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The Mechanisms of Fear Sensitization Caused by Acute Traumatic Stress: from Induction to Expression to Long-Lasting Reversal

Author

Perusini, Jennifer Nicole

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The Mechanisms of Fear Sensitization Caused by Acute Traumatic Stress: from Induction to Expression to Long-Lasting Reversal

A dissertation submitted in partial satisfaction of the

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Doctor of Philosophy in Psychology

by

Jennifer Nicole Perusini

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ABSTRACT OF THE DISSERTATION

The Mechanisms of Fear Sensitization Caused by Acute Traumatic Stress: from Induction to Expression to Long-Lasting Reversal

By

Jennifer Nicole Perusini

Doctor of Philosophy in Psychology

University of California, Los Angeles, 2014

Professor Michael S. Fanselow, Committee Chair

Fear is an adaptive response that is normally proportional to the level of imposed threat, which allows for a balance between defensive behavior and other behaviors necessary for survival. However, fear becomes maladaptive when the level is inappropriate to the level of imposed threat. Exposure to a severe stressor can alter future fear learning to become disproportionate to the actual level of threat, potentially leading to generalized fear to less threatening circumstances (Rau, DeCola, and Fanselow, 2005). Inappropriate fear regulation after severe stress is a hallmark of post-traumatic stress disorder (PTSD). The primary goal of the

experiments in this dissertation is to investigate the biological mechanisms that underlie both induction and expression of stress-enhanced fear learning (SEFL), a model developed and tested in rats to demonstrate that an acute footshock stressor nonassociatively and permanently enhances conditional fear learning.

In the SEFL procedure, rats are given 15 unsignaled footshocks in a certain context, and some time later, are given a single footshock in a novel context. When rats are tested for changes in freezing levels in the novel context in absence of a shock, they show exaggerated levels of freezing behavior, which is called SEFL. Many features of SEFL are similar to the symptoms of PTSD. Experiments in Chapter 1 of this dissertation show that corticosterone (CORT) is necessary to induce SEFL. This effect is demonstrated using intraperitoneal injections of metyrapone, a CORT synthesis blocker. Metyrapone before, but not after the 15 shocks dose-dependently attenuated SEFL and plasma CORT levels during the 15-shock stressor. Moreover, it is shown that the basolateral amygdala (BLA) must be functional during, but not after the 15-shock stressor. A glucocorticoid receptor (GR) antagonist infused into the BLA also attenuated SEFL; so, CORT acting on GRs in the BLA is necessary to induce SEFL.

Further work in Chapter 2 explored the mechanisms underlying expression of SEFL. CORT drove long-term alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor (AMPAR) subunit glutamate receptor 1 (GluA1), expression in the BLA, but not GluA2, or the glutamate N-methyl-D aspartate receptor (NMDAR) subunit GluN1. Infusing an AMPAR antagonist into the BLA after the severe stressor temporarily prevented sensitized fear expression. Experiments in Chapter 3 targeted GluA1 synthesis in the BLA using antisense oligonucleotide (ASO) treatments post-stress, which surprisingly reversed SEFL long-term. The most interesting finding in this set of experiments was that reversal of SEFL by ASO treatment

did not prevent fear learning or amygdala function, nor did it affect associative fear to the stressor context. Moreover, in Chapter 3 we examined the functional importance of increased GluA1 in the BLA after SEFL. This was accomplished with post-stressor intra-BLA infusions of a GluA2-lacking AMPAR blocker, IEM-1460, which reduced SEFL.

In conclusion, these results elucidate novel neurobiological mechanisms underlying sensitized behavioral responses observed using the SEFL model in rats, with potential relevance to PTSD treatments in humans. Specifically, the collective findings show that that CORT acts on GRs in the BLA during the stressor to upregulate the GluA1 subunit of the AMPAR long-term, which elucidates novel mechanisms for the induction and the expression of SEFL. Furthermore, the finding that a single ASO treatment directed at the AMPARs within the BLA restored normal fear responding is especially relevant for developing novel and potentially more effective treatments for PTSD. Clinical implications are discussed throughout the present work.

The dissertation of Jennifer Nicole Perusini is approved.

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Thomas Minor

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Igor Spigelman

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J. David Jentsch

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Michael S. Fanselow, Committee Chair

University of California, Los Angeles

2014

DEDICATION

This dissertation is dedicated to my mother, Iris.

To know her was to love her, and I try to honor

her memory and make her proud every day.

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VITA

2006	B.A., Neuroscience & Behavior Barnard College, Columbia University New York, NY
2011	M.A., Psychology, Behavioral Neuroscience University of California, Los Angeles
2011-2014	Teaching Assistant, UCLA
2013	Candidate of Philosophy, Psychology University of California, Los Angeles

PUBLICATIONS AND PRESENTATIONS

"The Corticosterone Synthesis Inhibitor, Metyrapone, Blocks Stress-Enhanced Fear Learning," UCLA Psychology Department, Master's Thesis, 2011.

"Mechanisms of Fear Sensitization Caused by Acute Traumatic Stress: From Induction to Expression to Potential Cure," UCLA Integrative Center for Learning and Memory, Junior Scientist Lecture, 2014.

Fanselow, M.S., Zelikowsky, M., Perusini, J., Rodriguez-Barrera, V., Hersman, S. (*Accepted October 2013).* Isomorphisms between Psychological Processes and Neural Mechanisms: From Stimulus elements to genetic markers of activity. *Neurobiology of Learning and Memory*.

Perusini, J.N., Meyer, E., Rau, V., Spigelman, I., Fanselow, M.S. (*Submitted to Journal of Neuroscience).* Mechanisms underlying induction of stress-enhanced fear learning: corticosterone during acute traumatic stress produces long-term glutamatergic receptor changes in the basolateral amygdala.

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Perusini, J. N., Rajbhandari, A., Condro, M. C., Moore, S. A., Chiang, B., Avershal, J., Waschek J., & Fanselow, M. S. (*In preparation for Neuron)* Targeting GluA1-containing AMPA receptors in the basolateral amygdala produces long-lasting reversal of stress-enhanced fear learning.

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Long, V. A., Rau, V. Perusini, J., & Fanselow, M. S. “An Anxiety Profile of Stress-Enhanced Fear Learning, a Rodent Model of Post-Traumatic Stress Disorder.” Poster presented at *Society for Neuroscience, 2011*.

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Perusini, J., Li, V., & Fanselow, M. S. “Early Life Stress & Behavioral Persistence: An Animal Model.” Poster presented at *Society for Neuroscience, 2012* and *UCLA Brain Research Institute Symposium, 2012.*

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INTRODUCTION

Predation is the most urgent threat to an animal's survival and future reproductive success. Because of this, strong behavioral systems have emerged to help animals defend themselves against predators. An animal's response to threat is a species-specific defense reaction, such as fight, flight, or freezing (Bolles, 1970). Fear is a mechanism that promotes an animal to engage in the defensive behavior most likely to ensure its survival (Fanselow, 1994; Fanselow and Lester, 1988). However, fear becomes maladaptive when the level is inappropriate to the intensity of imposed threat, and an imbalance occurs between defensive behaviors and other behaviors necessary for survival. Following an experience with a severe stressor, future fear learning becomes disproportionate to the actual threat and may generalize to other potential threats (Rau, DeCola, and Fanselow, 2005). This suggests that stress produces long-term changes in fear-learning circuitry. The primary goal of this dissertation is to investigate the biological mechanisms that underlie both the initiation and expression of stress-enhanced fear learning (SEFL), mechanisms that are poorly understood. Another goal of this dissertation work is to elucidate mechanisms that can produce an enduring reversal of the exaggerated fear responding by targeting specific aspects of the neural substrates that are involved in SEFL.

Post-traumatic stress disorder

When a fear response is disproportionate to the severity of a threat, it can interfere with behaviors serving other adaptive functions, and negative consequences can result (Fanselow and Lester, 1988). Inappropriate fear responses in humans can result in the development of anxiety disorders (Rosen and Schulkin, 1988), including post-traumatic stress disorder (PTSD). PTSD

develops in some individuals who experience a traumatic event and affects 4-7% of the United States population. Symptoms include avoiding stimuli associated with the traumatic event, constant re-experiencing of the event, and increased arousal, exhibited by exaggerated startle response (American Psychiatric Association, 2013). Under normal circumstances, these symptoms are adaptive for coping with the trauma (Bonne, et al., 2004; Charney, 2004; Christopher, 2004; Eberly, Harkness, and Engdahl, 1991). For instance, avoiding stimuli associated with the traumatic event lessens the probability of encountering the threat or others like it. Re-experiencing the trauma may help an individual learn from the event and develop more effective ways in responding to the event if encountered again. Lastly, hypervigilance may help increase awareness of surroundings and detect potential threats.

However, patients with PTSD lose normal daily functioning because these responses become dysfunctional and exaggerated. As a result, re-experiencing the event can lead to sleep disturbances, avoiding trauma-related cues can lead to a stifled life, and hypervigilance can lead to exhaustion (Eberly, Harkness, and Engdahl, 1991). One feature of PTSD is an exaggerated reaction to a mild stressor or reminder of the trauma, a response more suitable for the original traumatic event that is too intense for the current, normal conditions (Bremner, et al., 1995; Dykman, et al., 1997; Friedman, 1994). Additionally, PTSD is co-morbid with phobias and depression, and other reports have also shown that PTSD leads to a predisposition to drug and alcohol abuse (Goisman, et al., 1998; van Dam, et al., 2013; Dutton, et al., 2013; Stander, Thomsen, and Highfill-McRoy, 2014). Therefore, PTSD poses itself as a serious mental illness, and there is a need for developing novel and effective treatments for this disorder.

Pavlovian Fear Conditioning

Pavlovian fear conditioning has been used to model symptoms of exaggerated fear learning and responding in rodents (Rau, DeCola, and Fanselow, 2005) and in people with PTSD (Orr, et al., 2000; Peri, et al., 2000). When a person feels threatened, environmental stimuli may become aversely conditioned, and hyperactivity toward the perceived threat can develop. Therefore, it is likely that processes related to both associative and nonassociative fear learning may underlie anxiety disorders, like PTSD (Charney, et al., 1993). There is also a great understanding of the neural circuitry of Pavlovian fear conditioning, making it a useful tool for deriving the neural mechanisms of behavioral symptoms.

In a typical Pavlovian fear conditioning experiment, an aversive stimulus, such as footshock (the unconditional stimulus or US), is delivered to the animal, and is usually paired with a conditional stimulus (CS), which may be the context in which the animal is shocked or a discrete cue such as a tone. Pairing the CS and US will result in the CS becoming associated with a conditional response (CR). During testing the rodent subsequently freezes when played the tone CS or is placed back in the training context. Freezing is defined as the absence of movement except that necessary for respiration (Fanselow, 1980; 1994). The rodent exhibits freezing behavior because when under threat an animal's behavioral repertoire narrows to include only behaviors that serve a critical survival function, one of which is freezing to avoid detection by predators. These behaviors are referred to as species-specific defense reactions (Bolles, 1970). In addition to freezing, other CRs may develop; fear activates autonomic systems to produce changes in heart rate and blood pressure (Fendt and Fanselow, 1999), the stress response system is prompted to release stress hormones (Cordero, Merino, and Sandi, 1998; Cordero, et al., 2002), and endogenous opioids are secreted via the descending analgesic system (Fanselow, 1984).

The freezing response is a reliable measure of learned fear and the amount of freezing is tightly controlled by the intensity and number of shock USs (Fanselow and Bolles, 1979a); fear responding should be proportional to the level of the threat; that is, as the number and intensity of shocks increases, so does the level of freezing. However, exposure to a traumatic event can produce disproportionate fear responding no longer appropriate for the level of threat. For example, rats receiving one brief mild shock show around 25% freezing (Fanselow and Bolles, 1979a). However, when animals are first given a series of 15 footshocks in a different context prior to the single shock, they show 80-90% freezing to the single shock, more than three times what previously unstressed animals exhibit (Fanselow and Bolles, 1979b; Fanselow, DeCola, and Young, 1993). The single shock now elicits a disproportionate level of freezing, a level more appropriate to the original stress, the 15 footshocks.

A similar enhancement of fear responding is observed with prior exposure to other types of stressors, such as restraint stress (Cordero, et al., 2003). In this experiment, rats were given a two-hour session of restraint stress by placement in a confined space. Two days later, all rats underwent a fear conditioning procedure in which they received three context-shock pairings. In a context test the next day, restrained animals showed an increased percentage of freezing compared to non-restrained controls. Moreover, in another study, rats were exposed to inescapable tail shock and subsequently fear conditioned with a brief periorbital shock US and a white noise CS. Stressed rats exhibited significantly more conditioned eyeblink responses compared to unstressed controls (Shors, Weiss, and Thompson, 1992). These situations of inappropriate regulation of fear responding may be analogous to a common experience of PTSD patients in which a previous encounter with a traumatic stressor causes an exaggerated response

to future non-threatening stimuli (Bremner, et al., 1995; Dykman, Ackerman, and Newton, 1997).

Stress Response

Exposure to stress appears to sensitize the biological system involved in generating fear responses; therefore, it is likely that biological mediators of enhanced fear responses are stress hormones (Carrasco and Van de Kar, 2003; Johnson, et al., 1992). Stress initiates both the sympathetic nervous system and the activation of the neuroendocrine stress cascade, also called the hypothalamic-pituitary-adrenal (HPA) axis. The activation of these systems causes the body to undergo a set of responses that facilitate dealing with a challenge and restore homeostasis after the threat has passed. These responses include mobilizing energy and other resources to sustain the brain, heart, and muscles (i.e., increased blood pressure and heart rate), preparing the immune system, enhancing cognitive functioning, and inhibiting other behaviors that are unnecessary for survival (Christopher, 2004; Johnson, et al., 1992; Sapolsky, 2000).

Activation of the HPA axis is coordinated by the neuropeptide corticotropin-releasing hormone (CRH) released from the paraventricular nucleus of the hypothalamus (PVN), which is densely populated with CRH neurons and receptors (Antoni, 1986; Owens and Nemeroff, 1991; Chalmers, Lovenberg, and De Souza, 1995; Swanson, et al., 1983). CRH neurons in the amygdala help to modulate activation of the HPA axis. The amygdala is a key extra-hypothalamic processor and integrator of information about environmental threats, and inputs from the amygdala to the PVN facilitate HPA axis activation after a threat has been detected (Fanselow and Gale, 2003; Fanselow and LeDoux, 1999; Maren, 2003; Herman, et al., 2003). Stress-induced activation of CRH in the PVN causes the release of aderenocorticotropic hormone (ACTH) from the anterior pituitary gland. ACTH then travels through the bloodstream and

initiates glucocorticoid release from the adrenal cortex (Antoni, 1986; Owens and Nemeroff, 1991; Vale et al., 1981). Glucocorticoids (i.e., cortisol in humans, corticosterone in rodents; CORT) aid in energy mobilization and help to restore homeostasis via negative feedback mechanisms after a threat has passed (Munck, Guyre, and Holbrook, 1984; Sapolsky, Romero, and Munck, 2000). Because glucocorticoids are lipophilic, they can enter the brain and bind to receptors or cross neuronal cell membranes (De Kloet, et al., 1998; McEwen and Weiss, 1970). They bind to either mineralocorticoid or glucocorticoid receptors (MR or GR), which can translocate into the nucleus and alter gene transcription; some of these mechanisms help exert negative feedback control over the stress response (McEwen and Weiss, 1970; Reichardt and Schutz, 1998)

Mimicking HPA axis activation with CORT injection has shown to have consistent results with studies in which animals were exposed to stress before fear conditioning. Both chronic and acute administration of CORT enhances fear conditioning in rats (Thompson, et al., 2004; Cordero, et al., 2003). Animals that were given CORT injections for five days before fear conditioning and animals given just a single injection of CORT after fear conditioning both showed increased conditional fear during a context test, as opposed to vehicle-treated animals. Together, these animal studies suggest a role for stress hormones in mediating stress-induced enhancement of behavioral responding.

Altered HPA axis responsiveness may contribute to the sensitized responses PTSD patients experience to innocuous events that are perceived as threatening; that is, PTSD symptoms may develop by a sensitization process involving the HPA axis that causes less intense stressors to be perceived as stronger than they are (Rasmusson and Charney, 1997; Yehuda, 1997). The initial traumatic event activates the stress response, but upon receiving

reminders of the trauma or mild but similar stressors, the stress response reactivates. Repeated activation modifies the HPA axis negative feedback system and makes the stress response become more easily triggered. Sensitization results in a lowered activation threshold for subsequent stimuli, facilitating higher responding to neutral stimuli now perceived as threatening (Hagemen, Andersen, and Jorgensen, 2001; Rosen and Schulkin, 1998). This type of repeated activation is similar to the electrophysiological phenomenon of kindling, a process in which repeated subthreshold electrical stimulation eventually leads to a full-blown seizure (Goddard, McIntyre, and Leech, 1969). Moreover, re-experiencing aspects of the traumatic event sensitize fear systems leading to the exaggerated reactions as observed in PTSD patients (Hagemen, Andersen, and Jorgensen, 2001; Post, et al., 1997). This may occur via sensitization of nuclei in the fear circuit (discussed in the next section) by a kindling mechanism, mediated by stress hormones (Rosen and Schulkin, 1998). Animal studies show that kindling in regions such as the amygdala can have anxiogenic effects in behavioral tests like the elevated plus maze and open field (Helfer, et al., 1996; Nieminen, et al., 1992) and can increase fear potentiated startle (Rosen, et al., 1996). Sensitized activity in fear circuitry due to repeated activation of the stress response may contribute to dysregulation of the HPA axis and perhaps to PTSD symptomatology.

Neural systems involved in fear learning

The neural systems mediating associative fear learning are well-known, which further validates using such a procedure to model fear responses and related symptoms in PTSD (Fendt and Fanselow, 1999). During learning, sensory input relating to both the CS and US converge on the basolateral amygdala (BLA) complex, a subregion of the amygdala, where a CS-US association is encoded via long-term potentiation (LTP) at BLA synapses (Rogan, et al., 1997;

Kim and Jung, 2006). This plasticity is dependent upon excitatory N-methyl-D aspartate receptors (NMDAR; Fanselow and Kim, 1994) and is modulated through inhibitory GABAergic neurons (Ehrlich, et al., 2009; Makkar, et al., 2010). Under normal conditions, the BLA, consisting of lateral (LA) and basal (BL) nuclei, is critical for fear learning (Maren, 1998; Gale, et al., 2004; McDannald and Galarce, 2011). Specifically, the BLA is necessary for encoding the memory of the US as either pleasant or aversive, as well as storing it long-term (Fanselow and Gale, 2003; Gale, et al., 2004). Indeed, functional magnetic resonance imaging studies show enhanced amygdala activity in PTSD patients during encoding and exposure to negative stimuli (Brohawn, et al., 2010; Rauch, et al., 2000; Shin, Rauch, and Pitman, 2006).

Although the BLA is a very important node in the fear conditioning circuitry, there are different types of fear learning and memory, requiring multi-modal sensory input and processing. Therefore, fear conditioning is actually achieved by activating neural networks. Sensory input from an auditory CS is projected through the medial geniculate nucleus of the thalamus, or through a thalamocortical relay to the LA (Romanski and LeDoux, 1992). A flashing light CS is conveyed to the BLA through a lateral geniculate-cortical pathway (Merigan and Maunsell, 1990; Callaway, 2005). Spatial representation of contextual information is formed by the hippocampus, which projects to the BL; information about the footshock US is relayed to the LA via the spinothalamic tract (Maren and Fanselow, 1995; Fendt and Fanselow, 1999).

The BLA projects to CeA both directly and indirectly, via a link through the BL and intercalated cell masses that lie between these two regions (Pitkanen, et al., 1997; Pare et al., 2004). Specifically, BLA neurons project to the lateral subdivision of the CeA (CeL), which sends GABAergic projections to the medial subdivision of the CeA (CeM; Haubensak, et al.,

2010). The fear response (i.e., freezing) is controlled by projections from CeM to the periaqueductal grey (PAG; Fanselow, 1991). Besides the CeA, the BLA projects to the bed nucleus of the stria terminalis, which in turn projects to the PAG for fear responding (Walker, et al., 2003; Waddell, et al., 2006; Poulos, et al., 2010). Other important regions in the fear learning and memory network involve cortical regions such as the prefrontal cortex (Milad and Quirk, 2002; Santini et al., 2004). Descending projections of the medial prefrontal cortex modulate the behavioral outputs of this circuit— the prelimbic (PL) cortex projects to the BLA to enhance fear responding, while the infralimbic cortex (IL) indirectly projects to the CeM via intercalated cells to promote extinction of fear (Quirk, et al., 2003). Moreover, PL and IL receive amygdala projections originating mainly from the BL. IL and PL have opposing roles in expression of fear following extinction learning (Senn, et al., 2014), which suggests that these reciprocal connections influence the outcome of fear and extinction learning. Moreover, inputs from the ventral hippocampus onto the BA, either directly or indirectly through PL, mediate contextual control of fear and fear renewal after extinction (Orsini, et al., 2011).

Biochemical Substrates and of Fear Learning and their Associated Changes

Within the amygdala, glutamatergic modifications in excitatory neurotransmission, including glutamate receptor-regulated synaptic plasticity, have been implicated in fear conditioning. Glutamate NMDAR and alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPAR) within the amygdala participate in different components of fear learning, including acquisition, expression, and extinction (Kim and Fanselow, 1994; Walker and Davis, 2002; Jasnow, Cooper, and Huhman, 2004; Kim, et al., 1993). In particular, it has been shown that blockade of NMDAR in the BLA prevented acquisition of fear learning (Kim and Fanselow 1994). Moreover, intra-BLA infusions of an AMPAR antagonist blocked expression of

fear (Kim, et al., 1993). It has also been shown that altered activity patterns due to stress can change the distribution of AMPAR in the BLA, increasing the density of AMPAR on dendritic spines (Hubert, et al. 2013).

The neural structures, pathways, and systems discussed in this simplified overview are highly integrated. This is a requirement of the fear conditioning system that is advantageous to survival; the system is able to rapidly discern and encode relevant associative relationships over a multitude of environmental stimuli that could signal a major threat. Furthermore, an important survival system could not afford to rely upon a single locus of function in case of damage; there would need to be alternate and compensatory structures and pathways to continue operating. One implication of this complexity is that the temporal contiguity of the occurrence of the CS and US is not enough to establish a predictive relationship between a CS and US (Kamin, 1968). Instead, associative learning within a complex environment is dynamic so that CS-US associations are strengthened for selected stimuli, producing competition between flexible neural circuits for CS associations with the US (Fanselow, 2010).

Stress-enhanced Fear Learning model

We have developed a model using fear conditioning procedures to examine how exposure to a traumatic stressor can affect future responding (Rau, DeCola, and Fanselow, 2005). In the stress-enhanced fear learning (SEFL) procedure, animals are given a series of 15 randomized, unsignaled shocks in a distinct context. Animals are then given a single context- shock pairing in a novel context, with different grid floor, lighting, and scent from the stressor context. Animals exposed to the 15 shocks show an enhanced fear response to the single shock in the second context compared with animals that did not receive the 15 shocks. Prior to the single shock, previously stressed rats show no generalized freezing to the second context, arguing against an

account of associative generalization. Rather, the effect of the 15-shock stressor appears to be nonassociative, as it occurs in a novel situation and only after the animal receives a milder version of the previous trauma. Sensitization is a nonassociative process in which there is increased reactivity to a potent stimulus after repeated exposure to that stimulus, producing a lowered activation for subsequent stimulation (Groves and Thompson, 1970; Rosen and Schulkin, 1998). We believe this effect is similar to that experienced by PTSD patients in which exposure to a traumatic event causes sensitized reactions to less intense but similar stressors (Bremner, et al., 1995; Dykman, Ackerman, and Newton, 1997).

