behaviorally, CRF has also been linked to stress-induced changes such as impairments in cognitive tasks, such as temporal order memory and reversal learning, in acutely stressed rats (Uribe-Mariño et al., 2016; Schreiber et al., 2017). This behavioral deficit is thought to be modulated by CRF's actions in the mPFC, where CRF

activation leads to PKA signaling and cAMP phosphorylation, as blocking PKA attenuates the maladaptive behavioral response evoked by acute stress (Uribe-Mariño et al., 2016). CRF is also thought to play a role in anxiety behavior, primarily through the CRF(1) receptor, as antagonizing this receptors during post-stress behavioral tests is anxiolytic (Smith et al., 1998; Timpl et al., 1998; Takahashi, 2001; Bale et al., 2002). Additionally, stress-induced CRF is thought to play a role in the development of substance use disorder (SUD), which is often co-morbid with PTSD (Haass-Koffler and Bartlett, 2012). Finally, CRF is known to mediate neural plasticity in regions including the amygdala, where it plays a role in mediating glutamatergic transmission, which is a major player in the development of PTSD-like symptoms (Shekhar et al., 2005; Pollandt et al., 2006; Fu et al., 2007). The molecular and behavioral data implicating CRF as playing a role in stress-mediated changes in fear and anxiety poise it as a prime contender for regulating the PTSD-like symptoms seen in our acute stressor model. Subsequent work is needed to elucidate CRF's role in mediating one or many PTSD-like symptoms observed in our model.

Another stress modulator that could mediate the PTSD-like symptoms that arise following an acute stressor is norepinephrine (NE), also known as noradrenaline. NE is a catecholamine that binds to G-coupled protein receptors (GCPR's) to exert its influence on cellular function. These GCPR's act to either increase cAMP (beta-adrenergic receptors), decrease cAMP (alpha 2-adrenergic receptors) or activate phospholipase C (alpha 1-adrenergic receptors) (Stone, 1987; Stone et al., 1987). Prior work has indicated that a single bout of stress is sufficient to alter NE function in the brain, leading to alterations in electrophysiological properties of cells in the locus coeruleus (LC), the main source of NE in the brain, and an enhancement of tyrosine hydroxylase mRNA in the same brain region, which indicates a higher level of NE utilization (George et al., 2013). Broadly, NE is known to interact with both CRF and

CORT, and blocking beta-adrenergic receptors in the BLA can reverse a CORT-induced enhancement of memory consolidation (Roozendaal et al., 2006). Interestingly, the CeA is also a hub of noradrenergic innervation, and cells in the CeA express high levels of alpha 1-adrenergic receptors. One study found that antagonizing alpha 1-receptors in the CeA blocked stress-induced behavioral changes in a Social Interaction test, while antagonizing beta-receptors in the same region had no effect, consistent with a lack of beta-receptor expression in the CeA (Cecchi et al., 2002). Interestingly, the same study failed to find an effect of alpha 1-receptor antagonism on a separate measure of stress-induced anxiety, the EPM, indicating that these neural systems can differentially and selectively mediate some aspects of stress (Cecchi et al., 2002). A separate study, using repeated predator exposure as a form of stress, found long-lasting sensitization in the BLA that was dependent on both alpha 1-receptors and CRF 1-receptors, which together generated PTSD-like phenotypes including heightened startle and deficits in prepulse inhibition (PPI) (Rajbhandari et al., 2015). NE interacts with changes in glutamatergic transmission in the fear and anxiety circuit, as one group found that administering propranolol (a beta-receptor antagonist) reduces expression of GluA1 at the cell's surface in the lateral amygdala following reactivation of a fear memory (Zhou et al., 2015). There is additional evidence of NE's effects on stress and mediating behavioral symptoms of stress in humans. For instance, one group discovered that acute stress altered functional connectivity between brain regions including the amygdala, hypothalamus and dorsal anterior cingulate cortex heightened following an acute stressor (Hermans et al., 2011). This change in functional connectivity could be attenuated by administering propranolol, but interestingly, not by administering metyrapone, a CORT-synthesis blocker (Hermans et al., 2011).

