

Induction and Expression of Fear Sensitization Caused by Acute Traumatic Stress

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Fear promotes adaptive responses to threats. However, when the level of fear is not proportional to the level of threat, maladaptive fear-related behaviors characteristic of anxiety disorders result. Post-traumatic stress disorder develops in response to a traumatic event, and patients often show sensitized reactions to mild stressors associated with the trauma. Stress-enhanced fear learning (SEFL) is a rodent model of this sensitized responding, in which exposure to a 15-shock stressor nonassociatively enhances subsequent fear conditioning training with only a single trial. We examined the role of corticosterone (CORT) in SEFL. Administration of the CORT synthesis blocker metyrapone prior to the stressor, but not at time points after, attenuated SEFL. Moreover, CORT co-administered with metyrapone rescued SEFL. However, CORT alone without the stressor was not sufficient to produce SEFL. In these same animals, we then looked for correlates of SEFL in terms of changes in excitatory receptor expression. Western blot analysis of the basolateral amygdala (BLA) revealed an increase in the GluA1 AMPA receptor subunit that correlated with SEFL. Thus, CORT is permissive to trauma-induced changes in BLA function.

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INTRODUCTION

A single emotionally traumatic event can have a lifelong impact on the psychological well-being of an organism, including triggering anxiety-related disorders like post-traumatic stress disorder (PTSD). PTSD leads to hyperactivity to mild stressors, especially when it bears similarity to the traumatic event, and to a propensity to form new fears. PTSD patients often exhibit co-morbid depression and high rates of alcohol and drug abuse (Brady *et al*, 2000). Exposure therapy, the major mode of treatment for anxiety disorders, is relatively ineffective in treating PTSD (Hembree *et al*, 2004). Given the high prevalence of PTSD and its toll on society, it is essential to understand the mechanisms by which stress induces changes in brain function, as well as the mechanisms that perpetuate long-term expression of maladaptive behavior.

We have developed an animal model of acute stress that parallels many symptoms of PTSD (Rau *et al*, 2005; Rau and

Fanselow, 2009; Long and Fanselow, 2012). In this model, rats receive a series of 15 shocks, randomly distributed over 90 min. This experience produces behavioral changes lasting at least 90 days unabated (Rau and Fanselow, 2009). After this experience, rats acquire exaggerated contextual and cued fear in novel situations using a single shock. This reflects a permanent sensitization of fear-conditioning circuitry because it does not depend on an explicit memory of the stressful situation, does not reflect fear generalization from the stress situation, and is not affected by extinction of the stress context (Rau *et al*, 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. Therefore, we have labeled this phenomenon stress-enhanced fear learning (SEFL).

The primary goals of this review are to first describe PTSD and how it is a form of aberrant fear learning, as well as to illustrate SEFL as a robust animal model of PTSD in terms of phenotypic, biochemical, and neuroanatomical similarities. Moreover, we report the necessary conditions for the induction and expression of SEFL because such knowledge should suggest potential treatment targets for PTSD. We first describe the necessity of corticosterone (CORT) to, in part, cause SEFL during a severe stressor. Using the CORT

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synthesis blocker metyrapone, we determined that CORT changes during stress are necessary for SEFL induction. Moreover, we show that SEFL expression relies on long-term CORT-dependent changes in receptors mediating excitatory neurotransmission in the basolateral amygdala (BLA), a region crucial for fear learning and memory. Lastly, we discuss future directions of PTSD research that will be necessary in order to develop appropriate treatments and potential cures.

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, compromising function and fitness (Fanselow and Lester, 1988). Inappropriate fear responses in humans can result in the development of anxiety disorders (Rosen and Schulkin, 1998), including 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 responses (American Psychiatric Association, 2013). In threatening situations, these symptoms are adaptive for coping with the causes of trauma (Eberly *et al*, 1991; Bonne *et al*, 2004; Charney, 2004; Christopher, 2004). For instance, avoiding stimuli associated with the traumatic event lessens the probability of encountering the threat or others like it. 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 by occurring in situations that do not demand such intense reactions. 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 *et al*, 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 (Friedman, 1994; Bremner *et al*, 1995; Dykman *et al*, 1997). 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 *et al*, 2014). Therefore, PTSD poses itself as a serious mental illness, and there is a need for developing novel and effective treatments for this disorder.