There are several important features of SEFL indicating that it is a very long-lasting nonassociative enhancement of fear learning. Firstly, SEFL is indeed an enduring phenomenon. We have separated the 15 shock treatment from the single shock treatment by as long as 90 days with no diminution in the enhancement of conditioning (Rau and Fanselow, 2009). The presence of symptoms at least 30 days after trauma is required for a diagnosis of PTSD; hence, the longevity of SEFL is an important factor. Secondly, SEFL is not mediated by generalization between the two contexts. Imposing extinction of the stressor context before conditioning, while effective in eliminating fear of the stressor, has no impact on the enhancement of new conditioning. This may correspond to the reduced effectiveness of extinction in treating PTSD (i.e., Peri, et al., 2000). Additionally, blocking contextual fear learning to the stressor context by delivering an amnestic agent to the hippocampus during the 15 shocks does not alter the enhancement. This may correspond to observation of PTSD in patients that have amnesia for the traumatic episode (i.e., Krikorian, et al., 1998). Furthermore, while we have most often looked at the enhancement in new context fear learning, the fear enhancement is also found in auditory conditioning (Rau, DeCola, and Fanselow, 2005). Since there is no auditory cue during the 15

shocks, there is no basis for generalization to influence tone fear learning. Lastly, SEFL reflects a change in fear learning: the order of the 15 shocks and 1 shock matters. SEFL occurs only when the single shock conditioning is given after, not before, the stressor. If SEFL were due to summation of fear expression, (or, for that matter, generalization between contexts) order should not matter. If SEFL alters the fear learning circuit, the stressor would necessarily have to come before the 1-shock conditioning, and that is what is observed.

SEFL is extremely robust. Collapsing over many experiments we have trained well over 500 rats with just a single shock in the conditioning context that have either received or did not receive the prior 15-shock treatment. Over 90% of the rats receiving the 15-shock treatment freeze more than 2 standard deviations above the mean of the rats without prior stress. One could point to this robustness as a deviation from PTSD, where it is estimated that 10-25% of people who experience trauma go on to develop PTSD. However, in unpublished data, we did find that if we reduce the number of pre-shocks, a smaller percentage of rats meet the 2 standard deviation criterion. If we reduce the pre-shock treatment to 3 or 4 we find only 20% of the rats develop SEFL by this criteria—which is more in line with what is observed in human trauma cases. However, we use the 15-shock stressor because it simply makes experiments more tractable and efficient when we use parameters that cause more robust conversion to SEFL.

Animal Model Translation to PTSD

The SEFL model captures multiple aspects of PTSD, including exaggerated fear as seen through freezing, and blunted emotional reactivity, as measured through reactivity to the shock (the first of which will be the primary measure of the presented studies). These animal studies also show that HPA axis activation can enhance fear responding, and their results are consistent with the stress response of PTSD patients. For instance, Vietnam combat veterans diagnosed

with PTSD show increased levels of CRH in their cerebrospinal fluid (Baker, et al. 1999; Bremner, et al., 1997). However, there seems to be controversy about alterations in CORT levels. Both increased levels (Lemieux and Coe, 1995; Maes, et al., 1999) and decreased levels (Pitman and Orr, 1990; Boscarino, 1996; Mason, et al., 1986; Yehuda et al., 1990; 1995) of CORT have been found in PTSD patients compared to individuals without PTSD. The disagreement of these results suggests that a simple change in CORT levels in itself cannot explain the symptoms of the disorder. One theory is that PTSD patients experience enhanced negative feedback responding of the HPA axis during activation of the stress response, which would cause low CORT levels to be observed (Yehuda, 1997; 2001). Moreover, it is possible that CORT changes during stress are critical for the induction of exaggerated fear and that CORT level changes at other times are less critical to the behavior.

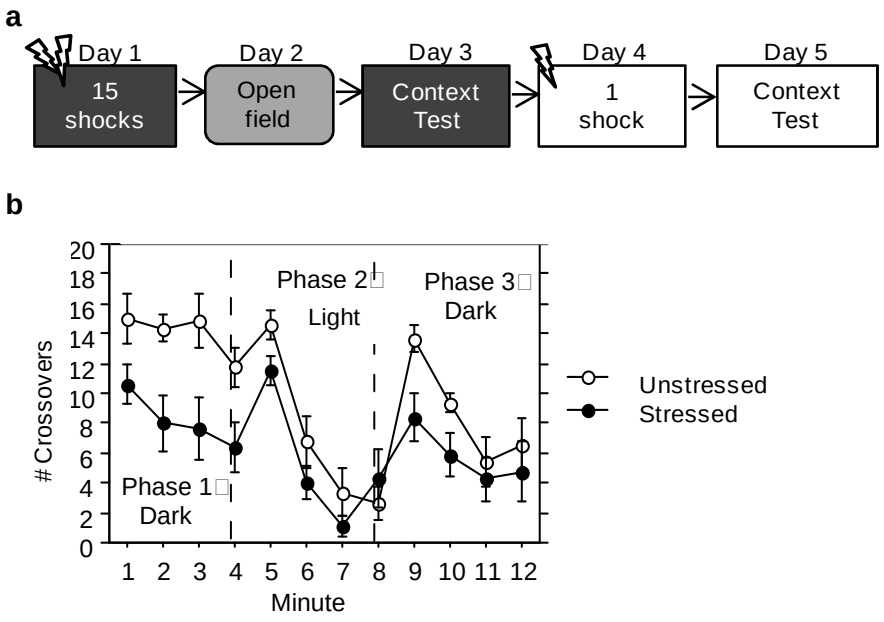

Figure 1: SEFL causes anxiety phenotype on the open field test. a. Experimental Design. We developed a modified version of the open field test that has been validated for anxiety testing (Godsil, et al., 2004; 2005). The open field test consisted of three phases: 1) four minutes of dark, 2) four minutes of light and 3) four minutes of dark. Locomotion, defined as the number of crossovers, was quantified during the 12-minute test. **b.** Open field test. Pre-exposure to shock significantly decreased the number of crossovers during phase 1, the first four dark minutes of the open field, $p < 0.005$. There was no effect of pre-exposure to shock on the number of crossovers during phase 2, minutes 5-8. Pre-exposure to shock decreased in the number of crossovers during phase 3, $p < 0.05$. Therefore, previously shocked rats showed reduced exploratory activity than controls when placed in a dark open field. When

bright lights turned on at one end, the rats retreated to the dark end and reduced activity similar to controls. However, when the lights went out, unlike controls, they remained in the dark corner and did not increase exploration. The open field test did not affect the context tests in either the stress or conditioning contexts (data not shown).

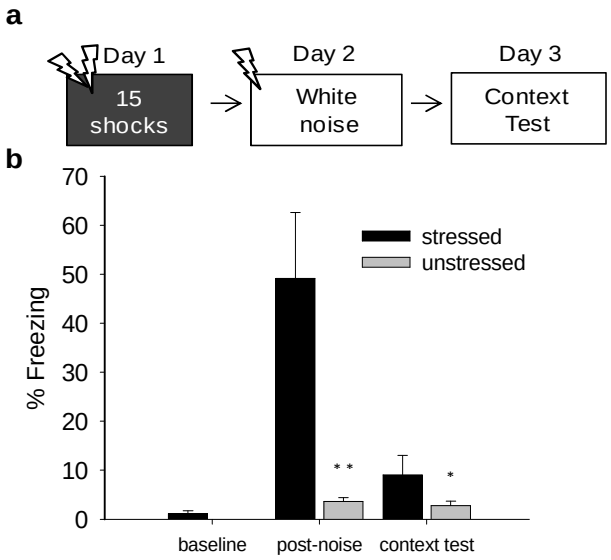

Figure 2: The 15-shock stressor causes exaggerated startle to white noise burst. a. Experimental design. Instead of 1-shock in conditioning context on Day 2, white noise (92dB, 1sec) was given. **b.** Freezing (+SEM) for baseline on Day 2, 5 minutes post-noise on Day 2, and context test on Day 3. Previously stressed rats showed a pronounced freezing response to a loud noise; this reaction was not seen in unstressed controls, **p < 0.01 (mixed factorial ANOVA). Upon return to the noise context the following day without noise, the stressed rats showed a small but statistically reliable increase in freezing relative to controls, *p <0.05

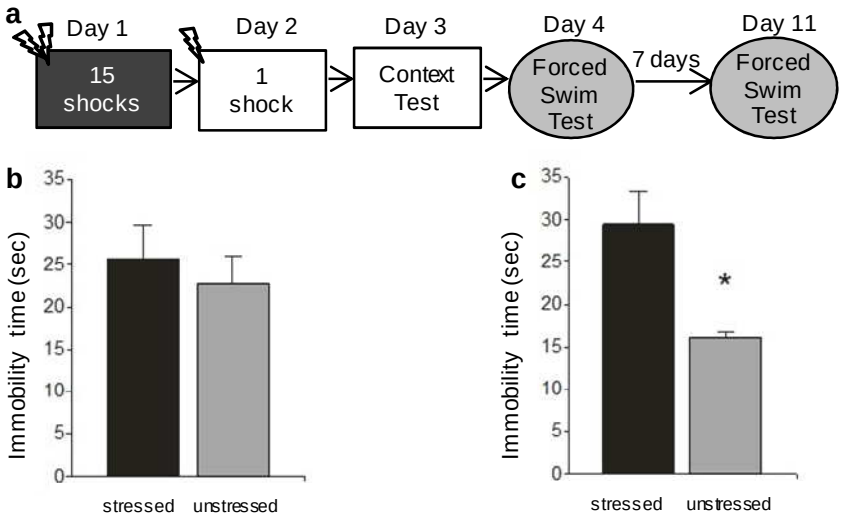

Figure 3: SEFL causes depression-like phenotype in forced swim test. a. Experimental design. Training (Day 4) and testing (Day 11) sessions for forced swim test were 5 minutes. Water temperature was 77 degrees F, and the apparatus dimensions are 74cm x 36.5 cm. **b.** Time spent immobilized (sec + SEM) on Day 4. **c.** Time spent immobilized (sec + SEM) on Day 11. * p<0.05 (one-way ANOVA).

Rats exhibiting SEFL also show decreased exploratory behavior in open fields (**Figure 1**), increased consumption of alcohol (Meyer, et al., 2013), potentiated startle reactivity **(Figure 2**), and a depression-like phenotype in the forced swim test (**Figure 3**). Moreover, SEFL causes an anxiety profile on the elevated plus maze, a long-lasting dysregulation of the diurnal cycle for CORT, and an increase of GR in the BLA (Poulos, et al., 2013). These findings show that SEFL behavior reflects several of the symptoms of PTSD (**Table 1**).

The Symptomatology of PTSD and SEFL

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 (comorbidity with simple phobia	Increased cued and contextual fear	Rau, et al., 2005
Anxiety	Anxiety; open field; elevated plus maze	Figure 1
Comorbid alcohol and drug abuse	Increased voluntary alcohol consumption	Meyer, et al., 2013
Symptoms present $\geq$30 days post trauma	$\geq$90 days	Rau & Fanselow, 2009
Increased startle reactivity	Hyper-reactivity to loud noise	Figure 2
Comorbid depression	Forced swim test	Figure 3

Table 1: Symptomatology of PTSD and SEFL. This table displays the overlap of symptoms of SEFL and PTSD, supporting the use of SEFL as a rodent model of PTSD.

Dissertation Objectives

The current work had three objectives: 1) to experimentally determine the biological mechanisms that cause the induction of SEFL; 2) to investigate the neural and biochemical changes that underlie the expression of SEFL; 3) to specifically target the primary SEFL expression mechanisms to reverse SEFL. Together, these studies inform us of the necessary conditions for the induction and expression of SEFL. In our findings, we produced a long-lasting reversal of SEFL, which immediately suggested a potential for treatment development for PTSD.

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CHAPTER ONE

Mechanisms underlying the Induction of Stress-Enhanced Fear Learning

Abstract

Fear is a mechanism that facilitates adaptive responses to threats. However, when the level of fear is not proportional to the level of threat, it is possible for an anxiety disorder to result. For example, post-traumatic stress disorder (PTSD) develops in response to a traumatic event, and patients often show sensitized reactions to mild stressors associated with the trauma. We have developed a model, called stress-enhanced fear learning (SEFL), to study such sensitized responding in animals, in which exposure to a 15-shock stressor nonassociatively enhances fear to a mild 1-shock stressor in another context. In this chapter, we examined the neural and biological mechanisms necessary for this sensitization to occur, including the role of stress hormones on the basolateral amygdala (BLA), a brain region responsible for fear and anxiety. In Experiment 1, we examined the role of corticosterone (CORT) in SEFL induction. Administration of the CORT synthesis blocker metyrapone prior to the 15-shock stressor, but not at time points after, attenuated SEFL. In Experiment 2, we determined the necessity of CORT in SEFL by co-administering metyrapone and CORT pre-stressor. CORT rescued SEFL from metyrapone, but CORT alone without the 15-shock stressor was not sufficient to produce SEFL. In Experiment 3, we examined the role of the BLA in SEFL with intra-BLA infusions of muscimol, a γ-aminobutyric acid type A ($GABA_A$) receptor agonist, pre- or post-stressor. The BLA must be functional during the 15 shocks but not after in order for SEFL to occur. Lastly, in Experiment 4, to determine if CORT's action was on the BLA, we infused the glucocorticoid receptor (GR) antagonist, mifepristone, directly into the BLA immediately prior to the 15-shock stressor, which attenuated SEFL. The data from these experiments indicate that CORT activation of GRs in the BLA is necessary for SEFL induction.

Introduction

Fear is an adaptive response that has evolved to protect animals and humans from danger. During a threatening situation, innate defense behaviors are used to avoid harm and promote future reproductive success (Bolles, 1970). Fear facilitates the use of defensive behaviors most appropriate to the threat, those that have the highest likelihood of promoting survival (Fanselow and Lester, 1988). However, fear is only adaptive when its level is appropriate to the level of the threat. Furthermore, fear should be limited to situations in which an actual threat is present. In humans, inappropriate activation of fear responses can result in the development of anxiety disorders (Rosen and Schulkin, 1998).

One such anxiety disorder is post-traumatic stress disorder (PTSD), which develops in some individuals who experience a traumatic event. Symptoms include persistent re-experiencing of the trauma, avoiding stimuli associated with the trauma, and signs of hyperarousal, like increased startle (American Psychiatric Association, 2013). Normally, these symptoms may be adaptive in dealing with the traumatic event (Bonne, et al., 2004; Charney, 2004; Christopher, 2004; Eberly, Harkness and Engdahl, 1991). As an example, avoiding stimuli associated with the event reduces the chances of encountering the threat. However, in PTSD, these formerly adaptive responses disrupt normal daily functioning in affected individuals (American Psychiatric Association, 2013).

We have previously developed a model using Pavlovian fear conditioning to examine how exposure to a traumatic stressor can affect subsequent fear conditioning (Rau, DeCola, and Fanselow, 2005; Rau and Fanselow 2009). In a typical fear conditioning procedure, a brief electrical current is delivered to the feet of an animal through the metal grid floor of a conditioning chamber. This footshock, or unconditional stimulus (US), is paired with a

conditional stimulus (CS), such as the context in which the animal is shocked. Pairing the CS and US will eventually result in the CS producing a conditional response (CR) in the absence of the US. Freezing behavior, defined as the absence of movement except that necessary for respiration, is an adaptive reaction of rats to a fearful stimulus (Fanselow, 1980; Fanselow and Lester, 1988). Freezing can be observed in response to stimuli associated with footshock, making it a valuable CR to assess an animal's fear to a CS in the laboratory (Blanchard and Blanchard, 1971; Bolles and Collier, 1976; Fanselow, 1980).

In this specific stress-enhanced fear learning (SEFL) procedure, animals are given a 15-shock stressor prior to fear conditioning with a single shock in another distinct context. Animals that have experienced this stressor show an enhanced fear response to the single shock in the second context compared to animals not given the stressor (90% freezing vs. 25% freezing). This enhancement is not due to generalization or summation of fear between the two contexts; animals can be extinguished in the stressor context or given an amnestic agent into the hippocampus during the stressor with no reduction in SEFL (Rau, DeCola, and Fanselow, 2005). This is also a long-lasting effect; the 15 shocks and 1 shock may be separated by as much as 90 days without any attenuation in freezing (Rau and Fanselow, 2009). Therefore, exposure to this 15-shock stressor appears to sensitize a biological system involved in generating fear responses.

Biological mediators of this effect may be stress hormones (Carrasco and Van de Kar, 2003; Johnson, et al., 1992). Stress initiates the activation of the neuroendocrine stress cascade, also called the hypothalamic-pituitary-adrenal (HPA) axis. Activation of the HPA axis is orchestrated by corticotrophin releasing hormone (CRH) neurons in the paraventricular nucleus of the hypothalamus (PVN; Antoni, 1986; Vale, et al., 1981). CRH neurons in the PVN travel through the portal capillary zone and project to the median eminence and anterior pituitary.

Stress-induced activation of CRH in the PVN causes the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which travels through the bloodstream and stimulates glucocorticoid release from the adrenal cortex (Antoni, 1986; Owens and Nemeroff, 1991; Vale, et al., 1981). Glucocorticoids (mainly corticosterone in the rat and cortisol in humans; CORT) aid in energy mobilization and help to restore homeostasis after a threat has passed by negative feedback mechanisms (Munck, Guyre, and Holbrook, 1984; Sapolsky, 2000).

Activation of stress hormones has been shown to increase fear responding. Chronic administration of CORT enhances fear conditioning in rats (Thompson, et al., 2004). Animals given CORT injections for five days before fear conditioning showed increased conditional fear during a context test on day six, compared to vehicle-treated animals (Thompson, et al 2004). Animals given just one injection of CORT after fear conditioning also show enhanced conditional freezing compared to vehicle-treated animals (Cordero, Merino, and Sandi, 1998; Hui, et al., 2004). Moreover, stress-induced HPA axis activation can enhance conditional freezing, as well as future HPA responsiveness. Exposure to a stressor produced an enhancement in conditional fear and increased CORT release during training (Cordero, et al., 2003). In this experiment, animals were given a two-hour session of restraint stress. Two days later, they were given three context-shock pairings. The following day, when returned to the context in which they had been shocked, previously restrained animals showed enhanced freezing compared to trained non-restrained control animals. In this study, CORT levels were sampled every 15 minutes for an hour after fear conditioning training. Previously restrained animals had elevated CORT levels for 45 minutes after training compared to non-restrained controls. Levels returned to baseline by 60 minutes post-training.

The amygdala is essential for fear conditioning and emotional learning in animals (Helmstetter, 1992; Sananes and Davis, 1992; Fanselow and Kim, 1994; Muller, et al., 1997; Gale, et al., 2004). Functional magnetic resonance imaging studies show enhanced amygdala activity in PTSD patients during encoding and exposure to negative stimuli (Shin, Rauch, and Pitman, 2006; Brohawn, et al., 2010; Rauch, et al., 2000). Inactivating the basolateral amygdala (BLA) demonstrates that it is a region necessary to encode and store fear memory (Fanselow and Kim, 1994; Wilensky, Schafe, and LeDoux, 1999; Schafe and LeDoux, 2000; Fanselow and Gale, 2003; Kim, et al., 2005).

Receptors for CORT are found in key areas involved in fear conditioning, including the amygdala (Chalmers, Lovenberg, and De Souza, 1995; Swanson, et al., 1983). During stress, CORT that is synthesized and released into the bloodstream enters the brain and binds to glucocorticoid receptors (GRs) or crosses neuronal cell membranes to alter gene transcription (McEwen and Weiss, 1970; McEwen, Weiss, and Schwartz, 1968). Strikingly, long-lasting changes in gene expression have been observed in the BLA of animals showing SEFL (Ponomarev, et al., 2010). Furthermore, studies show that stress leads to increased CORT and CRH, which in turn increases excitability in the BLA; this leads to increased transmission to areas that mediate fear responses (Braga, et al., 2004; Adamec, Blundell, and Burton, 2005; Rodriguez, et al., 2005; Roozendaal, McEwen, and Chattarji, 2009; Rosen and Schulkin, 1998). This may account for how mild stressors or traumatic reminders come to elicit sensitized reactions. These converging lines of evidence suggest roles for both CORT and the BLA in mediating sensitization of behavioral responding caused by stress exposure.

In Experiment 1A, we examined the role of CORT in SEFL. Animals were given an injection of the CORT synthesis blocker metyrapone prior to the 15-shock stressor, and freezing

was recorded in the 1-shock context test. Previous studies show that manipulations that decrease CORT levels such as systemic metyrapone administration (Cordero, et al., 2002), adrenalectomy (Pugh, et al., 1997), and administration of GR antagonists (Pugh, Fleshner, and Rudy, 1997) reduce conditional fear. Moreover, plasma CORT levels were measured immediately after the 15-shock stressor and after the context test in the 1-shock context. In Experiments 1B and 1C, metyrapone was given at two time points post-stressor, and freezing was recorded during the context test in the 1-shock context. If CORT is necessary for initiation of this sensitization, then metyrapone would work if administered pre-15 shock stressor. Previous data show that disrupting fear to the 15-shock stressor by a glutamate N-methyl-D-aspartate receptor (NMDAR) antagonist left sensitization intact, demonstrating that fear to the 15-shock stressor context is not necessary for sensitization (Rau, DeCola, and Fanselow, 2005). However, if stress-induced CORT release is initiating a biological state change in the animal, then blocking the increase in CORT caused by the stressor should attenuate fear in both contexts. Moreover, if CORT is also necessary for expression, and not just initiation, of this exaggerated fear, then metyrapone administered post-stressor would also attenuate sensitization.

In a follow-up experiment (Experiment 2), metyrapone was administered again prior to the SEFL procedure, and then 10 mg/kg of CORT was administered systemically immediately before the procedure in order to assess both the necessity and sufficiency of CORT. If CORT is necessary for sensitization, then metyrapone would prevent SEFL and CORT co-administration would rescue enhanced freezing to the 1-shock context. If CORT is also sufficient for SEFL, then CORT alone without the 15 shocks would also produce enhanced freezing. Prior studies show that animals that were given CORT injections for five days before fear conditioning and animals given just a single injection of CORT after fear conditioning both exhibit increased

conditional fear during a context test, as opposed to vehicle-treated animals (Thompson, et al., 2004; Cordero, et al., 2003).

To assess the amygdala's role in SEFL, in Experiment 3 we determined if BLA activity during stress was necessary for SEFL by inactivating the BLA with the γ-aminobutyric acid type A ($GABA_A$) agonist muscimol. Modifications in excitatory neurotransmission within the amygdala have been implicated in fear conditioning. In particular, NMDAR and alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPAR) within the amygdala participate in different components of fear learning, including acquisition, expression, and extinction (Fanselow and Kim, 1994; Walker and Davis, 2002; Jasnow, Cooper, and Huhman, 2004). The BLA was inactivated either pre- or post- stressor. We hypothesized that only functional inactivation pre-stressor would attenuate sensitization, given the importance of excitatory modification in the BLA during fear conditioning.

Lastly, given the results from Experiments 1 through 3, in Experiment 4 we infused the GR antagonist, mifepristone, directly into the BLA immediately prior to the 15-shock stressor in order to determine if CORT's action was on the BLA. Prior work has shown that systemic administration of GR antagonists impairs contextual fear conditioning (Pugh, Fleshner, and Rudy, 1997). However, given the importance of CORT and the BLA in fear learning, the BLA was a prime target for direct micro-infusion of a GR antagonist. We predicted that GR antagonism in the BLA would reduce sensitization, given the abundance of GR in the BLA and their ability to both alter gene transcription and increase excitability in this region.