A third candidate system that might be modulating some PTSD-like symptoms observed following an acute stressor is the endocannabinoid (eCB) system. There are two receptors of

the endocannabinoid system: the CB1 receptors, which are located primarily pre-synaptically and widely distributed across the brain, and the CB2 receptors, which are localized primarily in glial cells (Devane et al., 1988; Massi et al., 2008; Riebe and Wotjak, 2011). Interestingly, CB1 receptors are richly expressed in the BLA, but are noticeably absent in the CeA and the medial nucleus of the amygdala (Katona et al., 2001). Both CB1 and CB2 receptors are G-protein coupled receptors that are activated by eCBs, Two widely studied eCBs, N-arachidonylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG), are expressed post-synaptically by calcium activation, and cross the synapse to activate pre-synaptic CB1 receptors to modulate presynaptic cellular function (Riebe and Wotjak, 2011). Following stress, there is a reduction in AEA and an increase in 2-AG in the BLA (Patel et al., 2005). The eCB system also interacts with the HPA axis and glucocorticoids, as pharmacologically antagonizing CB1 receptors has been shown to augment CORT peripherally (Patel et al., 2004; Wade et al., 2006). Interestingly, eCBs play a role in forming fear memories through their actions on inhibitory interneurons in the BLA; CB1-receptor activation mitigates GABA release from these inhibitory interneurons onto neighboring pyramidal neurons in the BLA (Katona et al., 2001). It is possible that the typical actions of eCBs on CB1-receptors in forming fear memories is augmented following stress, leading to heightened expression of 2-AG activation of CB1-receptors, which disinhibits pyramidal neurons in the BLA, ultimately producing a sensitized amygdala (Di et al., 2016). There is evidence that the eCB system plays a role in mediating anxiety behaviors as well, as a deficiency of the CB1 receptor activity results in anxiogenic behaviors in a variety of tests including the elevated plus maze and open field test (Urigüen et al., 2004; Järbe et al., 2008; Komaki et al., 2015). Given the role of the eCB system in modulating fear and anxiety behaviors, and its dynamic regulation in response to stressors, this system could additionally be modulating one or more of the PTSD-like symptoms observed in our model of the disease.

The literature encompassing the behavioral and neural effects of stress on fear and anxiety behavior is large and varied, spanning many behavioral consequences and candidate neural systems. This dissertation focuses primarily on a few of these systems, specifically the contribution of KORs to stress-induced changes in behavior, and how glutamatergic transmission in the amygdala is affected by an acute stressor. However, these two systems are by no means the only neural pathways that are dynamically regulated by stress and could contribute to the PTSD-like symptoms we observe. I have introduced three additional systems worth considering as mediating various changes in fear and anxiety behavior: CRF, norepinephrine, and endocannabinoids. Each of these systems respond differentially to stressors, and have been shown to affect some of the behaviors seen in our model, including enhanced fear conditioning and heightened measures of anxiety and depression. As we continue to study our model of PTSD and characterize its neural mechanisms associated with behavioral consequences of stress, it is imperative to incorporate these other neuromodulator systems and consider the wide array of phenotypes observed following stress. One strength of our PTSD model is the wide array of phenotypes seen that mimic symptoms observed in the human population (see Table 1.1). To use this strength advantageously and unearth potential mechanisms of PTSD, it is of vital importance to consider as many neural systems and study the behavioral consequences of perturbing these systems on as many PTSD-like phenotypes as possible.

Taken together, this dissertation utilizes an animal model of PTSD to unearth the behavioral and neural mechanisms responsible for the development of this disease. The behavioral, pharmacological, and molecular manipulations presented here provide a framework by which subsequent studies can add to our understanding of PTSD's effects on both the brain and on producing maladaptive behaviors, particularly those of heightened fear, anxiety and

depression. Moving forward, as additional studies in our laboratory and others augment our understanding of PTSD, it is critical to consider how animal models of the disease converge and diverge in evidence, and how this important information can be used to ultimately progress our ability to diagnose and treat the disease in the human population.

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