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 (Johnson *et al*, 1992; Carrasco and Van de

Kar, 2003). Stress activates both the sympathetic nervous system and 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 (ie, increased blood pressure and heart rate), preparing the immune system, enhancing cognitive functioning, and inhibiting behaviors that are currently unnecessary for survival (Johnson *et al*, 1992; Sapolsky, 2000; Christopher, 2004).

Activation of the HPA axis is coordinated by stress-induced activation of corticotropin-releasing hormone in the paraventricular nucleus of the hypothalamus, which causes the release of adrenocorticotrophic hormone from the anterior pituitary gland. Adrenocorticotrophic hormone then travels through the bloodstream and initiates glucocorticoid release from the adrenal cortex (Vale *et al*, 1981; Antoni, 1986; Owens and Nemeroff, 1991). Glucocorticoids (ie, 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 *et al*, 1984; Sapolsky *et al*, 2000). Glucocorticoids are lipophilic and are actively transported into the brain, where they bind to receptors or cross-neuronal cell membranes (McEwen and Weiss, 1970; De Kloet *et al*, 1998). They bind to either mineralocorticoid or glucocorticoid receptors (GRs), 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 injections of CORT was shown to have consistent results in studies where animals were exposed to stress before fear conditioning. Both chronic and acute administration of CORT enhances fear conditioning in rats (Cordero *et al*, 2003; Thompson *et al*, 2004). Animals that were given CORT injections for 5 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 via 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 (Rosen and Schulkin, 1998; Hagemen *et al*, 2001). Moreover, re-experiencing aspects of the traumatic event sensitizes fear systems leading to exaggerated reactions, as observed in PTSD patients (Post *et al*, 1997; Hagemen *et al*, 2001). This may occur via sensitization of amygdalar nuclei (discussed in the next section), a process that is mediated by stress hormones (Rosen and Schulkin, 1998). 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 conditional stimulus (CS) and unconditional stimulus (US) converge on the BLA complex, a subregion of the amygdala, where a CS-US association is encoded via long-term potentiation at BLA synapses (Rogan *et al*, 1997; Kim and Jung, 2006). This plasticity is dependent upon excitatory N-methyl-D aspartate receptors (NMDAR) (Miserendino *et al*, 1990; Fanselow and Kim, 1994) and is modulated through inhibitory γ -aminobutyric acid (GABA)-ergic neurons (Ehrlich *et al*, 2009; Makkar *et al*, 2010). Under normal conditions, the BLA, consisting of lateral and basal nuclei, is critical for fear learning (Maren, 1998; Fanselow and LeDoux, 1999; 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 for 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 (Rauch *et al*, 2000; Shin *et al*, 2006; Brohawn *et al*, 2010).

The BLA projects to central amygdala both directly and indirectly, via a link through the 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 central amygdala, which sends GABAergic projections to the medial subdivision of the central amygdala (Haubensak *et al*, 2010). The fear response (ie, freezing) is controlled by projections from medial subdivision of the central amygdala to the periaqueductal grey (Fanselow, 1991). Besides the central amygdala, the BLA projects to the bed nucleus of the stria terminalis, which in turn projects to the periaqueductal grey for fear responding (Walker *et al*, 2003; Waddell *et al*, 2006).

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 cortex projects to the BLA to enhance fear responding, while the infralimbic cortex indirectly projects to the medial subdivision of the central amygdala via intercalated cells to promote extinction of fear (Quirk *et al*, 2003). Moreover, prelimbic and infralimbic cortices receive amygdala projections originating mainly from the basal amygdala. Infralimbic and prelimbic cortices have opposing roles in the 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 BLA, either directly or indirectly through the prelimbic cortex, mediate contextual control of fear and fear renewal after extinction (Orsini *et al*, 2011).