Experiment 1A: Pre- stressor metyrapone attenuates stress-enhanced fear learning

Experiment 1A Method

Subjects. A total of 102 experimentally naïve adult male Long Evans rats, approximately 300 g, were purchased from Harlan (Indianapolis, IN) for this experiment. Food and water were available *ad libitum* to the animals, and a 14:10-hour light-dark cycle, lights coming on at 6:00am, was maintained in the colony room. The rats were individually housed in stainless steel wire mesh cages and were handled daily for approximately 45 seconds for five days prior to the start of the experiments. All experimental procedures took place during the light cycle.

The procedures used in this experiment were in accordance with policy set and approved by the Institutional Animal Care and Use Committee of the University of California, Los Angeles.

Apparatus. Two contexts were used during the course of the experiments, the "stress" context and the "conditioning" context. These contexts differed in background noise, illumination, and odor. Chambers housed within the contexts differed in shape, size, color, and texture. In the stress context, two ceiling mounted 6-ft. fluorescent bulbs illuminated the room and a ventilation fan was used to provide background noise (65dB). Stress context chambers (28 x 21 x 21 cm) had a clear Plexiglas back wall, ceiling and front door and aluminum side walls. The floor, composed of 18 stainless steel rods (4mm in diameter), spaced 1.5 cm center to center, was wired to a shock generator and scrambler (Med Associates, Inc; St. Albans, VT). Chambers were wiped down with isopropyl alcohol (10%) and dried before and after each subject, and a Simple Green (50%) odor was placed in stainless steel pans inserted below the chamber to provide a distinctive smell in the context.

The conditioning context was lit by a single red 30W bulb, and no background noise was provided. Conditioning context chambers were initially the same size as stress context chambers,

but were made smaller by black triangular inserts, creating side walls at a 60 degree angle with the floor. Conditioning context chamber floors were composed of 17 stainless steel rods (4 mm in diameter) staggered into two rows spaced 1cm apart vertically and 2.6 cm apart horizontally, and they were wired to the shock generator as described for the stress context chambers. Conditioning context chambers were cleaned with acetic acid solution (1%) before and after each subject, and the same solution was placed in the pan underneath each chamber. The freezing behavior was analyzed using the VideoFreeze program (Med Associates, Inc).

Computer Scoring by VideoFreeze Program. The VideoFreeze program is a reliable way to analyze behavioral data that is comparable to hand scoring by a trained person (Anagnostaras, et al., 2010). A motion analysis algorithm was used to analyze the video stream in real time, recording at 30 frames per second, 320 x 240 pixels, 8-bit grayscale. A reference video sample was taken prior to placing the rats into the four chambers to calibrate the equipment. This reference sample established the amount of baseline noise in the video signal on a pixel-by-pixel basis, across multiple successive frames. Once the rats were placed in the chambers, successive video frames were continuously compared to each other and to the reference sample on a pixel by pixel basis. Any differences between pixels in the current video signal larger than those in the reference sample were interpreted as animal movement. These pixel differences were summed for each image frame, and this summation was counted as the Motion Index. The Motion Index is the number of pixels that have changed within 1 second that exceed video noise. When this Motion Index was below 50 for one second, an instance of freezing is scored.

Procedure. Rats were randomly assigned to one of two groups: those that received 15 shocks over a 90-minute period in the stress context ("stressed"), and those who remained in the chambers of this context for the same duration without receiving any shocks ("unstressed"). Both

groups were composed of four subgroups based on what drug dose was administered: vehicle, 50 mg/kg metyrapone, 100 mg/kg metyrapone, and 150 mg/kg metyrapone (Tocris Bioscience, Ellisville, MO). Drug dosages were determined from previous studies showing that 150 mg/kg metyrapone administration reduced inactivity caused by inescapable shock (Baez, Siriczman, and Volosin, 1996), and pre-training administration of 50 mg/kg metyrapone reduced contextual fear conditioning (Cordero, et al., 2002); 100 mg/kg was chosen as an intermediate dose. The vehicle for the drug was composed of 60% saline and 40% propylene glycol (Sigma Aldrich; St. Louis, MO). All groups had an n of 12, except for stress/vehicle (n=14), and unstressed/vehicle (n=16). One hour prior to trauma exposure, animals were given an intraperitoneal (i.p.) injection of one of the four doses. The volume of all injections was 1.0 ml/kg. Injections were given in the rats' housing area, and the rats remained in their homecages until the stress exposure.

One hour after injection, rats were transported to the stress context in their homecages. Stress exposure consisted of 15 shocks (1 mA, 1sec), with a variable shock interval of 240-480 seconds. Animals receiving no stress during exposure were placed in chambers for an equivalent amount of time as the stressed animals—90 minutes. Immediately after the stressor, animals were placed in a restraining tube for a maximum of 5 minutes and approximately 0.5 ml of tail blood was collected into heparinized tubes. Animals were then brought back to their homecages and returned to their housing area.

Animals were then given two days rest time in their homecages (Days 2 and 3). On Day 4, all 102 animals were given a context test in the stress context for 8 minutes. They were transported in the same manner as on Day 1, in their homecages. On Day 5, animals were given an 8 minute session in the novel conditioning context, in order to provide pre-exposure and to ensure the animals were not generalizing to this context. They were transported out of their

homecages and placed into a black container (Rubbermaid) that was partitioned with inserts (Plexiglas) into equivalent chambers (20 x 15 x 25 cm) to carry four animals. Lids were placed on the container so that the animals were transported in darkness to the conditioning context. On Day 6, all rats were given a single shock (1mA, 1 sec) in the conditioning context 180 seconds after placement in the chamber. The rats were transported in the same manner as Day 5, in the black partitioned container. Animals were removed from the chamber after an additional 300 seconds and brought back to their housing area and placed back in their homecages. On Day 7, animals were given an 8 minute context test in the 1-shock context. Animals were transported in the same manner as on Days 5 and 6, in the black container. 45 minutes after the context test, tail blood was collected again in the same manner as was for Day 1. See **Figure 4a** for a diagram of the procedure.

Behavioral Recordings. Pre-stress baseline freezing was recorded for all eight groups on Day 1, and percent freezing in the inter-shock interval between the first and second shocks for all four stressed groups, as well as all four unstressed groups as a control. Percent freezing was recorded for Days 4 and 7 using the VideoFreeze program. SEFL is indicated by high percent freezing, a reliable measure of learned fear (Fanselow, 1980; 1994), in the 1-shock conditioning context test.

Corticosterone Assay. CORT plasma levels were measured by enzyme immunoassay (AssayPro; Correlate-EIA corticosterone enzyme immunoassay kit). Blood was collected immediately after the context test in heparinized tubes and centrifuged (2000 g) for 15 minutes. Plasma was removed and stored in a deep freezer (-20 □C) until processed. Duplicate 0.5 ml samples were run.

Analysis. Two-way Analysis of Variance (ANOVA) was used to analyze percent freezing as influenced by both drug dose and stress condition in both Day 1 and Day 4 in the stress context and Day 7 in the conditioning context. Two-way ANOVA was used to compare plasma CORT levels between groups on both Days 1 and 7. Moreover, trend analysis was performed to determine if the data follow linear dose-dependent functions.

Experiment 1A Results and Discussion

One of four doses of metyrapone was administered 1 hour prior to the 15-shock stressor, as shown in **Figure 4a**, the diagram of the procedure. **Figure 4b** displays baseline freezing for all 8 groups pre-stress on Day 1 (mean $\pm$ SEM). While ANOVA showed that there was a drug main effect, $F(3, 94) = 17.185$, $p<0.0001$; the stress main effect and stress-drug interaction were not statistically significant. **Figure 4c** displays freezing during the inter-shock-interval (mean $\pm$ SEM) between shock 1 and shock 2 on Day 1. The stress main effect, drug main effect, and stress-drug interaction were all significant ($ps<0.005$).

Figure 4d displays the percent freezing for all eight groups in stress context on Day 4 (mean $\pm$ SEM). A two-way ANOVA showed a significant interaction between drug dose and stress treatment, $F(3, 94) = 17.236$, $p<0.0001$. Trend analysis showed a significant linear dose-dependent function of metyrapone in stressed animals, but not in unstressed animals, $F(1, 94) = 106.4$, $p<0.0001$.

The critical behavioral data is shown in **Figure 4e**, which displays percent freezing during the context test in the conditioning context on Day 7 (mean $\pm$ SEM), 24 hours after all animals received 1 footshock in this context. In vehicle controls, prior stress enhanced fear conditioning to the 1 shock that occurred 6 days later; metyrapone dose-dependently blocked this stress-

induced enhancement of fear learning. A significant interaction between drug dose and stress treatment was found, $F(3, 94) = 2.957$, $p<0.05$. Trend analysis indicated that pre-stress metyrapone dose-dependently reverses this increase in freezing in a linear fashion, $F(1, 94) = 12.796$, $p<0.0005$. This pattern was not seen in unstressed animals $F(1, 94) = 0.079$, $p>0.05$.

Plasma CORT levels (ng/mL) of all eight groups after the Day 1 stressor are displayed in **Figure 4f** (mean $\pm$ SEM). Similar to freezing behavior, metyrapone dose-dependently blocked the stress-induced increases in CORT. There was a significant main effect of stress, where stressed animals had higher CORT levels than did unstressed animals, $F(1, 94) = 11.509$, $p<0.001$. Trend analysis showed that in stressed animals, metyrapone linearly dose-dependently decreased plasma CORT levels after the 15-shock stressor on Day 1 in a linear fashion, $F(1, 94) = 5.19$ $p<0.05$. This trend did not appear in unstressed animals, $F(1, 94) = 0.080$, $p>0.05$. CORT levels during testing showed no reliable group differences, $ps>0.05$ (**Figure 4g**).

The results from this experiment suggest that blocking the rise in CORT induced by a 15-shock stressor attenuates sensitized freezing during both the 15-shock and the 1-shock context test, reducing both associative and nonassociative fear. From **Figures 4b** and **4c**, it is clear that metyrapone does impair locomotion; however all critical behavioral tests were performed drug-free. The rise in CORT appears to be essential to the production of fear enhancement. These results suggest that CORT may induce a state change in animals that sensitizes fear circuitry. Moreover, plasma CORT levels after the 15-shock stressor on Day 1 but not on Day 7 during the context test in the 1-shock context are dose-dependently decreased by metyrapone. This demonstrates that CORT increases at the time of stress, but not after, are critical for SEFL. This is consistent with other studies finding that blocking CORT synthesis during testing did not affect the expression of learned fear (Barrett and Gonzalez-Lima, 2004). Measurement of CORT

in PTSD patients is typically taken long after, not immediately after, the stressor, which may explain the inconsistencies in reported CORT levels in PTSD patients (Yehuda, et al., 1990; Maes, et al., 1998). While SEFL rats did not show changes in elicited CORT levels at the time of test, it should be noted that SEFL causes a dysregulation of the circadian rhythm in basal CORT levels (Poulos, et al., 2013). Experiments 1B and 1C investigated the effect of metyrapone either immediately after the 15-shock stressor or prior to a retrieval test in the 15-shock context.

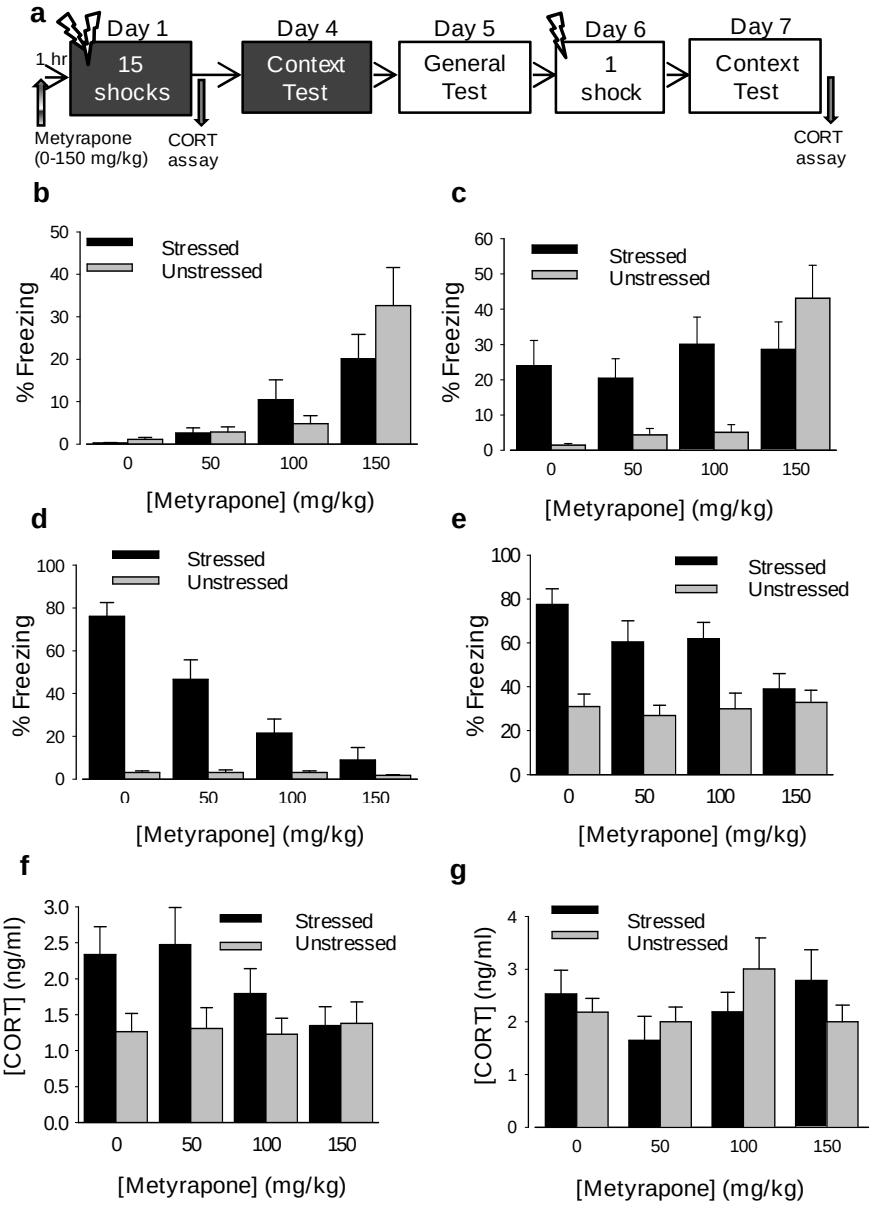

Figure 4. **Pre-stressor administration of metyrapone attenuates SEFL. a.** Experimental Design. Shock in both contexts: 1mA, 1sec. **b.** Baseline freezing data on Day 1 (mean ± SEM). While ANOVA shows that there was a drug main effect, $p<0.0001$, the stress main effect and stress-drug interaction were not statistically significant. **c.** The first inter-shock-interval between the first and second shock on Day 1. The stress main effect, drug main effect, and stress-drug interaction were all significant ($ps<0.005$). **d.** Context test in the stress context on Day 4: The mean ± SEM of freezing percentage in stress context on Day 4. Drug x stress, $p<0.0001$ (two-way ANOVA), linear trend analysis for stressed animals, $p<0.0001$. **e.** Freezing (mean + SEM) in conditioning context on Day 7. Drug x stress, $p<0.05$ (two-way ANOVA), linear trend analysis for stressed animals, $p<0.0005$. **f.** Plasma CORT levels (ng/mL; mean + SEM) after the 15-shock stressor on Day 1. Main effect of stress, $p<0.001$ (two-way ANOVA), linear trend analysis for stressed animals, $p<0.05$. **g.** Plasma CORT levels (ng/mL) found on Day 7, 1 hour after the context test in conditioning context (mean ± SEM). No significant differences were found for any main effect or interaction (two-way ANOVA).

Experiment 1B: Post-stressor metyrapone does not prevent stress-enhanced fear learning

To compare pre-stress administration and post-stress administration of metyrapone, as well as to test if the drug blocks consolidation of the fear memory, metyrapone was administered after the 15 shocks, and freezing was measured during the context test in the 1-shock context.

Experiment 1B Method

Subjects. A total of 20 experimentally naïve adult male Long Evans rats, approximately 300 g, were purchased from Harlan (Indianapolis, IN). Animals were housed and handled as described for Experiment 1A.

Procedure. The same apparatus and VideoFreeze program from Experiment 1A was used in this experiment. Rats were randomly assigned to one of four groups: those that received 15 shocks over a 90-minute period in the stress context with either a 150 mg/kg injection of metyrapone ("stressed/metyrapone"), or of vehicle ("stressed/vehicle"), and those who remained in the chambers of the stress context for the same duration without receiving any shocks, with injection of either 150 mg/kg metyrapone ("unstressed/metyrapone") or of vehicle ("unstressed/vehicle"). All parts of this procedure were kept constant from the behavior portion of Experiment 1A;

however the drug dose was given immediately after the Day 1 15-shock stressor. See **Figure 5a** for an outline of the procedure.

Behavioral Recordings. Percent freezing was recorded for Days 4 and 7 using the VideoFreeze program.

Analysis. Two-way ANOVA was used to analyze percent freezing as influenced by both drug dose and stress condition in both Day 4 in the stress context and Day 7 in the conditioning context.

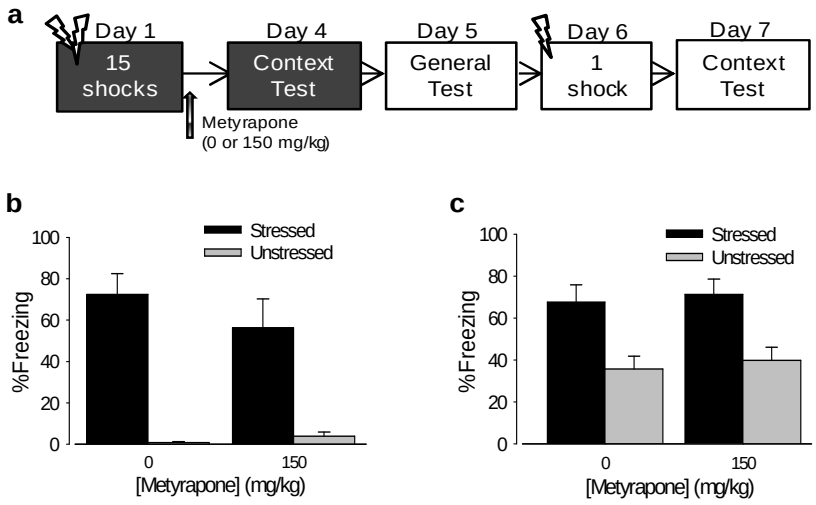

Figure 5. Post-stressor administration of metyrapone does not prevent SEFL. a. Experimental Design. Shock in both contexts: 1 mA, 1 sec. **b**. Mean (+ SEM) percentage freezing during the stress context test on Day 4. main effect of stress, $p<0.0001$ (two-way ANOVA). **c**. Mean (+ SEM) percentage freezing during the conditioning context test on Day 7. Main effect of stress, $p<0.001$, but not of drug (two-way ANOVA).

Experiment 1B Results and Discussion

The mean percent freezing (± SEM) in stress context on Day 4 is shown in **Figure 5b**. While there was a main effect of stress, $F(1, 16) = 51.761$, $p<0.0001$, there were no significant differences were found for drug-stress interaction or drug main effect.

Figure 5c displays the mean percent freezing (± SEM) on conditioning context on Day 7. There was a main effect of stress, $F(1, 16) = 20.394$, $p<0.001$, but no significant drug-stress interaction or drug main effect differences were found.

The results of Experiment 1B show that the animals that received injections of metyrapone after the 15-shock stressor still had a disproportionate amount of fear to the 1-shock context compared to animals that received metyrapone pre-stressor in Experiment 1A. This indicates that metyrapone administered immediately after the stressor did not block consolidation of the fear memory. Moreover, the results suggest that CORT must be on board during the stressor in order for sensitized fear to be expressed.

Experiment 1C: Pre-retrieval metyrapone does not prevent stress enhanced fear learning

Prior research has shown that extinction of fear in the stressor context does not eliminate SEFL (Rau, DeCola, and Fanselow, 2005; Rau and Fanselow, 2009). However it is possible that the stress memory has to reconsolidate once it is reactivated (Nader, et al., 2000). Such a retrieval could create a window of opportunity to block reconsolidation and alleviate SEFL. Therefore, three days following stress, metyrapone was injected prior to placement in the 15-shock context, and freezing was measured during the context test in the 1-shock context.

Experiment 1C Method

Subjects. A total of 24 experimentally naïve adult male Long Evans, approximately 300 g, were purchased from Harlan (Indianapolis, IN) for this experiment. Animals were housed and handled as described for Experiments 1A and 1B.

Procedure. The same apparatus and VideoFreeze program from Experiment 1A was used in this experiment. Rats were randomly assigned to one of four groups: those that received 15 shocks over a 90-minute period in the stress context with either a 150 mg/kg injection of metyrapone ("stressed/metyrapone"), or of vehicle ("stressed/vehicle"), and those who remained in the chambers of the stress context for the same duration without receiving any shocks, with injection of either 150 mg/kg metyrapone ("unstressed/metyrapone") or of vehicle ("unstressed/vehicle"). All parts of this procedure were kept constant from the behavior portion of Experiment 1A; however the drug dose was given 1 hour prior to the Day 4 context test in the 15-shock context, and an extra context test in this context was given in on Day 8, when the drug was no longer on board. See **Figure 6a** for additional details.

Behavioral Recordings. Percent freezing was recorded for Day 7 and Day 8 using the VideoFreeze program.

Analysis. Two-way ANOVA was used to analyze percent freezing as influenced by both drug dose and stress condition in both Day 7 in the conditioning context and Day 8 in the stress context.

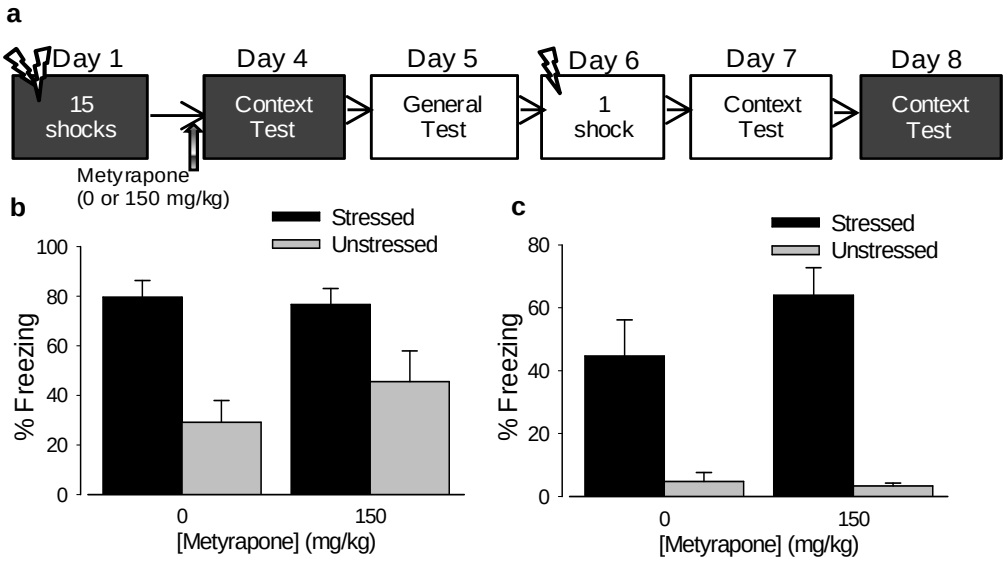

Figure 6. Pre-retrieval administration of metyrapone does not prevent SEFL. a. Experimental Design. Shock for both contexts: 1 mA, 1 sec. **b.** Mean + SEM of freezing percentage in the conditioning context on Day 7. Main effect of stress, $p<0.0001$ (two-way ANOVA) **c**. Mean + SEM freezing in the stress context on Day 8. Main effect of stress, $p<0.0001$, but not of drug (two-way ANOVA).

Experiment 1C Results and Discussion

The mean (± SEM) percent freezing in the conditioning context on Day 7 is shown in **Figure 6b.** While there was a main effect of stress $F(1, 20) = 21.087$, $p<0.0001$, there were no significant differences found for drug-stress interaction or drug main effect.

The mean ± SEM of freezing percentage in the stress context on Day 8 is shown during the 8-minute context test (**Figure 6c**). There was a significant main effect of stress, $F(1, 20) = 41.298$, $p<0.0001$. However, there was no significant main effect of drug condition, nor was there a drug by stress interaction.

The results from Experiment 1C demonstrate that stressed animals that received injections of metyrapone prior to a context test in the 15-shock stressor context still had a disproportionate amount of fear to the 1 shock in the novel context and therefore did not block sensitization. This means that when metyrapone was administered before retrieval of the fear memory, it did not

interfere with reconsolidation, and the exaggerated fear response is not attenuated. While disappointing with respect to a potential treatment, the data identify another example of how SEFL differs from standard associative fear conditioning. Like the results from Experiment 1B, these results suggest that CORT must be on board during the stressor in order to induce fear sensitization.

Experiment 2: Co-administration of metyrapone and corticosterone rescues stress-enhanced fear learning

Experiment 2 Method

Subjects. A total of 116 experimentally naïve adult male Long Evans rats, approximately 300 g, were purchased from Harlan (Indianapolis, IN). Animals were housed and handled as described for Experiment 1.

Procedure. Animals were given either a pre-training injection of 150 mg/kg metyrapone or vehicle. As in Experiments 1A and 1B, pre-stress injections were given 1 hour prior to the 15-shock stressor in the rats' housing area; 10 mg/kg CORT in 15% alcohol/ 85% saline solution (Sigma-Aldrich, St. Louis, MO) or saline was injected 10 minutes prior to the 15 shocks. After the 15 shocks on Day 1, all rats were taken back to their housing area. The remainder of the procedure follows Experiment 1. There were a total of 8 groups, and n per group ranged between 12 and 17. See **Figure 7a** for an outline of the procedure.

Behavioral Recordings: Freezing was recorded for Days 4 and 7 using the VideoFreeze program.

Analysis. An overall one-way ANOVA was performed to determine significance differences of freezing for both context tests. *A priori* planned comparisons were also made to determine if

CORT alone could induce SEFL in unstressed animals, and if CORT can rescue SEFL from stressed animals that received metyrapone (Gaito, 1965).

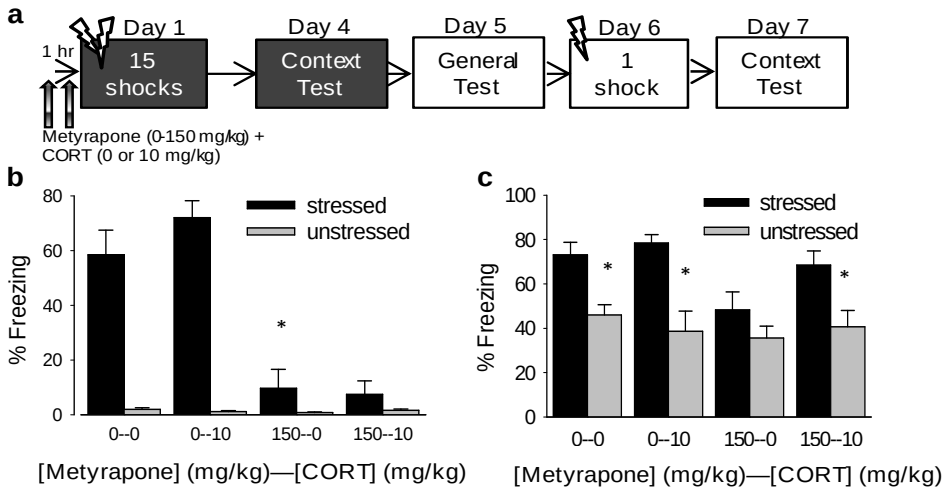

Figure 7: Co-administration of metyrapone and CORT rescues SEFL. a. Experimental Design for metyrapone and CORT co-administration. **b.** Freezing (mean ± SEM) in the stress context on Day 4. First digit in group designations = metyrapone dose (0 or 150 mg/kg); second= CORT dose (0 or 10 mg/kg). *p<.001 (overall one-way ANOVA, followed by planned contrasts). **c.** Freezing (mean + SEM) in conditioning context on Day 7. First digit in group designations = metyrapone dose (0 or 150 mg/kg); second = CORT dose (0 or 10 mg/kg). * p <0.01 (overall one-way ANOVA, followed by planned contrasts).

Experiment 2 Results and Discussion

The mean percent freezing (± SEM) in the stress context on Day 4 is shown in **Figure 7b**. A one-way ANOVA was performed, which indicated an overall difference between groups, $F(7, 108) = 36.355$, $p<0.0001$. The freezing levels for stressed/vehicle animals were significantly higher than for unstressed animals that received only CORT, which were not significantly different from unstressed controls $F(1, 108) = 60.54$, $p<0.0001$, and $F(1, 108) = 0.01$ $p>0.05$, respectively. Stressed/vehicle rats froze significantly more than did stressed/metyrapone animals, $F(1, 108) = 47.43$, $p<0.0001$, which showed no significant differences from either stressed/metyrapone/CORT animals or unstressed controls, ps >0.05.

Figure 7c shows the mean (± SEM) percentage of freezing in the conditioning context from Day 7. An overall one-way ANOVA indicated a reliable difference between groups, $F(7,108) = 6.619$, $p<0.0001$. SEFL was replicated in drug-free controls (Group 0—0), and metyrapone blocked this effect. In unstressed rats, CORT administration alone did not produce a SEFL-like response. *A priori* planned comparisons indicated that the freezing levels of the stressed/vehicle animals were significantly higher than that of unstressed/vehicle animals receiving CORT, $F(1, 108) = 11.891$, $p<0.001$; however, the latter group was not significantly different from unstressed controls, $F(1, 108) = 0.619$, $p>0.05$. Although CORT without stress did not generate SEFL, CORT did rescue SEFL from metyrapone in stressed rats. These animals froze significantly more than unstressed controls did, $F(1, 108) = 7.297$, $p<0.01$, and did not show any significant differences from stressed controls, $F(1, 108) = 0.254$, $p>0.05$.

While the CORT synthesis inhibitor metyrapone prevented SEFL, CORT administration alone did not mimic the effect of stress. However, SEFL was rescued from metyrapone by co-administration of CORT. Thus, changes in CORT are necessary but not sufficient for producing SEFL. This differs from the results from the 15-shock context test on Day 4, where co-administration of CORT and metyrapone did not rescue freezing, showing a further dissociation of associative and nonassociative fear. Having metyrapone on board during the 15-shock stressor may have disrupted consolidation of the memory of the 15 shocks, but perhaps having enhanced CORT on board during the stressor creates enough of a state change to enhance later nonassociative freezing. CORT seems to play a permissive role critical to SEFL-inducing changes elsewhere. Additionally, CORT's rescue of SEFL from metyrapone indicates that the drug's effect is likely caused by its ability to block CORT synthesis.

Experiment 3: Basolateral amygdala inactivation reduces stress-enhanced fear learning

Experiment 3 Method

Subjects. A total of 12 experimentally naïve adult male Long-Evans rats purchased from Harlan (Indianapolis, IN), weighing 250-300 g at the beginning of the experiment, were housed individually on a 14:10 hour light/dark cycle with free access to food and water. Housing and handling procedures followed those from Experiments 1 and 2.

Procedure. One week before surgery, rats were handled daily for 1-2 minutes. Rats were anesthetized (isoflurane: induction at 5%, maintenance 2.5%) before stereotaxic mounting (Kopf Instruments, Tujunga, CA), rat's were shaved (head), and were injected with ketoprofen (2 mg/kg, s.c.), and 0.9% sterile saline (approximately 0.3 mg/kg, s.c.). Body temperature was maintained during surgery using a water circulating heating pad. Before incision and retraction, scalps were cleaned with 70% ethyl alcohol and Betadine. Two holes were drilled into the skull for implantation of 26-gauge guide cannulae (Plastics One, Roanoke, VA, USA) aimed bilaterally at the BLA; coordinates (from bregma) were: anterior/posterior -3.1 mm, medial/lateral +/- 5.2 mm, dorsal/ventral -7.6 mm. Guide cannulae were secured with dental acrylic cemented to anchoring skull screws. "Dummy" cannulae were inserted into the guides to keep them clear, and they were replaced daily with clean ones. Recovery lasted 10-14 days; animals received daily injections of ketoprofen (2 mg/kg, i.p.) for two days and trimethoprim sulfa in their drinking water for five days post-surgery.

For habituation to infusion procedures, rats were transported to the infusion room and dummy cannulae were changed on the two days before experimental infusion. For infusions, 33-

gauge injector cannulae that extended 1 mm below the guides were inserted. Muscimol (Sigma-Aldrich, St. Louis, MO, USA) dissolved in artificial cerebrospinal fluid (ACSF; Fisher Scientific, Waltham, MA) to yield a solution of 1 mg/ml was back-loaded via 33-gauge infusion cannulae into polyethylene tubing connected to 10 ml Hamilton micro-syringes (Hamilton company, Reno, NV, USA), the infusion rate was 0.1 µl/minute to reach a volume of 0.25 µl/side, delivered via a Harvard #22 syringe pump (Harvard Apparatus, South Natick, MA, USA). The injector remained in place for 1-2 minutes allowing for complete diffusion; clean dummies were inserted into guide cannulae after infusions.

All animals underwent surgery to implant guide cannulae 10-14 days before the start of experiments. Animals were randomly assigned to one of three groups; pre-stressor muscimol animals received micro-infusions of muscimol 20 minutes *before* the 15 shocks, post-stressor muscimol animals underwent the 15 shocks first, receiving micro-infusions of muscimol 45-60 minutes *after* the stressor, or unstressed controls that received ACSF before being placed in the stress context for 90 minutes without shocks. The procedure was similar to Experiments 1 and 2, except that a shortened 3-day version was used. On Day 1, animals received 15 footshocks over 90 minutes. Twenty-four hours later, animals were placed into the novel context and received a single (1 mA, 1 sec) footshock. The test of SEFL occurred 24 hours later in the single shock context; freezing was assessed for 8 minutes. See **Figure 8a** for an outline of the procedure.

Behavioral Recordings: Freezing during the Day 3 context test was recorded using the VideoFreeze program.

Cannulae placement verification: Cannulae placements were confirmed after behavioral testing. Animals were deeply anesthetized, decapitated, brain tissue extracted, placed in 10%

buffered formalin phosphate (Fisher Scientific, Fair Lawn, NJ), later transferred to 30% sucrose, and subsequently rapidly frozen and sectioned at -20 °C. Sections (50 µm) were mounted and stained using cresyl violet (Sigma-Aldrich, St. Louis, MO). Sections were examined with a light microscope (Zeiss, Oberkochen, Germany) to verify cannulae placement.

Verification of correct bilateral cannulae placement are depicted on a schematic diagram in **Figure 8b**. After exclusion of animals with misplaced cannulae groups consisted of: pre-stressor muscimol (n=4), post-stressor muscimol (n=4), and unstressed controls (n=4).

Analysis. A one-way ANOVA was performed to determine between-group differences for freezing during the context test in the conditioning context. *A priori* planned comparisons were used to compare individual groups.

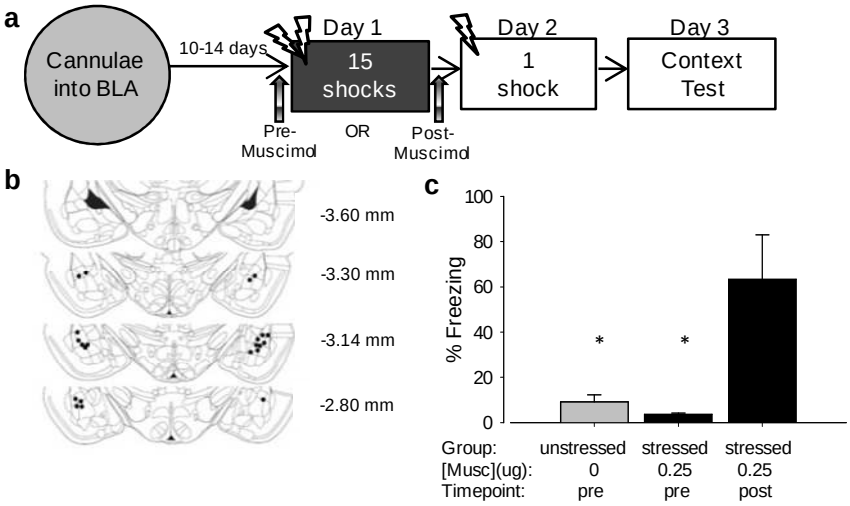

Figure 8. Basolateral amygdala inactivation prior to the stressor reduces SEFL a. Experimental Design. Shock in both the stress and conditioning context: 1mA, 1sec. **b.** Cannulae placement. **c.** Freezing (mean + SEM) in conditioning context on Day 3. *$p < 0.05$ (one-way ANOVA, followed by planned comparisons).

Experiment 3 Results and Discussion

In this experiment, the BLA was inactivated either prior to or after the 15-shock stressor, using a similar but shortened procedure as Experiment 1. **Figure 8b** depicts verification of correct bilateral cannulae placement on a schematic diagram. **Figure 8c** illustrates clear group differences on the context test in the conditioning context (mean $\pm$ SEM); the post-stressor muscimol group showed more fear than both the pre-stressor muscimol group and unstressed control group. This difference was confirmed statistically with a one-way ANOVA, $F(2, 9) = 8.17$, $p < 0.009$. *A priori* planned comparisons showed that post-stressor muscimol animals froze significantly more than did pre-stressor muscimol animals, $F(1, 9) = 13.5$ $p<0.003$; however, pre-stressor muscimol animals did not show significantly different freezing levels compared to unstressed controls, $F(1, 9) = 0.13$, $p=0.72$.

Inactivation of the BLA during the stressor reduced SEFL, while inactivation following the stressor did not. During the context test in the conditioning context, we found that animals that received pre-stressor muscimol infusions to inactivate the BLA showed an appropriate level of fear for the single context-shock pairing they experienced and have similar freezing levels to the unstressed controls. Conversely, post-stressor BLA inactivated animals displayed exaggerated fear manifested as high levels of freezing in the single shock environment—a response more appropriate to the stressor environment, and similar to SEFL observed in other studies (Rau, DeCola, and Fanselow, 2005; Rau and Fanselow, 2009). Therefore, the BLA must be functional during the stressor for SEFL to occur.

Experiment 4: Intra-basolateral amygdala glucocorticoid receptor antagonism attenuates stress-enhanced fear learning

Experiment 4 Method

Subjects. A total of 17 experimentally naïve adult male Long-Evans rats, purchased from Harlan (Indianapolis, IN), weighing 250-300 g at the beginning of the experiment, were housed individually on a 14:10-hour light/dark cycle with free access to food and water, following the same housing and handling procedures as Experiments 1-3.

Procedure. All animals underwent surgery to implant guide cannulae into the BLA 10-14 days before the start of experiments, using an identical procedure to Experiment 3. Mifepristone was dissolved in 80% ACSF ad 20% dimethyl sulfate (DMSO). Animals either received micro-infusions of 0.5 µg mifepristone or vehicle 10 minutes *before* the 15 shocks. A third group consisted of unstressed animals that received vehicle before the stressor. The animals then underwent the shortened 3-day SEFL procedure as in Experiment 3. Freezing in the conditioning context was recorded on Day 3. After exclusion of animals with misplaced cannulae groups consisted of: stressed/mifepristone (n = 5), stressed/vehicle (n = 7), and unstressed/vehicle (n=5). Cannulae placements were confirmed after behavioral testing, and sections were examined with a light microscope (Zeiss, Oberkochen, Germany) to verify cannulae placement. See **Figure 9a** for an outline of the procedure.

Behavioral Recordings. Freezing was recorded during the Day 3 context test using the VideoFreeze program.

Analysis. A one-way ANOVA was performed to determine between-group differences for freezing during the conditioning context test. *A priori* planned comparisons were used to compare freezing across individual groups.

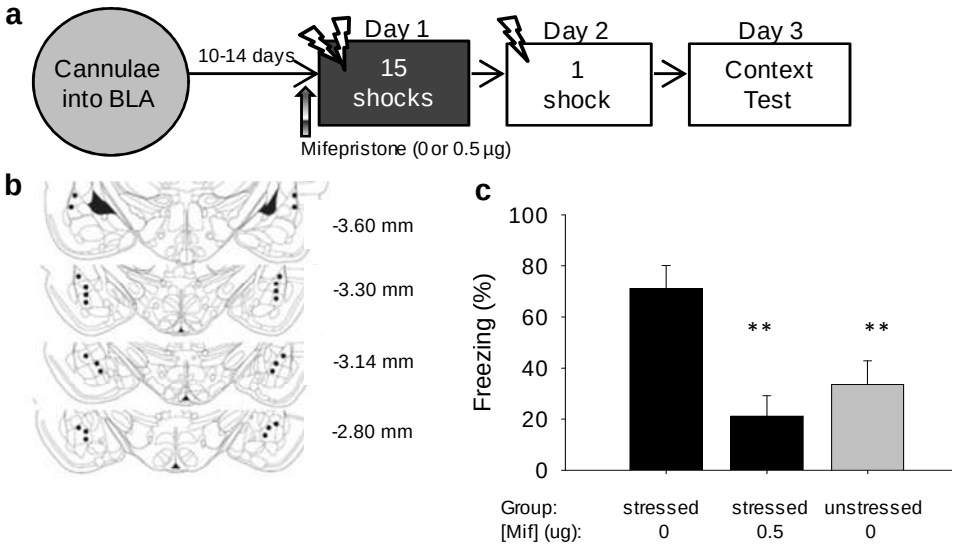

Figure 9. Intra-basolateral infusions of mifepristone pre-stressor reduce SEFL. **a.** Experimental Design for mifepristone infusions. Shock in both contexts: 1 mA, 1 sec. **b.** Cannulae placement. **c.** Freezing (mean + SEM) in conditioning context on Day 3. **p<.005 (one-way ANOVA).

Experiment 4 Results and Discussion

This experiment sought to neuroanatomically localize the effect of CORT by giving intra-BLA infusions of mifepristone. **Figure 9a** shows the experimental procedure, and **Figure 9b** shows verification of correct bilateral cannulae placement on a schematic diagram. **Figure 9c** shows freezing in the conditioning context test on Day 3. A one-way ANOVA confirmed statistically significant group differences in freezing, $F(2, 14) = 8.349$, $p<0.005$. *A priori* planned comparisons showed that stressed/vehicle animals froze significantly more than did stressed/mifepristone animals, $F(1, 14) = 16.42$, $p<0.001$; however, stressed/mifepristone animals did not show significantly different freezing levels from unstressed/vehicle controls, $F(1, 14) = 1.95$, $p=0.17$.

Blocking GR in the BLA prior to the 15-shock stressor attenuates SEFL. During the conditioning context test, stressed animals that received vehicle showed exaggerated freezing

levels; however, animals that received intra-BLA mifepristone infusions prior to the stressor showed attenuated fear compared to the stressed/vehicle group that is appropriate to the level of receiving a single shock (Day 3; **Figure 9c**). This suggests that the specific effects of CORT on BLA GRs are imperative for SEFL induction.

General Discussion

This set of experiments replicates previous findings that exposure to a 15-shock stressor in one context produces an enhancement of conditional fear to a second novel context in which a single footshock was given (Rau, DeCola, and Fanselow, 2005). Moreover, these experiments show that both blocking CORT synthesis with metyrapone and inactivating the BLA prior to stress prevented future sensitization of fear learning. Results from Experiment 1 suggest that CORT increases *during* the 15-shock stressor are necessary for the enhancement of conditional fear to the single shock. During drug-free tests in the conditioning context, freezing followed a graded dose response function, indicating that metyrapone blocked both associative fear learning and nonassociative enhancement of future fear learning. In other words, metyrapone dose-dependently blocked the ability for the shock to directly condition fear to a context paired with shock, and also prevented the long-term sensitization of future fear learning (i.e., SEFL).

While CORT synthesis is necessary for the induction of SEFL, CORT seems to play no role in the consolidation or expression of SEFL because post-stressor or pre-test administration of metyrapone had no effect on freezing and CORT levels at the time of test did not differ between stressed and unstressed rats (Experiments 1B and 1C). While metyrapone prevented SEFL, CORT administration without stress did not mimic the sensitizing effect of stress (Experiment 4). However, SEFL was rescued from metyrapone by co-administration of CORT.

Thus, changes in CORT during the stressor are necessary but not sufficient for producing SEFL. Moreover, CORT rescued SEFL but not fear conditioning from metyrapone (**Figure 7b-c**); animals that received metyrapone and CORT showed little fear to the original stress context, but fear was significantly increased in the same group in the conditioning context. Therefore, CORT plays a role in initiating SEFL, i.e., nonassociative fear, but not associative fear; another action of metyrapone may be affecting the latter. The results point out another dissociation between SEFL, which we see as a form of sensitization, and typical associative fear conditioning.

The plasma CORT levels after the 15-shock stressor (Experiment 1A, Day 1) also displayed a linear dose-dependent reduction. The increase in plasma CORT in vehicle/stressed animals after the 15-shock exposure is consistent with other studies showing that footshock stress causes a rise in CORT (Thompson, et al., 2004; Anderson, et al., 2004; Pitman, Ottenweller, and Natelson; 1990). However, plasma CORT levels from Day 7 yielded no significant interactions or main effects. This is consistent with other studies finding that blocking CORT synthesis during testing did not affect the expression of learned fear (Barrett and Gonzalez-Lima, 2004). CORT levels from this time point did not reflect SEFL, as indicated by high freezing rates. This CORT level finding is relevant to the controversy over CORT levels reported in PTSD patients. To induce SEFL, there must be CORT fluctuations during the 15-shock stressor that are blocked by metyrapone. However, the CORT levels at time points after the stressor are not critical for SEFL. The relevant parameter is CORT changes during stress and not at other time points. Measurement of CORT in PTSD patients is typically taken well-after, not immediately after, trauma.

Moreover, our findings from Experiment 3 indicate that functional activity in the BLA *during* the 15-shock stressor is necessary for later stress enhancements of fear learning to occur.

Inactivation of the BLA during the stressor reduced SEFL, while inactivation following the stressor did not. During the context test in the conditioning context, we found that animals that received pre-stressor muscimol infusions to inactivate the BLA showed an appropriate level of fear for the single context-shock pairing they experienced. Conversely, post-stressor BLA-inactivated animals displayed exaggerated fear learning, manifested as high levels of freezing in the single shock environment. The BLA is relevant because receives and integrates information about the environment from many different brain sites (Maren, 2001), and it acts as the locus of association between both context and tone CS's and the footshock US (Davis, 1992; Fanselow and LeDoux 1999; Maren, 2003; Maren and Fanselow, 1996). The BLA sends projections to the central nucleus of the amygdala (CeA), which in turn sends projections to areas that generate defensive responses, such as the periacqueductal gray (PAG; LeDoux, 1993; Fanselow, 1991; 1994). The ventral PAG is involved in mediating freezing behavior (De Oca, et al., 1998; Fanselow 1991). In addition to Pavlovian fear conditioning, the BLA, according to these results, is also crucial for fear sensitization.

It was previously suggested that exposure to a stressor causes a state change in the animal that produces a long-lasting sensitization of fear circuitry. The results of these experiments thus far suggest that stress hormones, namely CORT, mediate sensitization of this circuitry. Again, CORT levels were found to be high after the 15 shocks, but not after the context test in the 1-shock context, even though conditional freezing was enhanced. This suggests that the CORT increase after the stressor is the event that initiates the sensitization process in the brain. Perhaps sensitization is no longer reflected peripherally but centrally, via CORT interactions in the brain. CORT cell bodies and GRs are found in brain regions critical for mediating fear conditioning, such as the BLA (Chalmers, Lovenberg, and De Souza, 1995; Swanson, et al., 1983). Results

from Experiment 4 suggest that CORT's actions are on GRs in the BLA, which are necessary for SEFL induction to occur. As previously mentioned, increased CORT during stress increases amygdalar excitability, most likely via GR binding (Rosen and Schulkin, 1998).

The experiments in this chapter elucidate the critical components of induction of stress-induced enhancement of fear. More specifically, a severe stressor initiates the HPA axis stress response to increase CORT levels. CORT acts centrally in the BLA, which must also be functional during the stressor, by binding to GRs. CORT synthesis blocking by metyrapone administration after the stressor did not affect fear sensitization, and CORT levels were not increased at any time point after the 15-shock stressor. The next chapter will address the underlying mechanisms of expression of enhanced fear.

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CHAPTER TWO

Mechanisms underlying Expression of Stress-Enhanced Fear Learning

Abstract

Pavlovian fear conditioning has been used to effectively model symptoms of exaggerated fear learning and responding in rodents and in people with post-traumatic stress disorder (PTSD). When a person detects a threat, it is generally believed that environmental stimuli become aversely conditioned and hypervigilance for perceived sources of threat can develop. Therefore it is likely that processes related to associative learning and fear sensitization underlie anxiety disorders, like PTSD. There is a good understanding of the neural circuitry of Pavlovian fear conditioning, making it a useful tool for deriving the neural mechanisms of behavioral symptoms. During learning, sensory input relating to the conditional stimulus (CS) and unconditional stimulus (US) converge on the basolateral amygdala (BLA) where a CS-US association is encoded via long-term potentiation (LTP) at BLA synapses. This process is dependent on both N-methyl-D aspartate (NMDA) and alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors. These receptors within the amygdala participate in different components of fear learning, including acquisition, expression, and extinction. To explore for potential mechanisms of expression of stress-enhanced fear learning (SEFL), we looked for changes in glutamatergic receptor subunits in the BLA as an increase in excitatory neurotransmission in this structure could enhance fear conditioning. Western blot analysis of BLA tissue revealed an increase in the GluA1 AMPA receptor subunit but not the GluA2 AMPA receptor subunit or GluN1 NMDA receptor subunit. Moreover, NBQX, an AMPA receptor antagonist given at time points after the 15-shock stressor attenuates SEFL. Since facilitating glutamatergic activity at AMPA receptors enhances the rate of fear conditioning and SEFL occurs predominantly by increasing the rate of fear conditioning, the increase in GluA1 subunits is a highly plausible mechanism for the expression of SEFL.

Introduction

Fear is normally proportional to the level of imposed threat, which allows for a balance between defensive behavior and other behaviors necessary for survival (Fanselow, 1984). However, fear is no longer adaptive when its expression is unsuitable for the level of current threat. Following experience with a severe stressor, future fear learning becomes exaggerated and generalizes to other potential threats (Rau, DeCola, and Fanselow, 2005). We have developed a procedure to study this phenomenon and its neurobiological underpinnings, in which exposure to a 15-shock stressor enhances responding to a 1-shock contextual fear conditioning procedure.

Exposure to stress appears to sensitize the neural systems involved in generating fear responses. Stress initiates the activation of the neuroendocrine stress cascade, also called the hypothalamic-pituitary-adrenal (HPA) axis, which culminates in glucocorticoid release from the adrenal cortex (Antoni, 1986). Glucocorticoids (i.e., corticosterone in rodents [CORT], cortisol in humans) aid in energy mobilization and help to restore homeostasis via negative feedback mechanisms after a threat has passed (Munck, Guyre, and Holbrook, 1984).

Regions important to Pavlovian fear conditioning both affect and are affected by HPA axis activation (Korte, 2001; Herman, et al., 2005). The basolateral amygdala (BLA) is critical for fear learning and expression (Helmstetter, 1992; Sananes and Davis, 1992; Fanselow and Kim, 1994; Muller, et al., 1997; Gale, et al., 2004). Lesioning or inactivating the BLA demonstrates that it is a region necessary for both encoding and storage of a fear memory (Fanselow and Kim, 1994; Wilensky, Schafe, and LeDoux, 1999; Schafe and LeDoux, 2000; Fanselow and Gale, 2003; Kim, et al., 2005). The BLA is excited, either by a threatening unconditional stimulus (US) or fear-evoking conditional stimulus (CS), and it drives secretion of

CORT via the hypothalamus (Helmstetter, 1992; Sananes and Davis, 1992; Fanselow and Kim, 1994; Muller, et al., 1997; Gale, et al., 2004; Herman, et al., 2005). Systemic CORT crosses the blood brain barrier and thus feeds back on receptors in the hippocampus (Herman, et al., 2005) and BLA (Arriza, et al., 1988; Duvarci and Pare, 2007). It is important that the HPA axis be turned off rapidly following a stressor because the catabolic effects of CORT suppress homeostatic functions and can be damaging (Bethune, 1974). CORT, both stress-induced and artificial, enhances BLA excitation (Duvarci and Pare, 2007; Whitehead, et al., 2013), and feedback of CORT on the hippocampus and other sites in the HPA axis serve to halt further release of CORT (Herman, et al., 2005). The modulatory effects of CORT are largely exerted on fear learning and consolidation.

Prior research has shown that the BLA plays an important role in stress-induced fear responses (Braga, et al., 2004; Adamec, Blundell, and Burton, 2005; Rodriguez Manzanares, et al., 2005; Roozendaal, McEwen, and Chattarji, 2009). Receptors for CORT are found in key areas involved in fear conditioning, particularly the BLA (Chalmers, Lovenberg, and De Souza, 1995; Swanson, et al., 1983). While CORT action in the hippocampus is directed toward suppression of the HPA axis, in the BLA, CORT binds to glucocorticoid receptors (GRs), nuclear receptors that may alter gene transcription (McEwen and Weiss, 1970; McEwen, Weiss and Schwartz, 1968). Strikingly, long-lasting changes in gene expression have been observed in the BLA of animals showing SEFL (Ponomarev, et al., 2010).

Within the amygdala, glutamatergic modifications in excitatory neurotransmission, including glutamate receptor-regulated synaptic plasticity, have been implicated in fear conditioning. Glutamate N-methyl-D aspartate receptors (NMDAR) and alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPAR) within the amygdala are involved in

different components of fear learning (Kim and Fanselow, 1994; Miserendino, et al., 1990; Walker and Davis, 2002 Jasnow, Cooper, and Huhman, 2004; Kim, et al., 1993). In particular, it has been shown that blockade of NMDAR in the BLA prevents acquisition of fear learning (Fanselow and Kim, 1994), while intra-BLA infusions of an AMPAR antagonist blocks expression of fear (Kim, et al., 1993). Additionally, excitatory neurotransmission in the BLA could also enhance fear conditioning (Fanselow, DeCola, and Young, 1993). It has also been shown that altered activity patterns due to stress can change the distribution of AMPAR in the BLA, increasing the density of AMPAR on dendritic spines (Hubert, et al., 2013).

Chapter 1 focused on uncovering the mechanisms involved in initiating SEFL. In this chapter, to explore potential mechanisms of SEFL expression, we first probed for changes in glutamatergic receptor subunits in the BLA in Experiment 1. Rats received either metyrapone, a CORT synthesis blocker, or vehicle one hour before the 15-shock stressor. The findings from Experiment 1 in Chapter 1 show that pre-stress metyrapone dose-dependently attenuated SEFL. Two weeks after the test in the conditioning context, Western blot analysis of BLA samples was performed in order to assess the relative abundance of AMPAR and NMDAR subunits in the BLA after SEFL and metyrapone treatments, i.e., GluA1 and GluA2 subunits of the AMPAR and GluN1 subunit of the NMDAR. Given the importance of AMPARs for the expression of fear and NMDARs for the induction of SEFL, we hypothesized that AMPAR subunits, but not NMDAR subunits, would show an increase in concentration due to the stressor. This timepoint also corresponds to the period where we previously reported SEFL-induced changes in gene expression (Ponomarev, et al., 2010). Additionally, the two-week interval should eliminate short-term influences of behavioral testing.

Given the results from Experiment 1 and the importance of AMPARs in fear expression, in Experiment 2, AMPAR antagonists were infused into the BLA either immediately prior to the single shock in the conditioning context or prior to the conditioning context test. Because changes in AMPAR function within the BLA play a causal role in the expression of SEFL, we predicted that AMPAR blockade should temporarily prevent the expression of SEFL.

Experiment 1: Increases in GluA1 after stress-enhanced fear learning are prevented by metyrapone

Experiment 1 Method

Subjects. Experimentally naïve adult male Long Evans rats, approximately 300 g, were purchased from Harlan (Indianapolis, IN). Food and water were available *ad libitum* to the animals, and a 14:10-hour light-dark cycle, lights coming on at 6:00 am, was maintained in the colony room. The rats were individually housed in stainless steel wire mesh cages and were handled daily for approximately 45 seconds for five days prior to the start of the experiments. All experimental procedures took place during the light cycle.

The procedures used in this experiment were in accordance with policy set and approved by the Institutional Animal Care and Use Committee of the University of California, Los Angeles.

Apparatus. Two contexts were used during the course of the experiments, the “stress” context and the “conditioning” context. These contexts differed in background noise, illumination, and odor. Chambers housed within the contexts differed in shape, size, color, and grid floor texture. In the stress context, lighting consisted of two ceiling mounted 6-ft. fluorescent bulbs; a ventilation fan was used to provide background noise (65dB). Stress context chambers (28 x 21 x

21 cm) had a clear Plexiglas back wall, ceiling and front door and aluminum side walls. The floor, composed of 18 stainless steel rods (4mm in diameter), spaced 1.5 cm center to center, was wired to a shock generator and scrambler (Med Associates, Inc; St. Albans, VT). Chambers were wiped down with isopropyl alcohol (10%) and dried before and after each subject, and a Simple Green (50%) odor was placed in stainless steel pans inserted below the chamber to provide a distinctive smell in the context.

The conditioning context was lit by a single red 30W bulb, and no background noise was provided. Conditioning context chambers were initially the same size as stress context chambers, but were made smaller by black triangular inserts, creating side walls at a 60 degree angle with the floor. Floors were composed of 17 stainless steel rods (4 mm in diameter) staggered into two rows spaced 1 cm apart vertically and 2.6 cm apart horizontally, and they were wired to the shock generator as described for the stress context chambers. Conditioning context chambers were cleaned with acetic acid solution (1%) before and after each subject, and the same solution was placed in the pan underneath each chamber.

Procedure. Rats were randomly placed in one of four groups: “stressed control” animals (GluN1 n=7, GluA2 n= 10, GluA1 n=11), “unstressed control” animals (GluN1 n=8, GluA2 n= 10, GluA1 n=10), “stressed/metyrapone” (GluN1 n=11, GluA2 n=6, GluA1 n=8) or “unstressed/metyrapone” (GluN1 n=12, GluA2 n=6 GluA1 n=7). Rats from Experiment 1A, Chapter 1 were used in this experiment, from the group that received the highest dose of metyrapone (150 mg/kg). For the control groups, rats that both received vehicle and were untreated were included; for both the stressed and unstressed conditions, vehicle and untreated animals did not show significantly different optical density (OD) ratios for each of the subunits probed, ps>0.05. The vehicle for the drug was composed of 60% saline and 40% propylene

glycol (Sigma Aldrich; St. Louis, MO). One hour prior to 15-shock stressor exposure, animals were given an intraperitoneal (i.p.) injection of metyrapone. The volume of all injections was 1.0 ml/kg. Injections were given in the rats' housing area, and the rats remained in their homecages until the stressor exposure procedure.

One hour after injection, rats were transported to the stress context in their homecages. Stress exposure consisted of 15 shocks (1 mA, 1sec), with a variable shock interval of 240-480 seconds. Animals receiving no stress during exposure were placed in chambers for an equivalent amount of time as the stressed animals—90 minutes. Animals were then brought back to their homecages and returned to their housing area.

Animals were then given two days rest time in their homecages (Days 2 and 3). On Day 4, all animals were given a context test in the stress context for 8 minutes. They were transported in the same manner as on Day 1, in their home cages. On Day 5, animals were given an 8 minute session in the novel conditioning context, in order to provide pre-exposure and to ensure the animals were not generalizing to this context. They were transported out of their homecages and placed into a black container (Rubbermaid) that was partitioned with inserts (Plexiglas) into equivalent chambers (20 x 15 x 25 cm) to carry four animals. Lids were placed on the container so that the animals were transported in darkness to the conditioning context. On Day 6, all rats were given a single shock (1mA, 1 sec) in the conditioning context 180 seconds after placement in the chamber. The rats were transported in the same manner as Day 5, in the black partitioned container. Animals were removed from the chamber after an additional 300 seconds and brought back to their housing area and placed back in their homecages. On Day 7, animals were given an 8 minute context test in the 1-shock context. Animals were transported in the same manner as on Days 5 and 6, in the black container.

Two weeks after the Day 7 conditioning context test, control rats and rats that received 150 mg/kg metyrapone were euthanized and Western blots were used to probe for subunit concentrations of AMPAR and NMDAR. We chose this time point because SEFL is very long lasting (e.g., >90 days, Rau and Fanselow, 2009) and we were interested in determining the neural correlates of SEFL that reflected these long-term changes. This timepoint also corresponded to the period where we previously reported SEFL-induced changes in gene expression using microarray (Ponomarev, et al., 2010). See **Figure 10A** for a diagram of the procedure.

Western blot analysis. Rats were euthanized, 400 mm thick coronal brain slices were made from which the BLA was microdissected, and immediately frozen at -80 °C. Tissue was then thawed, and homogenized in ice cold buffer containing 1% SDS, 1 mM EDTA, and 10 mM Tris, pH 8.0. Protein concentrations, measured by D_C protein assay system (Bio-Rad, Hercules, CA). Samples (15 µg/lane) were loaded in 10 or 15 lane pre-cast 4 to 20% gradient SDS-polyacrylamide gels (Bio-Rad, Hercules, CA), and separated under reducing conditions using the Bio-Rad Mini–Protean 3 Cell system. Proteins were transferred to PVDF membranes (Immun-Blot PVDF membrane, 0.2 mm, Bio-Rad) by wet transfer (BioRad, Hercules, CA). Blots were probed with anti-peptide GluA1 (C-terminus epitope- AB1504; 1:1000 dilution; EMD Millipore, Temecula, CA), anti-peptide GluA2 (rabbit polyclonal, AB1768-I; 1:6000 dilution; Millipore, Temecula, CA), and GluN1 (aa 834-938; 1:1000 dilution; Upstate: EMD Millipore, Temecula, CA) antibodies, followed by HRP-conjugated secondary (Goat anti-rabbit; EMD Millipore, Temecula, CA) antibody (1:2000 dilution). Bands were detected by GE ECL prime or ECL2 Western blot detection kit (GE Healthcare Biosciences, Pittsburgh, PA) and images were captured using the LAS-3000 digital imaging system (Fujifilm, Tokyo, Japan) or developed onto

film (GE Healthcare, Biosciences). An anti-peptide glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotech, Santa Cruz, CA) antibody (1:2000 dilution) was used as a loading control. Bands corresponding to the appropriate subunit were analyzed, and optical density (OD) measurements were compared by densitometry using ImageJ (NIH, Bethesda, MD).

Analysis. Between-group differences in OD ratios were evaluated using two-way Analysis of Variance (ANOVA). *A priori* planned comparisons were made between stressed animals that underwent metyrapone treatment and stressed and unstressed controls (Gaito, 1965).

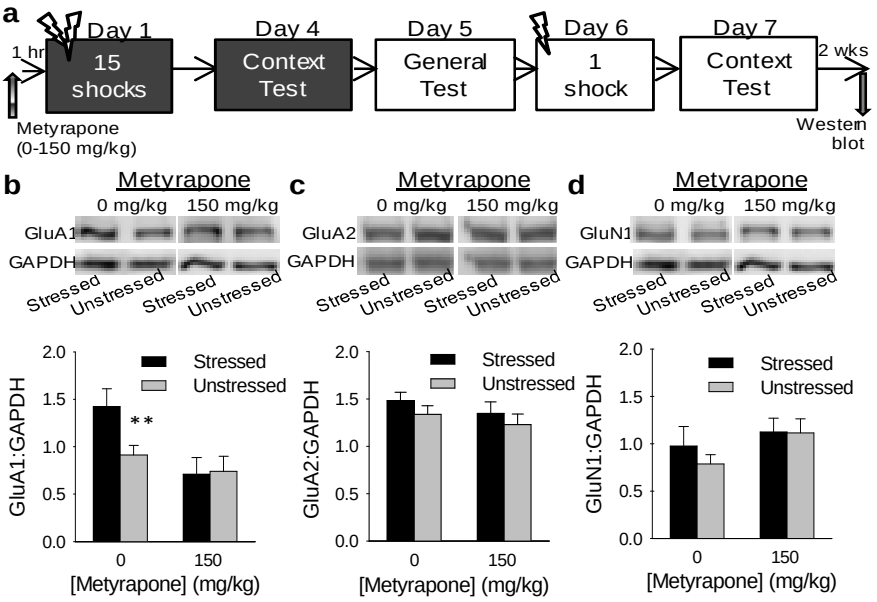

Figure 10. Increases in GluA1 after SEFL are prevented by metyrapone. a. Experimental Design. Shock for both contexts: 1mA, 1sec **b.** Representative Western blot images of GluA1 and GAPDH from the BLA of stressed and unstressed rats receiving vehicle or metyrapone. **GRAPH:** Mean GluA1: GAPDH optical density ratios (+ SEM). Main effect of drug, $p<0.005$, $^{**}p<0.01$ (two-way ANOVA, followed by *a priori* planned comparisons). **c.** Representative Western blot images of GluA2 and GAPDH from the BLA of stressed and unstressed rats receiving vehicle or metyrapone. **GRAPH:** Mean GluA2: GAPDH optical density ratios (+ SEM). **d.** Representative Western blot images of GluN1 and GAPDH from the BLA of stressed and unstressed rats that received vehicle or metyrapone. **GRAPH:** Mean GluN1: GAPDH optical density ratios (+SEM).

Experiment 1 Results and Discussion

In this experiment, Western blot analyses were performed on the BLA of animals receiving metyrapone prior to the 15-shock stressor, in order to assess the changes in abundance of AMPAR and NMDAR subunits after a severe stressor and metyrapone treatment. **Figure 10b** shows representative images of GluA1 and GAPDH from the BLA of stressed and unstressed rats that received pre-stress administration of either metyrapone or vehicle. The graph in **Figure 10b** shows the mean GluA1: GAPDH OD ratios (± SEM). There was a main effect of drug $F(1, 32) = 9.011$, $p<0.005$, but not of stress or stress by drug interaction. *A priori* planned comparisons indicated that the OD ratios of the stressed control animals were significantly higher than of unstressed control animals, $F(1, 32) = 5.03$, $p<0.05$. However, there was no significant difference between the OD ratios of stressed and unstressed animals that received metyrapone, $F(1, 32) = 0.0008$, $p>0.05$. Stressed control animals also showed a significantly increased OD ratio compared to stressed/metyrapone animals, $F(1, 32) = 8.03$, $p<0.01$, but there was no difference between OD ratios of stressed/metyrapone and unstressed controls, $F(1, 32) = 0.636$, $p>0.05$.

Figure 10c shows representative images of GluA2 and GAPDH from the BLA of stressed and unstressed rats that received pre-stress administration of either metyrapone or vehicle. The graph in **Figure 10c** shows the mean GluA2: GAPDH OD ratios (± SEM). There was no significant main effect of stress, drug, or a stress by drug interaction, $ps>0.05$.

Figure 10d shows representative images of GluN1 and GAPDH from the BLA of stressed and unstressed rats that received pre-stress administration of either metyrapone or vehicle. The graph in **Figure 10d** shows the mean GluN1: GAPDH OD ratios (± SEM). There was no significant main effect of stress, drug, or a stress by drug interaction, $ps>0.05$.

Western blot analysis of BLA tissue two weeks after SEFL revealed an increase in the GluA1 AMPAR subunit but not the GluA2 AMPAR subunit or GluN1 NMDAR subunit. Importantly, this is the same time point at which SEFL rats show pronounced changes in gene expression as indicated by microarray (Ponomarev, et al., 2010). Within the amygdala, NMDAR are important for acquisition of fear, while AMPAR are more critical for expression of fear (Fanselow and Kim, 1994; Kim, et al., 1993; Miserendino, et al., 1991; Walker and Davis, 2002; Hubert, et al., 2013). Therefore, two weeks after experience with the severe stressor, an increase in AMPAR subunits but not NMDAR subunits is consistent with such findings. Since facilitating glutamatergic activity at AMPAR enhances the rate of fear conditioning (Rogan, et al., 1997) and SEFL occurs predominantly by increasing the rate of fear conditioning (Fanselow, DeCola, and Young, 1993), the increase in GluA1 subunits is a highly plausible mechanism for the expression of SEFL.

Experiment 2: Intra-Basolateral Amygdala Infusions of NBQX at Two Time-points After the 15-Shock Stressor Attenuate Stress-Enhanced Fear Learning

Experiment 2 Method

Subjects. A total of 40 experimentally naïve adult male Long-Evans rats purchased from Harlan (Indianapolis, IN), weighing 250-300g at the beginning of the experiment, were housed individually on a 14:10-hour light/dark cycle with free access to food and water. Animals were housed and handled using the same procedure as in Experiment 1.

Procedure. One week before surgery rats were handled daily for 1-2 minutes. Rats were anesthetized (isoflurane: induction at 5%, maintenance 2.5%) before stereotaxic mounting (Kopf Instruments, Tujunga, CA), rat's were shaved (head), and were injected with ketoprofen (2

mg/kg, s.c.), and 0.9% sterile saline (aprox. 0.3 mg/kg, s.c.). Body temperature was maintained during surgery using a heating pad. Before incision and retraction, scalps were cleaned with 70% ethyl alcohol and Betadine. Two holes were drilled into the skull for implantation of 26-gauge guide cannulae (Plastics One, Roanoke, VA, USA) aimed bilaterally at the BLA; coordinates (from bregma) were: anterior/posterior -3.1 mm, medial/lateral +/- 5.2 mm, dorsal/ventral -7.6 mm. Guide cannulae were secured with dental acrylic cemented to anchoring skull screws. "Dummy" cannulae were inserted into the guides to prevent dust from entering, and they were replaced daily with clean ones. Recovery lasted 10-14 days; animals received daily injections of ketoprofen (2 mg/kg, i.p.) for two days and trimethoprim sulfa in their drinking water for five days post-surgery.

After 10-14 days of recovery from surgery, rats were transported to the infusion room and dummy cannulae were changed on the two days before experimental infusion to habituate them to infusion procedures. For infusions, 33-gauge injector cannulae that extended 1 mm below the guides were inserted. 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in artificial cerebrospinal fluid (ACSF; Fisher Scientific, Waltham, MA), and three different concentrations were made: 8.0 mg/ml, 4.0 mg/ml and 2.0 mg/ml. NBQX was back-loaded via 33-gauge infusion cannulae into polyethylene tubing connected to 10 ml Hamilton micro-syringes (Hamilton company, Reno, NV, USA). The infusion rate was 0.1 µl/minute to reach a volume of 0.25 µl/side, delivering either 2.0 µg, 1.0 µg, or 0.5 µg, respectively, via a Harvard #22 syringe pump (Harvard Apparatus, South Natick, MA, USA). The injector remained in place for 1-2 minutes allowing for complete diffusion; clean dummies were inserted into guide cannulae after infusions.

The procedure was similar to Experiment 1, except that a shortened 3-day version was used. On Day 1, animals received 15 footshocks over 90 minutes. Twenty-four hours later, animals were placed into the novel context and received a single (1 mA, 1 sec) footshock after 180 seconds. Animals were infused with vehicle, 0.5 µg/side or 1.0 µg/side immediately prior to the single shock in the novel context (Day 2). There were 4 groups total: stressed/vehicle (n=4), stressed/0.5 µg (n=6), stressed/1.0 µg (n=5), and unstressed/vehicle (n=8; "stressed" and "unstressed" refers to the 15-shock stressor). The test of SEFL occurred 24 hours later in the single shock context; freezing was assessed for 8 minutes.

In a separate group of animals, animals received the 15-shock stressor and 1 shock (Days 1 and 2) and were infused with either vehicle, 1.0 µg/side or 2.0 µg/side of NBQX immediately prior to the conditioning context test (Day 3). A fourth group consisting of unstressed animals receiving vehicle was included. All groups had an n=4. Freezing was assessed for 8 minutes during the Day 3 context test. A second context test in the conditioning context was given 24 hours later (Day 4) when NBQX was no longer on board, and freezing was, again, recorded. See **Figures 11a** and **11d** for outlines of the procedures.

Behavioral Recordings. Percent freezing was recorded during the Day 3 and Day 4 context tests using the VideoFreeze program. SEFL is indicated by high percent freezing, a reliable measure of learned fear (Fanselow, 1980; 1994) in the 1-shock conditioning context.

Computer Scoring by VideoFreeze Program. The VideoFreeze Program, which is comparable to hand scoring, was used to analyze behavioral data (Anagnostaras, et al., 2010). A motion analysis algorithm was used to analyze the video stream in real time, recording at 30 frames per second, 320 x 240 pixels, 8-bit grayscale. A reference video sample was taken prior to placing

the rats into the four chambers to calibrate the equipment. This reference sample established the amount of baseline noise in the video signal on a pixel-by-pixel basis, across multiple successive frames. Once the rats were placed in the chambers, successive video frames were continuously compared to each other and to the reference sample on a pixel by pixel basis. Any differences between pixels in the current video signal larger than those in the reference sample were interpreted as animal movement. These pixel differences were summed for each image frame, and this summation was counted as the Motion Index. The Motion Index is the number of pixels that have changed within 1 second that exceed video noise. When this Motion Index was below 50 for one second, an instance of freezing was scored.

Cannulae placement verification: Cannulae placements were confirmed after behavioral testing. Animals were deeply anesthetized, decapitated, brain tissue extracted, placed in 10% buffered formalin phosphate (Fisher Scientific, Fair Lawn, NJ), later transferred to 30% sucrose, and subsequently rapidly frozen and sectioned at -20 °C. Sections (50 µm) were mounted and stained using cresyl violet (Sigma-Aldrich, St. Louis, MO). Sections were examined with a light microscope (Zeiss, Oberkochen, Germany) to verify cannulae placement.

Analysis. A one-way ANOVA was performed for the first experiment to determine between-group differences for freezing during the context test in the conditioning context. *A priori* planned comparisons were made between stressed animals that were infused with vehicle and animals infused with NBQX. A mixed factorial ANOVA was performed for the second experiment, followed by planned comparisons.

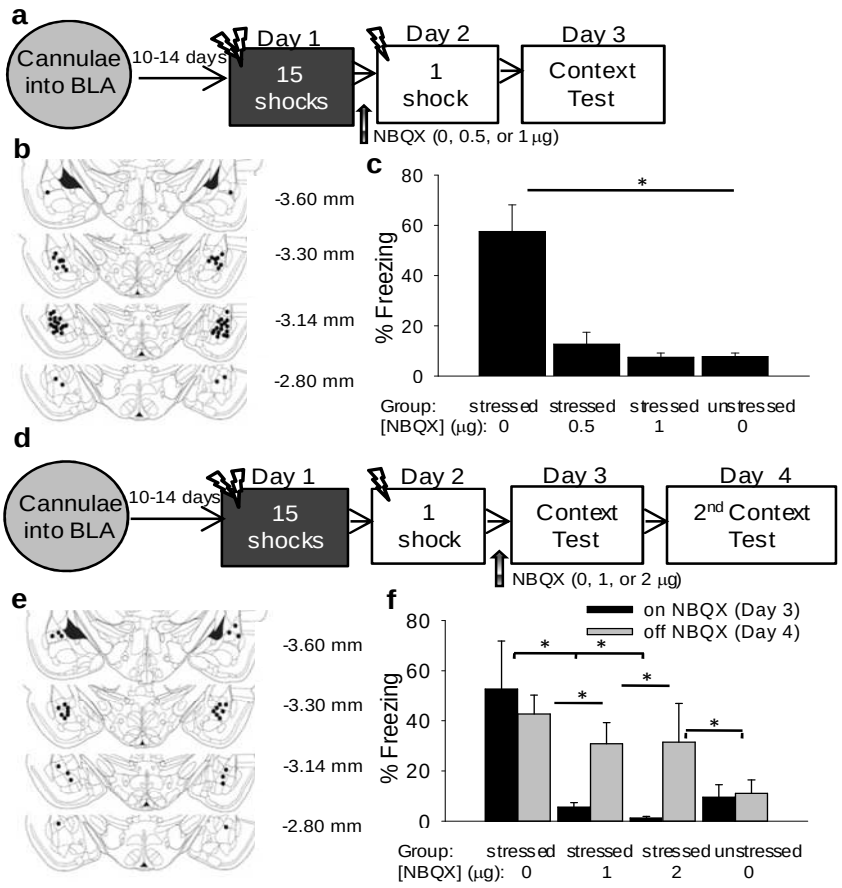

Figure 11. Intra-basolateral amygdala infusions of NBQX after the 15-shock stressor attenuate SEFL. a. Experimental Design for pre-1 shock infusions. Shock in both contexts: 1 mA, 1 sec. **b.** Cannulae placement **c.** Freezing (mean + SEM) in conditioning context test on Day 3, $p<0.0001$ (overall one-way ANOVA, followed by *a priori* planned comparisons). **d.** Experimental Design for pre-context test infusions. **e.** Cannulae placement. **f.** Freezing (mean + SEM) in both the on and off drug conditioning context tests on Days 3 and 4, respectively. $p < 0.005$. * $p<0.05$ (mixed ANOVA, followed by *a priori* planned comparisons).

Experiment 2 Results and Discussion

In this experiment, infusions of NBQX into the BLA were given either immediately prior to the single shock in the conditioning context or to the conditioning context test, using the shortened 3-day procedure. **Figures 11b** and **11e** depict verifications of correct bilateral cannulae placement on a schematic diagram. The graph in **Figure 11c** depicts mean (± SEM) freezing in conditioning context (Day 3) for animals that were infused with either vehicle or NBQX (0.5 µg or 1.0 µg/ side) immediately before the single shock (Day 2). Stressed animals that received both

doses of NBQX showed significantly lower freezing than vehicle-treated animals. This difference was confirmed statistically with a one-way ANOVA, $F(3, 19) = 25.799$, $p<0.0001$. *A priori* planned comparisons showed that stressed animals that received vehicle froze significantly more than animals that received both 0.5 µg and 1.0 µg doses of NBQX, $F(1, 19) = 44.26$, $p<0.0001$, and $F(1, 19) = 50.99$, $p<0.0001$, respectively.

Mean conditioning context freezing (± SEM) for animals that were infused with either vehicle or NBQX (1.0 µg or 2.0 µg/side) prior to the context test in the conditioning context is shown in **Figure 11f.** This graph depicts freezing in conditioning context while the drug was on board (Day 3), as well as 24 hours later when the drug was no longer present (Day 4). There was a significant effect of context test day, where animals froze significantly more during the context test off the drug than 24 hours prior while on the drug, $F(1, 12) = 5.13$, $p<0.05$. There was also a significant drug by test day interaction, $F(3, 12) = 3.40$, $p<0.05$. Planned comparisons showed that during the first context test on drug, vehicle-treated animals showed significantly more freezing than animals infused with either the 1.0 µg and 2.0 µg dose, as well as the unstressed vehicle group, Fs (1, 12)= 12.34, 14.53, and 10.32, respectively, $ps<0.05$. During the second context test off NBQX, stressed animals that received either dose of NBQX did not show a significant difference in freezing compared to vehicle-treated animals, Fs (1, 12) = 1.07, and 0.95, respectively, $ps>0.05$. Furthermore, animals treated with 1.0 µg NBQX had significantly higher freezing levels on Day 4 off drug than on Day 3 while on drug, $F(1, 12) = 5.78$, $p<0.03$; animals treated with 2.0 µg NBQX also had significantly more freezing on Day 4 than on Day 3, $F(1, 12) = 9.48$, $p<0.009$.

Stressed animals that received vehicle immediately before the single shock in the novel conditioning context (Day 2) showed significantly more freezing than NBQX-treated animals

during the drug-free context test. Moreover, stressed animals that received NBQX immediately before the context test (Day 3) also showed a reduction in SEFL compared to stressed/vehicle animals. However, when these rats were placed back in the conditioning context for a second test off NBQX the fear returned to the sensitized level (Day 4); therefore, this effect was temporary. This suggests that AMPARs in the BLA are necessary for SEFL expression, as targeting them with an AMPAR antagonist after the stressor temporarily attenuated SEFL.

General Discussion

These experiments elucidate the mechanisms that underlie expression of stress-induced enhancement of fear. Chapter 1 focused on the involvement of stress hormones in mediating the initiation of SEFL, and it also implicated the BLA as a critical structure for this observed fear sensitization. CORT may act centrally in the brain and pass through neuronal cell membranes to alter gene transcription (McEwen and Weiss, 1970; McEwen, Weiss and Schwartz, 1968). Because the co-occurrence of increased HPA axis activity and glutamate receptor changes within the BLA is observed after a stressor, their roles and interactions may be crucial in determining the mechanisms underlying SEFL, which is what the work of this chapter focused on.

The results from Experiment 1 interestingly show that besides preventing SEFL, metyrapone prevented the elevation in GluA1 in the BLA after SEFL and returned its expression levels to that of unstressed controls (**Figure 10b**). By contrast, there were no differences in GluA2 or GluN1 levels in stressed- or metyrapone-treated groups (**Figures 10c-d**). During learning, sensory input relating to the CS and US converge on the BLA where a CS-US association is encoded via long-term potentiation (LTP) at BLA synapses, dependent upon glutamate receptor-regulated synaptic plasticity (Rogan, et al., 1997; Kim and Jung, 2006).

NMDAR are important for acquisition of fear, while AMPAR, specifically GluA1-containing AMPAR are more critical for expression of fear via these LTP mechanisms (Fanselow and Kim, 1994; Kim, et al., 1993; Walker and Davis, 2002; Hubert, et al., 2013). Therefore, an increase in AMPAR subunits but not NMDAR subunits three weeks after the stressor is consistent with these findings. Similar results have been found in the hippocampus, where surface increases in GluA1 but not GluA2 occurred after restraint stress (Whitehead, et al., 2013). AMPAR lacking GluA2 have a high relative calcium permeability, whereas permeability of AMPAR containing GluA2 is very low; the former enhances cell excitability (Hollmann, et al., 1991). However, this long-term upregulation of GluA1 two weeks after the stressor is inconsistent with prior results showing that expression of calcium-permeable AMPARs peaks 24 hours after conditioning and subsides by 1 week. This shows a dissociation between SEFL, which we view as non-associative sensitization, and Pavlovian fear conditioning (Jarome et al., 2012).

If changes in AMPAR function within the BLA play a causal role in the expression of SEFL, then AMPAR blockade should prevent the expression of SEFL. Therefore, we applied the AMPAR antagonist NBQX to the BLA either immediately prior to the single shock in the conditioning context or prior to the conditioning context test (**Figures 11c and f**). Stressed animals that received vehicle immediately before the single shock in the novel conditioning context (Day 2) showed significantly higher freezing levels than that of NBQX-treated animals during the drug-free context test. Stressed animals that received NBQX immediately before the conditioning context test (Day 3) also showed an attenuation in SEFL, compared to animals that received the stressor and vehicle. When NBQX-treated rats were placed back in the conditioning context for a second test off NBQX, the fear returned to the sensitized level (Day 4). This

suggests that AMPARs in the BLA are necessary for SEFL expression, and targeting them with an AMPAR antagonist temporarily attenuates SEFL expression.

Increased availability of the GluA1 protein might lead to an overall increase in AMPARs or an increase in the proportion of calcium permeable AMPAR, either of which could lead to an increase in the LTP that supports fear learning (Fanselow and Kim, 1994; Miserendino, et al., 1990; Walker and Davis, 2002; Rogan, et al., 1997; Liu and Cull-Candy, 2000; He, et al., 2009). Induction and maintenance of LTP requires insertion of GluA1-containing AMPARs in the synapse (Kessels and Malinow, 2009; Hanley, 2010). However, NBQX is a general AMPAR antagonist. Therefore, to further validate the importance of GluA1 in SEFL, we examined the post-stress effects of antisense oligonucleotides (ASO) to block translation of GluA1 in the BLA in the final chapter.

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CHAPTER THREE

Neurobiological Targets to Produce Long-Lasting Reversal of Stress-Enhanced Fear Learning

Abstract

Inappropriate fear regulation after severe stress is a hallmark of post-traumatic stress disorder (PTSD). We developed a model called stress-enhanced fear learning (SEFL), in which an acute footshock stressor nonassociatively and permanently enhanced conditional fear learning in rats. SEFL is accompanied by several additional symptoms relevant to PTSD. However, the mechanisms of SEFL are poorly understood. In Chapters 1 and 2, we demonstrated that corticosterone (CORT) acting at glucocorticoid receptors (GRs) in the basolateral amygdala (BLA) is necessary to induce SEFL. Moreover, we showed that CORT drives long-term alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor subunit, GluA1 expression in the BLA. Targeting AMPA receptors with NBQX successfully attenuated SEFL, though this effect was temporary.

While Chapters 1 and 2 suggested potential *treatments* of SEFL, a true *cure* must permanently alleviate the condition without chronic administration. In our attempt to further validate the importance of GluA1 in SEFL, we first used antisense oligonucleotides (ASO) to block translation of GluA1 in the BLA. GluA1 ASO, which we validated with Western blot analysis, missense oligonucleotides (MSO), or vehicle was infused into the BLA after the 15-shock stressor and before the 1 shock. Surprisingly, one GluA1 ASO treatment showed a long-lasting reversal of SEFL. Importantly, ASO treatment did not eliminate the associative fear memory of the 15-shock context or the effects of a future stressor, indicating that ASO did not permanently disrupt normal amygdala function. Associative fear of the actual stress context may be beneficial as it would lead to evasion of a truly dangerous situation. If such associative fear to the stress context is contributing to negative symptomatology, then exposure treatment may still be necessary. We next explored the functional importance of GluA1 increases in the BLA after

SEFL by examining the post-stress effects of IEM-1460, a selective GluA2-lacking AMPA receptor blocker. Intra-BLA infusions of IEM-1460 post-stress reduced SEFL, showing that calcium permeable, GluA1- containing AMPA receptors are important for SEFL expression and can be targeted to reverse SEFL. These treatments, when put together with the treatment of the nonassociative effects of stress described here, suggest hope for those suffering from severe PTSD.

Introduction

At the center of the neural circuitry for fear learning is the amygdala (Helmstetter, 1992; Muller, et al., 1997; Gale, et al., 2004). Sensory and associative information projects directly and indirectly to the basolateral amygdala (BLA) complex. In Pavlovian fear conditioning, the BLA acts as a locus for the association between context conditional stimulus (CS) and footshock unconditional stimulus (US; Davis, 1992; Fanselow and LeDoux, 1999) The BLA sends projections to the central nucleus of the amygdala (CeA), which in turn projects to areas, such as the periaqueductal gray (PAG) that generate defense responses (Fanselow, 1991; 1994). The amygdalar fibers to the ventral PAG are involved in mediating freezing behavior (Maren and Fanselow, 1996; Fendt and Fanselow, 1999), while the amygdalar connections to the dorsal PAG mediate reactivity to the noxious stimulus (Fanselow, 1991).

Within the amygdala, changes in excitatory neurotransmission, including glutamate receptor-regulated synaptic plasticity, are crucial for fear conditioning. Glutamate N-methyl-D aspartate receptors (NMDAR) and alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPAR) within the BLA participate in different components of fear learning (Fanselow and Kim, 1994; Kim, et al., 1993; Walker and Davis, 2002; Hubert, et al., 2013). In particular, it has been shown that blockade of NMDAR in the BLA prevents acquisition of fear learning (Fanselow and Kim, 1994), whereas intra-BLA infusions of an AMPAR antagonist blocks expression of fear (Hubert, et al., 2013).

The BLA also plays an important role in stress-induced fear responses (Braga, et al., 2004; Adamec, Blundell, and Burton, 2005; Rodriguez Manzanares, et al., 2005; Roozendaal, McEwen, and Chattarji, 2009). Inappropriate fear responding in humans may manifest into anxiety disorders, such as post-traumatic stress disorder (PTSD). Indeed, human brain imaging

studies also show enhanced amygdala activity in PTSD patients during encoding and exposure to negative stimuli (Rauch, et al., 2000; Shin, et al., 2006; Brohawn, et al., 2010). In rodent studies, it has been shown that altered activity patterns due to stress can change the distribution of AMPAR in the BLA, increasing the density of AMPAR on dendritic spines; AMPAR moving from dendritic stores into spines may be in part responsible for the persistent behavioral alterations observed following severe stressors (Hubert, et al., 2013). Moreover, adrenal stress hormones (i.e., corticosterone [CORT]) increase BLA excitability; using electrophysiological techniques, it has been shown that both stress-induced CORT and administration of CORT concentrations appropriate for a stressor enhance the intrinsic excitability of principal BLA cells via glucocorticoid receptors (GRs; Duvarci and Pare, 2007; Whitehead, et al., 2013). Given that CORT, both stress-induced and administered, exaggerates responding to subsequent fear conditioning procedures (Cordero, Merino, and Sandi, 1998; Cordero, et al., 2003; Thompson, et al., 2004) and that CORT also increases BLA excitability, it is likely that these mechanisms work together to produce sensitization of fear observed after a severe stressor.

We have created a model of stress-enhanced fear learning (SEFL) in rodents in order to demonstrate that an acute stressor nonassociatively and permanently enhances conditional fear learning (Rau, DeCola, and Fanselow, 2005; Rau and Fanselow, 2009). In Chapter 1, we showed that increases in systemic CORT during the initial stressor are critical for sensitized fear, which can be blocked with metyrapone, a CORT synthesis blocker. It has been shown that systemic CORT crosses the blood brain barrier and takes on a central role by acting on receptors in the BLA (Duvarci and Pare, 2007). This was also reinforced in Experiment 4, Chapter 1; blocking GRs in the BLA with mifepristone blocked enhancement of fear after a severe stressor. In Chapter 2, we showed that three weeks after a severe stressor, there was a CORT-dependent

increase in the GluA1 subunit of the AMPAR in the BLA, but not of GluA2 or subunits of the NMDAR. This is consistent with prior studies showing the importance of AMPAR, particularly GluA2-lacking, calcium permeable AMPAR, in expression of fear via long-term potentiation (LTP), while NMDAR is more critical for acquisition of fear (Whitehead, et al., 2013; Fanselow and Kim, 1994; Kim, et al., 1993). Targeting AMPAR in the BLA after the stressor with the antagonist NBQX blunted the enhanced fear conditioning seen after a severe stressor.

However, metyrapone and mifepristone from Chapter 1 prevented SEFL only if given prior to stress, which severely limits its applicability as a potential treatment. Additionally, the effect of NBQX in reducing exaggerated fear to the conditioning context was temporary, and NBQX does not specifically target GluA1-containing AMPARs. While this is a successful *treatment* of SEFL, a true *cure* must permanently alleviate the condition without chronic administration.

In principal neurons throughout the brain, AMPARs containing GluA2 predominate (Liu and Zukin, 2007). However, fear learning and experience causes a switch in certain brain regions, like the amygdala, from GluA2-containing to GluA2-lacking, calcium permeable AMPARs to the synapse (Clem and Huganir, 2010; Jarome et al., 2012; Savtchouk and Liu, 2011; Whitehead, et al., 2013). Induction and maintenance of LTP requires insertion of GluA1-containing AMPARs in the synapse by both phosphorylation and endosomal recycling that appears to involve transmembrane AMPAR proteins that hold AMPARs at the synapse (Kessels and Malinow, 2009; Hanley, 2010). We believe that calcium-permeable AMPAR support nonassociative plasticity. The data presented in this chapter suggest that either preventing the upregulation of GluA1 after the stressor or perhaps blocking this synaptic AMPAR maintenance

at the synapse will permanently prevent SEFL from developing following a stressor, but still leaves the brain capable of learning normal, protective, fear.

In Experiment 1, we used antisense oligonucleotides (ASO) to block stress-induced translation of GluA1 in the BLA. GluA1 ASO, which we validated with Western blot analysis, missense oligonucleotides (MSO), or vehicle was infused into the BLA after the 15-shock stressor and before the 1 shock. Given the Western blot data from Experiment 1, Chapter 2, showing a sustained increase in GluA1 in the BLA after the stressor, as well as the NBQX data from Experiment 2, Chapter 2, we hypothesized that stressed animals receiving GluA1 ASO at these timepoints would show significantly lower freezing than vehicle-treated and MSO-infused animals.

In Experiment 2, we infused a cohort of animals with only one dose of GluA1 ASO into the BLA, either after the 15-shock stressor or before the 1-shock 24 hours later. Given the data showing that GluA1 after fear conditioning peaks after 24 hours (Jarome, et al., 2012), and the 15-shock stressor and 1-shock are 24 hours apart, we hypothesized that just one dose at either timepoint would be sufficient to reduce enhanced freezing to the 1-shock context. We tested this same cohort of animals in the 1-shock context ASO-free 7 days after the first context test. If the enhanced freezing is still reduced even though antisense lasts 10-12 hours (Kurreck, et al., 2007), then this means that just a single dose of ASO somehow reset the BLA to a less excitable state. Lastly, this same cohort of animals was given a second severe stressor ("re-SEFLed"). If animals show enhanced freezing to the context test in the 1-shock context, then this demonstrates that the amygdala was still functional and that fear conditioning was not impaired.

Lastly, in our attempt to further validate the functional role of GluA1 in SEFL, in Experiment 3, we examined the post-stress effects of *N*,*N*,*H*,-Trimethyl-5-[(tricyclo[3.3.1.13,7]dec-1-ylmethyl)amino]-1-pentanaminiumbromidehydrobromide (IEM-1460), a voltage-dependent open-channel blocker of AMPAR that displays selectivity between subtypes, blocking GluA2 subunit-lacking (calcium-permeable) receptors more potently than GluA2-containing receptors. IEM-1460 was infused into the BLA prior to the single shock in the novel context. Again, given the Western blot data from Experiment 1, Chapter 2 showing that GluA1 but not GluA2 was increased after SEFL, we expected to see a decrease in SEFL with drug infusion.

Experiment 1: Intra-BLA infusions of GluA1 antisense oligonucleotides reduce stress-enhanced fear learning

In this experiment we assessed the importance of GluA1 upregulation after a severe stressor by blocking GluA1 translation with ASO in the BLA. Using this method, we were able to specifically target GluA1-containing AMPARs that were upregulated as a result of the stressor.

Experiment 1 Method

Subjects. A total of 34 experimentally naïve adult male Long-Evans rats, purchased from Harlan (Indianapolis, IN), weighing 250-300 g at the beginning of the experiment, were used in this experiment. Food and water were available *ad* libitum to the animals, and a 14:10-hour light-dark cycle, lights coming on at 6:00am, was maintained in the colony room. The rats were individually housed in stainless steel wire mesh cages and were handled daily for approximately

45 seconds for five days prior to the start of the experiments. All experimental procedures took place during the light cycle.

The procedures used in this experiment were in accordance with policy set and approved by the Institutional Animal Care and Use Committee of the University of California, Los Angeles.

Apparatus. Two contexts were used during the course of the experiments, the "stress" context and the "conditioning" context. These contexts differed in background noise, illumination, and odor. Chambers housed within the contexts differed in shape, size, color, and texture. In the stress context, two ceiling mounted 6-ft. fluorescent bulbs illuminated the room and a ventilation fan was used to provide background noise (65dB). Stress context chambers (28 x 21 x 21 cm) had a clear Plexiglas back wall, ceiling and front door and aluminum side walls. The floor, composed of 18 stainless steel rods (4mm in diameter), spaced 1.5 cm center to center, was wired to a shock generator and scrambler (Med Associates, Inc; St. Albans, VT). Chambers were wiped down with isopropyl alcohol (10%) and dried before and after each subject, and a Simple Green (50%) odor was placed in stainless steel pans inserted below the chamber to provide a distinctive smell in the context.

The conditioning context was lit by a single red 30W bulb, and no background noise was provided. Conditioning context chambers were initially the same size as stress context chambers, but were made smaller by black triangular inserts, creating side walls at a 60 degree angle with the floor. Conditioning context chamber floors were composed of 17 stainless steel rods (4 mm in diameter) staggered into two rows spaced 1cm apart vertically and 2.6 cm apart horizontally, and they were wired to the shock generator as described for the stress context chambers. These

chambers were cleaned with acetic acid solution (1%) before and after each subject, and the same solution was placed in the pan underneath each chamber. The behavior was analyzed using the VideoFreeze program (Med Associates, Inc).

Computer Scoring by VideoFreeze Program. The VideoFreeze Program was used to analyze behavioral data (Anagostaras, et al., 2010). A motion analysis algorithm was used to analyze the video stream in real time, recording at 30 frames per second, 320 x 240 pixels, 8-bit grayscale. A reference video sample was taken prior to placing the rats into the four chambers to calibrate the equipment. This reference sample established the amount of baseline noise in the video signal on a pixel-by-pixel basis, across multiple successive frames. Once the rats were placed in the chambers, successive video frames were continuously compared to each other and to the reference sample on a pixel by pixel basis. Any differences between pixels in the current video signal larger than those in the reference sample were interpreted as animal movement. These pixel differences were summed for each image frame, and this summation was counted as the Motion Index. The Motion Index is the number of pixels that have changed within 1 second that exceed video noise. When this Motion Index was below 50 for one second, an instance of freezing is scored.

GluA1 antisense oligodeoxynucleotide design and treatment. Oligodeoxynucleotides (19 bases in length) were synthesized (Operon, Huntsville, AL) based on the GluA1 cDNA (GenBank accession number NM031608) listed in www.ncbi.nlm.nih.gov. The specific ASO (5’-T*A*A*GCATCACGTAAGG*A*T*C-3’; phosphorothiate-DNA chimera) was complementary to positions 1249–1268 of rat GluA1 cDNA. A MSO (“scrambled” antisense) used as a control, was also prepared in which the bases of the GluA1 oligonucleotide were randomized (5’-A*G*C*GTATCACAGTATA*G*A*C-3’; phosphorothiate- DNA chimera).

This control sequence revealed no other rodent sequence homology using the BLAST (basic local alignment search tool) search. This procedure is based on a previous *in vitro* study using the same sequences (Hefferan, et al., 2007). The ASO or MSO was resuspended in sterile ACSF to a concentration of 2 nmol/ml.

Procedure. One week before surgery, rats were handled daily for 1-2 minutes. Rats were anesthetized (isoflurane: induction at 5%, maintenance 2.5%) before stereotaxic mounting (Kopf Instruments, Tujunga, CA), their heads were shaved, and they were injected with ketoprofen (2 mg/kg, s.c.), and 0.9% sterile saline (aprox. 0.3 mg/kg, s.c.). Body temperature was maintained during surgery using a heating pad. Before incision and retraction, scalps were cleaned with 70% ethyl alcohol and Betadine. Two holes were drilled into the skull for implantation of 26-gauge guide cannulae (Plastics One, Roanoke, VA, USA) aimed bilaterally at the BLA; coordinates (from bregma) were: anterior/posterior -3.1 mm, medial/lateral +/- 5.2 mm, dorsal/ventral -7.6 mm. Guide cannulae were secured with dental acrylic cemented to anchoring skull screws. "Dummy" cannulae were inserted into the guides to keep cannulae clear. Recovery lasted 10-14 days; animals received daily injections of ketoprofen (2 mg/kg, i.p.) for two days and trimethoprim sulfa in their drinking water for five days post-surgery.

Rats were transported to the infusion room and dummy cannulae were changed on the two days before experimental infusion to habituate them to infusion procedures. Stress exposure consisted of 15 shocks (1 mA, 1sec), with a variable shock interval of 240-480 seconds. One hour later, rats were transported to the infusion room to receive the drug infusions. For infusions, 33-gauge injector cannulae that extended 1 mm below the guides were inserted. The infusion rate was 0.1 µl/minute to reach a volume of 0.25 µl/side, delivered via a Harvard #22 syringe pump (Harvard Apparatus, South Natick, MA, USA). The injector remained in place for 1-2 minutes

allowing for complete diffusion; clean dummies were placed back into the cannulae after infusions. Rats were randomly assigned to one of four groups: those that received 15 shocks over a 90-minute period in the stress context and got GluA1 ASO ("stressed/ASO", n=11), those that received 15 shocks and received MSO ("stressed/MSO", n=11), those that received 15 shocks and received ACSF ("stressed/ACSF", n=6), and those who remained in the chambers of this context for the same duration without receiving any shocks and received ACSF ("unstressed/ ACSF", n=6).

The next day, animals received a second infusion of ASO, MSO, or ACSF, identical to the ones received the day before. One hour later, they were then transported out of their homecages in a black container (Rubbermaid) that was partitioned with inserts (Plexiglas) into equivalent chambers (20 x 15 x 25 cm) to carry four animals. Lids were placed on the container so that the animals were transported in darkness to the conditioning context. All rats were given a single shock (1mA, 1 sec) in the conditioning context 180 seconds after placement in the chamber. Animals were removed from the chamber after an additional 60 seconds and brought back to their housing area and placed back in their homecages.

On Day 3, animals were given an 8 minute context test in the 1-shock conditioning context. Animals were transported in the same manner as on Day 2 in the black container. Two doses were given because the half-life of phosphorothioate ASO is between 10-12 hours, and GluA1 expression after stress peaks at 24 hours (Kurreck, et al., 2002; Jarome et al., 2012). For ASO and MSO groups, half of the animals were taken for cannulae placement and half were taken to validate the GluA1 ASO *in vivo* via Western blotting. See **Figure 13a** for a diagram of the procedure.

Behavioral Recordings. Percent freezing was recorded for Day 3 using the VideoFreeze program. SEFL is indicated by high percent freezing, a reliable measure of learned fear (Fanselow, 1980; 1994), in the conditioning context test after the 15-shock stressor.

Western blot analysis. Rats were euthanized, 400 mm thick coronal brain slices were made from which the BLA was microdissected, and immediately frozen at -80 °C. Tissue was then thawed, and homogenized in ice cold buffer containing 1% SDS, 1 mM EDTA, and 10 mM Tris, pH 8.0. Protein concentrations, measured by D_C protein assay system (Bio-Rad, Hercules, CA). Samples (15 µg/lane) were loaded in 10 or 15 lane pre-cast 4 to 20% gradient SDS-polyacrylamide gels (Bio-Rad, Hercules, CA), and separated under reducing conditions using the Bio-Rad Mini–Protean 3 Cell system. Proteins were transferred to PVDF membranes (Immun-Blot PVDF membrane, 0.2 mm, Bio-Rad) by wet transfer (BioRad, Hercules, CA). Blots were probed with anti-peptide GluA1 (C-terminus epitope- AB1504; 1:1000 dilution; EMD Millipore, Temecula, CA), followed by HRP-conjugated secondary (Goat anti-rabbit; EMD Millipore, Temecula, CA) antibody (1:2000 dilution), bands were detected by GE ECL prime or ECL2 Western blot detection kit (GE Healthcare Biosciences, Pittsburgh, PA) and images were developed onto film (GE Healthcare, Biosciences). An anti-peptide glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotech, Santa Cruz, CA) antibody (1:2000 dilution) was used as a loading control. Bands corresponding to the appropriate subunit were analyzed, and optical density (OD) measurements were compared by densitometry using ImageJ (NIH, Bethesda, MD).

Cannulae placement verification: Cannulae placements were confirmed after behavioral testing. Animals were deeply anesthetized, decapitated, brain tissue extracted, placed in 10% buffered formalin phosphate (Fisher Scientific, Fair Lawn, NJ), later transferred to 30% sucrose,

and subsequently rapidly frozen and sectioned at -20 °C. Sections (50 µm) were mounted and stained using cresyl violet (Sigma-Aldrich, St. Louis, MO). Sections were examined with a light microscope (Zeiss, Oberkochen, Germany) to verify cannulae placement.

Analysis. A one-way ANOVA was used to determine group differences for Western blot optical density (OD). An overall one-way ANOVA was performed to determine between-group differences for freezing during the context test in the conditioning context. *A priori* planned comparisons were made between stressed animals that were infused with vehicle or MSO and animals infused with ASO (Gaito, 1965).

Experiment 1 Results and Discussion

The experimental design is shown in **Figure 12a**, and verification of correct bilateral cannulae placement are depicted on the schematic diagram in **Figure 12b**. Representative Western Blot images of GluA1 and GAPDH from the BLA of both treatment groups: MSO and ASO, and mean GluA1: GAPDH optical density ratios ($\pm$ SEM) are shown in **Figure 12c-d.** There was a main effect of antisense treatment, $F(1, 9) = 16.61$, $p<0.005$, where the BLA of MSO-treated animals contained significantly more GluA1 than did the BLA of ASO-treated animals.

The conditioning context test for animals infused with GluA1 ASO, MSO, or ACSF at two time points between the 15-shock stressor and 1-shock conditioning are shown in **Figure 12e**. An overall one-way ANOVA confirmed statistically significant group differences, $F(3, 30) = 4.195$, $p<0.05$. *A priori* planned comparisons showed that stressed animals that received MSO froze significantly more than stressed animals that received ASO and unstressed animals, $F(1, 30) = 5.27$, $p<0.05$, and $F(1, 30) = 6.82$, $p<0.05$, respectively; however, this group did not freeze

more than stressed/ACSF animals, $p>0.05$. Stressed/ASO animals also did not show significant differences in freezing from unstressed/ACSF animals, $p>0.05$.

The results from Experiment 2 show that intra-BLA infusions of GluA1 antisense after the 15-shock stressor reversed SEFL. Stressed animals that received GluA1 ASO at either time point showed significantly lower freezing than ACSF-treated and MSO-infused animals, which was at a level appropriate for a single shock. This differed from the experiment with NBQX infusions (Experiment 2, Chapter 2). While NBQX also reduced freezing post-stressor, it did not discern between newly-made or established AMPARs, nor did it specifically bind to GluA1-containining AMPARs. Therefore, the reduction in freezing was completely diminished, far past the level of freezing elicited with just a single shock (25%). It is possible that GluA1 ASO reduced freezing to the single shock level because targeted translation of new GluA1, and therefore production of viable AMPARs, in the BLA caused by stress-induced release of CORT, but did not affect AMPARs already established before the stressor.

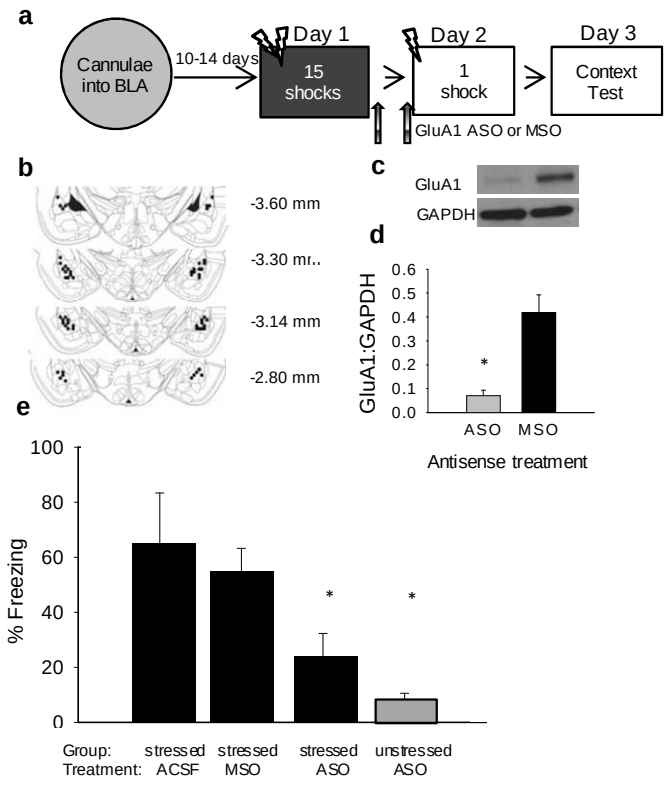

Figure 12. Intra-basolateral amygdala infusions of GluA1 antisense after the 15-shock stressor reverse SEFL. **a.** Experimental Design. **b.** Cannulae placement. **c.** Representative Western blot images of GluA1 and GAPDH from the BLA of stressed/MSO and stressed/ASO rats. **d.** Mean GluA1: GAPDH optical density ratios (+ SEM). *p<0.05 (one-way ANOVA). **e.** Mean (+SEM) percent freezing in the conditioning context on Day 3. * p<0.05 (one-way ANOVA followed by planned comparisons).

Experiment 2: A single infusion of GluA1 ASO into the BLA produces a long-lasting reversal of stress-enhanced fear learning and does not prevent future fear conditioning.

Given the effectiveness of ASO in reversing SEFL, we wanted to replicate the experiment with just a single dose and to determine how long-lasting the ASO effect was and how it may affect future fear conditioning.

Experiment 2 Method

Subjects. Six experimentally naïve adult male Long-Evans rats, purchased from Harlan (Indianapolis, IN), weighing 250-300g at the beginning of the experiment, were housed

individually on a 14:10 hour light/dark cycle with free access to food and water. Housing and handling were the same as in Experiment 1.

Procedure. All animals underwent surgery to implant guide cannulae into the BLA 10-14 days before the start of experiments. All behavioral, surgical, and infusion procedures are identical to those of Experiment 1. Animals either received BLA micro-infusions of 2 nmol/side of ASO either one hour after the 15 shocks or one hour before the 1-shock 24 hours later. The animals then underwent the same 3-day SEFL procedure as in Experiment 1, with an extra test day in the 1-shock context 10 days after the stressor. Freezing in the conditioning context was recorded on Day 3 during the 8 minute context test. The animals remained in their homecages ASO-free until Day 10, 7 days after the last context test. These animals were placed back in the 1-shock context test for a second 8 minute test. Then, the same animals were placed back in the stress context to receive another 15-shock stressor on Day 11, a single shock in the conditioning context on Day 12, and an 8 minute context test on Day 13.

After exclusion of animals with misplaced cannulae groups consisted of: Day 1 infusions (n = 3), Day 2 infusions (n = 3). See **Figure 13a** for the procedure.

Behavioral Recording. Freezing was recorded during the context tests on Day 3, Day 10, and Day 13 using the VideoFreeze program. Additionally, baseline freezing was recorded in the stress context on Day 1, and again on Day 11.

Analysis. A repeated-measures ANOVA was run to compare context test freezing of the one dose ASO animals on Day 3, Day 10, and Day 13. A one-way ANOVA was performed in order to compare context test freezing on Day 3 of animals that received one infusion of ASO to animals previously infused with two doses of ASO, as well as to compare freezing of re-

conditioned animals on Day 13 to MSO-treated animals. A paired-samples t-test was performed in order to compare baseline freezing in the stress context on Day 1 and on Day 11 before the 15 shocks.

Experiment 2 Results and Discussion

The experimental design for Experiment 2 is shown in **Figure 13a**, and verification of correct bilateral cannulae placement are depicted on a schematic diagram in **Figure 13b**. **Figure 13c** depicts the mean (±SEM) percent freezing in the conditioning context of animals that just received a single dose of ASO between the 15 shocks and single shock, on Days 3, 10, and 13. The two groups- those that received ASO after the 15 shocks and those that received ASO before the single shock- did not show any significant differences on Day 3 (Day 1: Mean= 29.13, SEM= 10.03; Day 2: 31.61, SEM= 12.89; $p>0.05$) and were therefore collapsed into a single group. The freezing levels of animals given just one dose of ASO on either Day 3 or Day 10 showed comparable freezing levels to animals treated with both doses, $F(1, 15) = 0.143$, $p>0.05$, and $F(1, 15)= 1.25$, $p>0.05$, respectively. A repeated-measures ANOVA confirmed significant group freezing differences $F(2, 10) = 10.52$, $p<0.01$. After animals were given the 3-day SEFL procedure again on Days 11-13, freezing levels in the context test (Day 13) were significantly higher than both Day 3 and Day 10 freezing, $F(1, 10)= 5.1$, $p<0.05$, and $F(1, 10)= 26.08$, $p<0.005$, respectively. This freezing level for Day 13 was comparable to animals that we have previously tested given MSO, $F(1, 15)= 0.39$, $p>0.05$.

The mean (± SEM) percent freezing in the stress context test on Day 1 (baseline) and on Day 11 prior to re-conditioning is depicted in **Figure 13d**. A paired-samples t-test confirmed that

these animals showed significantly higher baseline freezing levels on Day 11, the second time they were put into the stress context, than on Day 1, $t(5)= 3.22$, $p<0.05$

The results from Experiment 2 demonstrate that just a single dose of ASO between the 15 shocks and 1 shock was sufficient to reduce fear sensitization. Moreover, we found, unanticipatedly, that GluA1 ASO produced a *long-lasting* reversal of SEFL. While ASO lasts 12-24 hours, it may have interrupted the cycle of GluA1 upregulation that is normally long-lasting, and this was enough to also produce an enduring decrease in fear sensitization. Results also show that animals that received GluA1 ASO could still be fear conditioned and that ASO did not prevent a severe stressor from causing fear sensitization. Importantly, ASO treatment did not eliminate the associative fear memory of the 15-shock context or the effects of a future stressor, indicating that ASO did not permanently disrupt normal amygdala function. Associative fear of the actual stress context may be beneficial as it would lead to evasion of a truly dangerous situation.

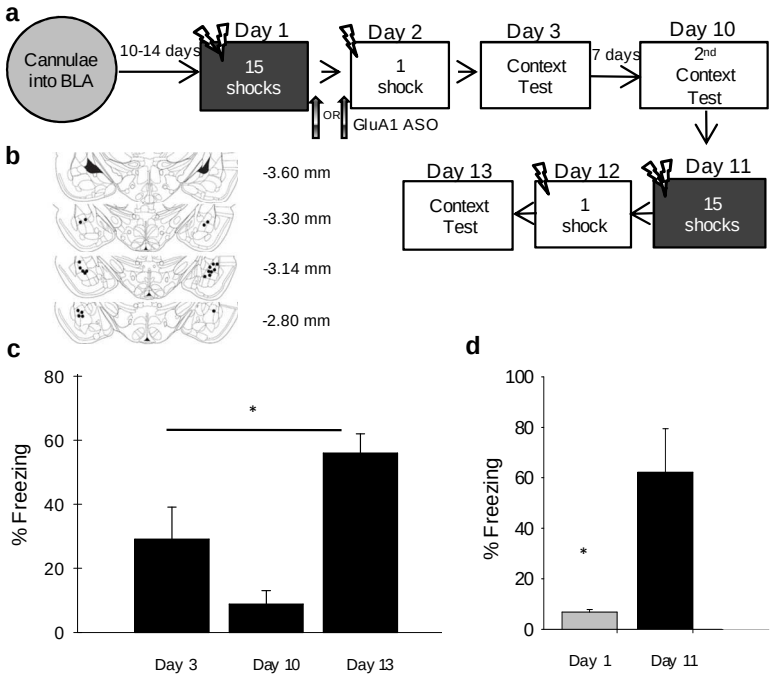

Figure 13. A single intra-basolateral amygdala infusion of GluA1 antisense produces a long-lasting reversal of sensitization. a. Experimental Design. **b.** Cannulae placement. **c.** Mean (± SEM) percent freezing of 1-dose ASO animals in the conditioning context on Days 3, 10, and 13. * $p<0.01$ (repeated-measures ANOVA). **d.** Mean (+ SEM) percent freezing of 1-dose ASO animals in the stress context on Day 1 (baseline) and on Day 11 prior to re-conditioning (the 3 minute before first shock). The high levels of freezing when the rats were returned to the stress context indicates that ASO treatment did not eliminate normal associative fear of the stress context.*$p<0.05$ (paired-samples t-test).

Experiment 3: Intra-amygdalar infusions of IEM-1460 post-stressor reverse stress-enhanced fear learning

This experiment sought to determine the functional role of the increase of GluA1, as well as the *lack* of increase of GluA2 in the BLA after SEFL by targeting calcium permeable AMPARs.

Experiment 1 Method

Subjects. A total of 15 experimentally naïve adult male Long Evans rats, approximately 300g, purchased from Harlan (Indianapolis, IN), were housed according to Experiments 1 and 2, on a 14:10 light cycle.i

Procedure. All animals underwent surgery to implant guide cannulae into the BLA 10-14 days before the start of the experiment. The surgical, behavioral, and infusion procedures were identical to those of Experiments 1 and 2. Stress exposure consisted of 15 shocks (1 mA, 1sec), with a variable shock interval of 240-480 seconds. Twenty-four hours later, rats were transported to the infusion room to receive the drug 10 minutes prior to the 1 shock, rats received their infusions. For infusions, 33-gauge injector cannulae that extended 1 mm below the guides were inserted. IEM-1460 (Tocris Bioscience, Ellisville, MO) was dissolved in artificial cerebrospinal fluid (ACSF; Fisher Scientific, Waltham, MA) and back-loaded via 33-gauge infusion cannulae into polyethylene tubing connected to 10 ml Hamilton micro-syringes (Hamilton company, Reno, NV, USA). The infusion rate was 0.1µl/minute to reach a volume of 0.25 µl/side,

delivered via a Harvard #22 syringe pump (Harvard Apparatus, South Natick, MA, USA). The injector remained in place for 1-2 minutes allowing for complete diffusion; clean dummies were placed back into the cannulae after infusions. Rats were randomly assigned to one of three groups: those that received 15 shocks over a 90-minute period in the stress context that got IEM-1460 ("stressed/IEM"), those that received 15 shocks and got ACSF ("stressed/ACSF"), and those that did not receive 15 shocks but just context exposure over 90 minutes and received vehicle ("unstressed/ACSF"). They were then transported out of their homecages in a black container (Rubbermaid) that was partitioned with inserts (Plexiglas) into equivalent chambers (20 x 15 x 25 cm) to carry four animals. Lids were placed on the container so that the animals were transported in darkness to the conditioning context. All rats were given a single shock (1mA, 1 sec) in the conditioning context 180 seconds after placement in the chamber. Animals were removed from the chamber after an additional 60 seconds and brought back to their housing area and placed back in their homecages. On Day 3, animals were given an 8 minute context test in the 1-shock conditioning context. Animals were transported in the same manner as on Day 2 in the black container. See **Figure 14a** for a diagram of the procedure.

Behavioral Recordings. Percent freezing was recorded for Day 3 using the VideoFreeze program. SEFL is indicated by high percent freezing, a reliable measure of learned fear (Fanselow, 1980; 1994), in the conditioning context test after the 15-shock stressor

Cannulae placement verification: Cannulae placements were confirmed after behavioral testing. Animals were deeply anesthetized, decapitated, brain tissue extracted, placed in 10% buffered formalin phosphate (Fisher Scientific, Fair Lawn, NJ), later transferred to 30% sucrose, and subsequently rapidly frozen and sectioned at -20 °C. Sections (50 µm) were mounted and

stained using cresyl violet (Sigma-Aldrich, St. Louis, MO). Sections were examined with a light microscope (Zeiss, Oberkochen, Germany) to verify cannulae placement.

Analysis. A one-way ANOVA was performed to determine between-group differences for freezing during the context test in the conditioning context, followed by *a priori* planned comparisons.

Experiment 3 Results and Discussion

The experimental design is shown in **Figure 14a,** and verification of correct bilateral cannulae placement are depicted on a schematic diagram in **Figure 14b**.

The conditioning context test for animals infused with IEM-1460 are shown in **Figure 14c**. A one-way ANOVA confirmed statistically significant group freezing differences, $F(2, 12) = 9.25$, $p<0.004$. *A priori* planned comparisons showed that the stressed/vehicle animals froze significantly more than did stressed/IEM animals and did unstressed controls $F(1, 12) = 12.95$, $p<0.002$ and $F(1, 12) = 7.12$, $p<0.05$, respectively. However, stressed/IEM animals did not show statistically significant freezing levels compared to unstressed/ vehicle controls, $F(1, 12) = 0.04$, $p=0.83$.

The results from Experiment 3 show that intra-BLA infusions of IEM-1460 prior to the single shock in the conditioning context test reduced SEFL. Stressed animals that received vehicle showed significantly higher freezing levels than did IEM-1460-treated animals. While NBQX (Experiment 2, Chapter 2) also reduced freezing post-stressor, it does not bind to specific types of AMPARs. The use of IEM-1460 specifically targets GluA2-lacking, calcium permeable AMPARs, and the results from this experiment reinforce the hypothesis that GluA1-containing AMPARs are essential for the expression of SEFL. The results also allow us to speculate over

the role of GluA2-containing AMPARs in SEFL. Given the large reduction in freezing caused by IEM-1460, GluA2-containing AMPARs may not be essential for expression of sensitized fear. This may be why increases in GluA2 were not detected after the severe stressor in the BLA, which would reinforce the Western blot data from Experiment 1, Chapter 2. This is consistent with prior studies showing that there are synaptic increases in GluA1 but not GluA2 after stress (Savtchouk and Liu, 2011; Whitehead, et al., 2013).

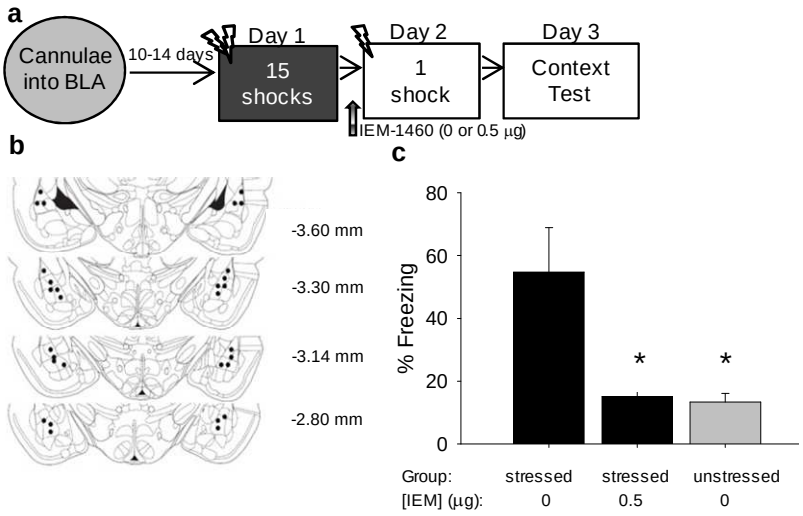

Figure 14: Intra-basolateral amygdala infusions of IEM-1460 after the 15-shock stressor attenuate SEFL. a. Experimental Design. Shock in both contexts: 1 mA, 1 sec. **b.** Cannulae placement verification. **c.** Freezing (mean + SEM) during the conditioning context test on Days 3. $p < 0.005$. * $p<0.05$ (one-way ANOVA, followed by *a priori* planned comparisons)

General Discussion

This set of experiments targeted the CORT-induced increase in GluA1 translation in the BLA after SEFL, as determined by Experiment 1, Chapter 2, using both GluA1 ASO and calcium permeable AMPAR blockers. The purpose of these experiments in Chapter 3 was to investigate the mechanisms that reverse SEFL as potential therapeutic targets, based on mechanisms of induction and expression outlined in Chapters 1 and 2. While several drugs were

shown to alleviate SEFL in the first two chapters, they would require chronic administration to keep SEFL attenuated, and their affects were not necessarily specific to nonassociative fear but associative fear to the stressor context as well (e.g., metyrapone).

We examined the post-stress effects of ASO to block translation of GluA1 in the BLA (and hence, SEFL) in Experiment 1. Stressed animals that received ACSF or MSO infusions showed significantly higher freezing levels than did GluA1 ASO at either time point. This level of freezing in ASO-treated animals is comparable to that seen after administration of just a single shock. These findings potentially implicate that GluA1 ASO reduced freezing to the 1-shock level because it targeted translation of new GluA1 (and therefore production of viable AMPARs) in the BLA caused by stress-induced release of CORT, but did not affect AMPARs already established before the stressor. This further validates the idea that fear conditioning alters GluA1 trafficking in the BLA; previous data show that blocking GluA1- containing AMPAR incorporation into the synapse blocks fear conditioning (Rumpel, et al., 2005). In Experiment 2, we determined that the decrease in sensitized freezing seen after GluA1 ASO administration lasted at least 10 days, well after the last infusion of ASO had been metabolized in the rats' neuronal systems, and did not prevent fear conditioning. Importantly, we also showed that ASO treatment did not eliminate the associative fear memory of the 15-shock context or the effects of a future stressor, indicating that ASO did not permanently disrupt normal amygdala function (Experiment 2; **Figure 14d**).

As shown in Chapters 1 and 2, brain regions implicated in fear circuitry, such as the BLA, both affect and are affected by hypothalamic-pituitary-adrenal (HPA) axis activation (Korte, 2001; Herman, et al., 2005). When the BLA is excited, either by a threatening unconditional stimulus (US) or fear-evoking conditional stimulus (CS), it drives secretion of

CORT via the hypothalamus (Herman, et al., 2005). Systemic CORT crosses the blood brain barrier and thus feeds back on GR receptors in the hippocampus (Herman, et al., 2005) and BLA (Arriza, et al., 1988; Duvarci and Pare, 2007). CORT enhances BLA excitation (Duvarci and Pare, 2007; Whitehead, et al., 2013) possibly via upregulation of GluA1-containing AMPARs, an idea that was explored in this chapter.

The functional importance of increased GluA1 in the BLA after SEFL was determined using IEM-1460, a drug that blocks GluA2-lacking AMPARs, which was infused into the BLA prior to the single shock in the novel context (Experiment 3). Stressed animals that received vehicle showed significantly higher freezing than did animals that received the drug. This suggests that GluA1-containing, but not GluA2- containing AMPARs, are critical for the expression of SEFL. In principal neurons throughout the brain, AMPARs containing GluA2 predominate (Liu and Zukin, 2007). However, fear learning and experience causes a switch in certain brain regions, like the amygdala, from GluA2-containing to GluA2-lacking, calcium permeable AMPARs to the synapse (Clem and Huganir, 2010; Jarome et al., 2012; Savtchouk and Liu, 2011; Whitehead, et al., 2013). Our data supports is consistent with these previous studies.

Induction and maintenance of LTP requires insertion of GluA1-containing AMPARs in the synapse by both phosphorylation and endosomal recycling. This requires transmembrane AMPAR proteins (TARPs) that maintain the levels of AMPARs at the synapse (Kessels and Malinow, 2009; Hanley, 2010). Prior data show that stress-induced glucocorticoids increase GluA1-containing AMPAR at the synapse via protein kinase A (PKA) phosphorylation mechanisms (Whitehead, et al., 2013). Relevant to these previous studies, the data from this chapter suggest that either preventing the upregulation of CORT-dependent GluA1 after the 15-

shock stressor or perhaps blocking the synaptic AMPAR maintenance permanently prevented SEFL from developing but still left the brain capable of learning normal, protective, fear. Associative fear toward the actual stress context is important to help the animal evade a dangerous situation. Therefore, blocking stress-induced translation of GluA1 suggests a potential route to explore for an effective, novel treatment, and perhaps eventually a cure, for PTSD.

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DISCUSSION

Fear is an adaptive response that is normally proportional to the level of imposed threat, which allows for a balance between defensive behavior and other behaviors necessary for survival (Fanselow, 1984). However, following experience with a severe stressor, future fear learning becomes disproportionate to the actual threat and is generalized to other potential threats (Rau, DeCola, and Fanselow, 2005). Inappropriate fear responses in humans can manifest in the form of anxiety disorders. Post-traumatic stress disorder (PTSD) is one such anxiety disorder.

PTSD develops in some individuals who experience a traumatic event. Individuals diagnosed with PTSD experience significant functional impairment, including increased risk for unemployment, disrupted relationships, and diminished physical health, which, in addition to the extensive suffering, creates a huge economic impact. Symptoms include 1) intrusive recollection, in which the traumatic event is persistently re-experienced as intrusive recollections or re-living of the event, including perceptions, hallucinations, intense psychological distress after exposure to even mild cues related to the traumatic event; 2) avoidance/numbing, as demonstrated by blunted emotional reactivity; and 3) hyper-arousal symptoms, as exhibited by sleep difficulties, irritability, impaired concentration, hyper-vigilance, and exaggerated startle response (American Psychological Association, 2013). Under conditions of threat, these symptoms are adaptive for coping with dangerous situations, but in the absence of a real threat, it can produce pathological conditions, including PTSD (Bonne, et al., 2004; Charney, et al., 2004; Christopher, 2004; Eberly, Harkness, and Engdahl, 1991). Avoiding stimuli associated with the threat, for instance,

lessens the probability of future encounters. However, patients with PTSD lose normal daily functioning under objectively safe conditions and display exaggerated behavioral responses.

One aspect PTSD is excessive behavioral responding to cues that may be associated with the trauma and are otherwise neutral or mild (Bremner, et al., 1995; Dykman, Ackerman and Newton, 1997). This excessive behavioral response likely represents a dysregulation of the fear response, whereby the level of fear is not appropriate to the level of threat. Our laboratory developed a model called stress-enhanced fear learning (SEFL) in order to study the dysregulation of fear at the level of neuronal substrates (Rau, DeCola, and Fanselow, 2005). As described throughout this dissertation, in the SEFL model, rats receive a series of 15 shocks that are randomly distributed over 90 minutes, and this traumatic experience produces behavioral changes that last at least 90 days unabated (Rau and Fanselow, 2009). After this experience, rats display highly exaggerated contextual and cued fear conditioning behavior in novel situations when exposed to a mild shock. This increase in fear conditioning reflects a permanent sensitization of fear circuitry, as it does not depend on an explicit memory of the stressful situation, does not reflect generalization of fear from the stress situation, nor does extinction of the traumatic stress context affect conditioning (Rau, DeCola, and Fanselow, 2005; Rau and Fanselow, 2009; Long and Fanselow, 2012).

From a clinical standpoint, nonassociative sensitization of fear is particularly problematic as it is not bound by the trauma context or stimuli specifically associated with it. Rats exhibiting SEFL also show decreased exploratory behavior in open fields (**Figure 1**), increased consumption of alcohol (Meyer, et al., 2013), potentiated startle reactivity (**Figure 2**), and a depression-like phenotype in the forced swim test (**Figure 3**). Moreover, SEFL causes an anxiety-like profile on the elevated plus maze, a long-lasting dysregulation of the diurnal cycle

for corticosterone (CORT), and an increase of glucocorticoid receptors (GR) in the basolateral amygdala (BLA; Poulos, et al., 2013). These sets of findings indicate that SEFL behavior captures several of the symptoms of PTSD (**Table 1).** The experiments in this dissertation were focused on understanding the neuronal mechanisms underlying SEFL. Most importantly, we were able to produce a long-lasting reversal of SEFL, suggesting possible strategies for treatment development for targeting the enhanced fear response in PTSD. Specifically, the experiments in this dissertation were designed to investigate three main aspects of SEFL, 1) Induction, 2) Expression, and 3) Reversal.

INDUCTION: Corticosterone action upon the amygdala during stress is necessary for the development of SEFL

Many of the long-term consequences of stress are mediated by the hypothalamic-pituitary-adrenal (HPA) axis activation and the ensuing release of CORT. Therefore, in Chapter 1, to determine the role of CORT on SEFL, one of four doses of metyrapone, a CORT synthesis blocker, was administered one hour prior to the 15-shock stressor in the SEFL procedure. The critical behavioral data in Chapter 1 (**Figure 4e**) displays percent freezing during the context test in the conditioning context on Day 7; all animals received one footshock in this novel context 24 hours prior. The 15-shock stress enhanced later fear conditioning to this context when paired with a single shock (i.e., SEFL occurred), and, importantly, metyrapone dose-dependently blocked this stress-induced enhancement of fear learning. A similar trend emerged when we tested rats for associative fear of the 15-shock stress context test (Day 4, **Figure 4d**). Similar to freezing behavior, pre-stress administration of metyrapone dose-dependently blunted the stress-induced rise in plasma CORT levels after the 15-shock stressor on Day 1, as expected (**Figure 4f**). However, CORT levels during testing (Day 7) showed no reliable group differences (**Figure**

4g). Moreover, metyrapone delivered at various time points after the 15-shock stressor did not decrease freezing in the conditioning context (**Figures 5-6**). Taken together, these results demonstrate that CORT levels at the time of stress, but not after, are critical for SEFL. Measurement of CORT in PTSD patients is typically taken well-after, not immediately after, trauma, one reason for differences in results observed in measurement of CORT levels in PTSD patients (Maes, et al., 1998; Yehuda, et al., 1990).

In order to validate that metyrapone's effect was mediated by its action on CORT, we determined if CORT administration rescued the fear response. Additionally, we determined if CORT administration in the absence of shock was sufficient to produce SEFL. Either 0 or 150mg/kg metyrapone, followed by either 0 or 10mg/kg CORT, was administered pre-stressor. In unstressed rats, CORT administration alone did not produce a SEFL-like response on Day 7; therefore, CORT administration without stress did not mimic the sensitizing effect of stress (**Figure 7c**). Although CORT without stress did not induce SEFL, CORT did rescue SEFL from metyrapone administration in stressed rats. Thus, changes in CORT during the stressor are necessary but not sufficient for producing SEFL. Moreover, CORT rescued SEFL but not fear conditioning from metyrapone (**Figure 7b**); animals that received a co-administration of metyrapone and CORT showed little fear to the original stress context on Day 4, but fear was significantly increased in the same group in the conditioning context. Therefore, CORT plays a role in initiating SEFL, i.e., nonassociative fear, but not associative fear; another action of metyrapone may be to affect the latter. These results further dissociate SEFL, which we see as a form of sensitization, from a typical associative fear conditioning.

In addition to neuroendocrine changes via the HPA axis under stress, there are also changes in a brain region crucial for fear conditioning and emotional learning in animals, the

amygdala (Helmstetter, 1992; Sananes and Davis, 1992; Fanselow and Kim, 1994; Muller, et al., 1997; Gale, et al., 2004). Lesioning or inactivating a sub-region of the amygdala—the BLA—demonstrates that this region is necessary for encoding and storing associative fear memory (Fanselow and Kim, 1994; Wilensky, Schafe, and LeDoux, 1999; Schafe and LeDoux, 2000; Fanselow and Gale, 2003; Kim, et al., 2005). Furthermore, the BLA plays an important role in stress-induced fear responses (Adamec, Blundell, and Burton, 2005; Rodriguez Manzanares, et al., 2005; Roozendaal, McEwen, and Chattarji, 2009). Animals with SEFL show long-lasting changes in gene expression in the BLA (Ponomarev, et al., 2010). Therefore, in order to determine the role of BLA in SEFL, we inactivated the BLA using the γ-aminobutyric acid type A receptor ($GABA_AR$) agonist muscimol either prior to or after the 15-shock stressor. The pre-stressor muscimol group showed less freezing to the conditioning context than the post-stressor muscimol group (Day 3; **Figure 8c**). This indicates that functional activity in the BLA *during* the 15-shock stressor is necessary for later stress enhancements of fear learning to occur.

In the final study exploring the induction of SEFL, we sought to neuroanatomically localize the effect of CORT. Infusions of mifepristone, a GR antagonist, into the BLA were made immediately prior to the 15-shock stressor. Stressed animals that received intra-BLA mifepristone infusions prior to the stressor showed significantly lower freezing than vehicle-treated animals (Day 3; **Figure 9c**). This suggests that the specific effects of CORT on BLA GRs are imperative for SEFL induction. Taken together, the studies conducted in this chapter aimed to understand the roles of brain substrates involved in induction of SEFL, and we showed that CORT action within the BLA is necessary for SEFL induction.

EXPRESSION: Increased GluA1 in amygdala is necessary for the expression of SEFL

Glutamatergic modifications in the excitatory neurotransmission within the amygdala have also been implicated in fear conditioning. In particular, glutamate N-methyl-D-aspartate receptors (NMDAR) and alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPAR) within the amygdala participate in different components of fear learning, including acquisition, expression, and extinction (Fanselow and Kim, 1994; Walker and Davis, 2002; Jasnow, et al., 2004). Increased excitatory neurotransmission in the BLA could enhance fear conditioning (Rogan, Staubli, and LeDoux, 1997). Therefore, to explore potential mechanisms involved in SEFL expression, we investigated changes in glutamatergic receptor subunits in the BLA after SEFL in Chapter 2. Rats received 150 mg/kg metyrapone one hour before the 15-shock stressor; vehicle-and untreated rats served as controls. Two weeks after the context test in the conditioning context, Western blot analysis was performed in order to assess the relative abundance of AMPAR and NMDAR subunits in the BLA after SEFL and metyrapone treatments. The amount of GluA1 in the BLA of stressed control animals was significantly higher than in the unstressed control animals. Interestingly, besides preventing SEFL, metyrapone prevented the elevation in GluA1 and returned its expression levels to that of unstressed controls (**Figure 10b**). There were no differences in GluA2 or GluN1 levels in stressed- or metyrapone-treated groups (**Figure 10c-d**). Since facilitating glutamatergic activity at AMPAR enhances the rate of fear conditioning (Rogan, Staubli, and LeDoux, 1997) and SEFL occurs predominantly by increasing the rate of fear conditioning (Fanselow, DeCola, and Young, 1993), the long-term increase in GluA1 subunits is a highly plausible mechanism for the expression of SEFL.

Because of the increase in GluA1 seen after the 15-shock stressor, we sought to block AMPARs in the BLA in order to prevent SEFL. Infusions of NBQX into the BLA were given

either immediately prior to the single shock in the conditioning context or prior to the conditioning context test. Stressed animals that received NBQX immediately before the 1 shock in the conditioning context (Day 2) showed significantly lower freezing than vehicle-treated animals during the context test (**Figure 11c).** Stressed animals that received NBQX immediately before the context test (Day 3) showed significantly lower freezing when NBQX was present. During the second context test, when NBQX was no longer present, stressed animals that had received NBQX the day before showed rescued, enhanced freezing (Day 4, **Figure 11f**). Overall, these findings suggest that AMPARs in the BLA are necessary for SEFL expression, and targeting them with an AMPAR antagonist temporarily attenuated SEFL expression. However, the return of enhanced fear in these rats indicates that the AMPAR antagonist infusion did not permanently alter amygdala function. Therefore, although an AMPAR antagonist can be used to prevent SEFL short-term, it cannot be used as a long-term treatment strategy.

REVERSAL: Infusion of GluA1 antisense oligonucleotides in the basolateral amygdala produces a long-lasting reversal of SEFL

Metyrapone prevented SEFL only when given prior to stress, which severely limits it applicability as a potential treatment. The effect of NBQX in reducing exaggerated fear to the conditioning context was temporary, and furthermore, NBQX does not specifically target GluA1-containing AMPARs. While NBQX may be used as a successful treatment of SEFL, it does not permanently alleviate the condition without chronic administration. In our attempt to further validate the importance of GluA1 in SEFL, in Chapter 3 we blocked translation of GluA1 in the BLA using antisense oligonucleotides (ASO). GluA1 ASO, which we validated with Western blot analysis (**Figure 12c-d)**, missense oligonucleotides (MSO), or vehicle was infused twice into the BLA after the 15-shock stressor and before the 1 shock. Stressed animals that

received GluA1 ASO showed significantly lower freezing than vehicle- and MSO-infused animals, which was at a level appropriate for 1 shock. Moreover, we found, unanticipatedly, that just a single infusion of GluA1 ASO between the stressor and single shock produced a *long-lasting* reversal of SEFL (**Figure 13c**). Induction and maintenance of long-term potentiation (LTP) requires insertion of GluA1-containing AMPARs in the synapse. While the process is induced by stress hormones (i.e., glucocorticoids), maintenance of AMPARs at the synapse involve both protein kinase A (PKA)-mediated phosphorylation and endosomal recycling (Whitehead, et al., 2013; Kessels and Malinow, 2009; Hanley, 2010). Our data suggest that either preventing the upregulation of GluA1 after the 15-shock stressor or perhaps blocking this synaptic AMPAR maintenance prevents SEFL from developing following a stressor, but still leaves the brain capable of learning normal, protective, fear. ASO treatment did not eliminate the associative fear memory of the 15-shock context or the effects of a future stressor, indicating that ASO did not permanently disrupt normal amygdala function (**Figure 13d**). Associative fear toward the actual stress context is critical for survival, as it would leave the reaction of evading a truly dangerous situation intact. If such associative fear to the trauma context is contributing to negative symptomatology, however, then exposure treatment may still be necessary. Fortunately, behavioral neuroscience is also leading to developments that enhance exposure therapy (Monfils, et al., 2009; Davis, et al., 2006; Zelikowsky, et al., 2014). Hence, the findings of these experiments, suggest a potential for developing novel treatments for PTSD.

We lastly sought to determine the functional role of the increase in the BLA after SEFL, as well as the *lack* of increase of GluA2. Therefore, we examined post-stress effects of IEM-1460, a GluA2-lacking, calcium permeable AMPAR blocker. This drug, when infused into the BLA after the 15-shock stressor, significantly reduced SEFL (**Figure 12c**). This demonstrates

that specifically GluA1-containing AMPAR are important for SEFL. This data is consistent with other studies showing the importance of GluA1-, but not GluA2-containing AMPARs in the synapse for fear expression (Clem and Huganir, 2010; Jarome et al., 2012; Savtchouk and Liu, 2011; Whitehead, et al., 2013).

In conclusion, the findings of this dissertation have outlined a clear set of mechanisms that underlie induction and expression of fear sensitization caused by an acute traumatic stressor; that is, stress-induced CORT in the BLA, mediated through GRs, increases GluA1-containing AMPAR in the BLA long-term. Moreover, the brain circuitry and neurotransmitter systems that mediate Pavlovian fear conditioning also mediate SEFL. These studies are the first of its kind to show that stress-induced GluA1-containing AMPAR increases in the BLA not only are important for fear expression but for expression of SEFL. These GluA1 increases may be targeted using ASO to produce an enduring reversal of this enhanced fear responding, which suggests hope for those suffering from severe PTSD.

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