BIOCHEMICAL SUBSTRATES 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 (AMPA) within the amygdala participate in different components of fear learning, including acquisition, expression, and extinction (Kim *et al*, 1993; Fanselow and Kim, 1994; Walker and Davis, 2002; Jasnow *et al*, 2004). In particular, it has been shown that blockade of NMDAR in the BLA prevented acquisition of fear learning (Miserendino *et al*, 1990; Fanselow and Kim, 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 these sections 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; Fanselow, 1998). 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 *et al*, 2005). In the 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 (Poulos *et al*, 2015). 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 *et al*, 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 for establishing its relevance to PTSD. The most obvious associative interpretation of SEFL is generalization between the stress and test contexts. However, there are multiple converging lines of evidence that SEFL is not mediated by generalization between the two contexts. As mentioned earlier, there is no evidence of fear in the conditioning context prior to the single shock. Additionally, eliminating fear of the stress context does not impact SEFL. For example, imposing extinction of the stressor context before conditioning, while effective in eliminating fear of the stressor context, has no impact on the enhancement of new conditioning. This may correspond to the reduced effectiveness of extinction in treating PTSD (eg, Peri *et al*, 2000) in that it suggests that extinguishing fear of trauma-related events will not reduce the nonassociative effects of trauma. Additionally, blocking contextual fear learning to the stressor context by delivering an amnesic agent to the hippocampus during the 15 shocks does not alter the enhancement. This may correspond to observation of PTSD in patients who have amnesia for the traumatic episode (eg, Krikorian and Layton, 1998).

Furthermore, whereas we have most often looked at the enhancement in new context fear learning, the fear enhancement is also found in auditory conditioning (Rau *et al*, 2005). As 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 single 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), the order should not matter. If SEFL alters the fear learning circuit, the stressor would necessarily have to come before the single-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 (eg, Rau *et al*, 2005; Rau and Fanselow, 2009). 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, based on previous research and in our own unpublished data, we find that if we reduce the number of pre-shocks, a smaller percentage of rats meet the 2 standard deviation criterion (Fanselow and Bolles, 1979). If we reduce the pre-shock treatment to three or four, 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 a 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 corticotropin-releasing hormone 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*, 1998) and decreased levels (Mason *et al*, 1986; Pitman and Orr, 1990; Yehuda *et al*, 1990, 1995; Boscarino, 1996) of CORT have been found in PTSD patients compared with 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). Using the SEFL model, we discovered that one long-term consequence of exposure to the stressor is a dysregulation in the diurnal rhythm of basal CORT. Rather than having a single peak shortly after dark, rats showed a double peak once during the light cycle and once at dark. Therefore, the stressed rats were either elevated or suppressed relative to controls depending on the hour of the day at which CORT was determined. Additionally, as will be argued below, it is possible that CORT changes during stress are the critical changes for the induction of exaggerated fear and that CORT

level changes at other times, such as after the trauma, are less critical to the sensitized behavior. Obviously, CORT levels are rarely taken during a traumatic episode in humans.

Rats exhibiting SEFL also show decreased exploratory behavior in open fields (Figure 1a and b), increased consumption of alcohol (Meyer *et al*, 2013), potentiated startle reactivity (Figure 1c and d), and a depression-like phenotype in the forced swim test (Figure 1e and f). 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).

STRESS-ENHANCED FEAR INDUCTION

In this section, we describe the neural and biological mechanisms necessary for stress sensitization to occur, including the role of stress hormones in the BLA. In the first experiment, we examined the role of 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. Next, we determined the necessity of CORT in SEFL by co-administering metyrapone and CORT pre-stressor. CORT restored SEFL after metyrapone administration, but CORT alone without the

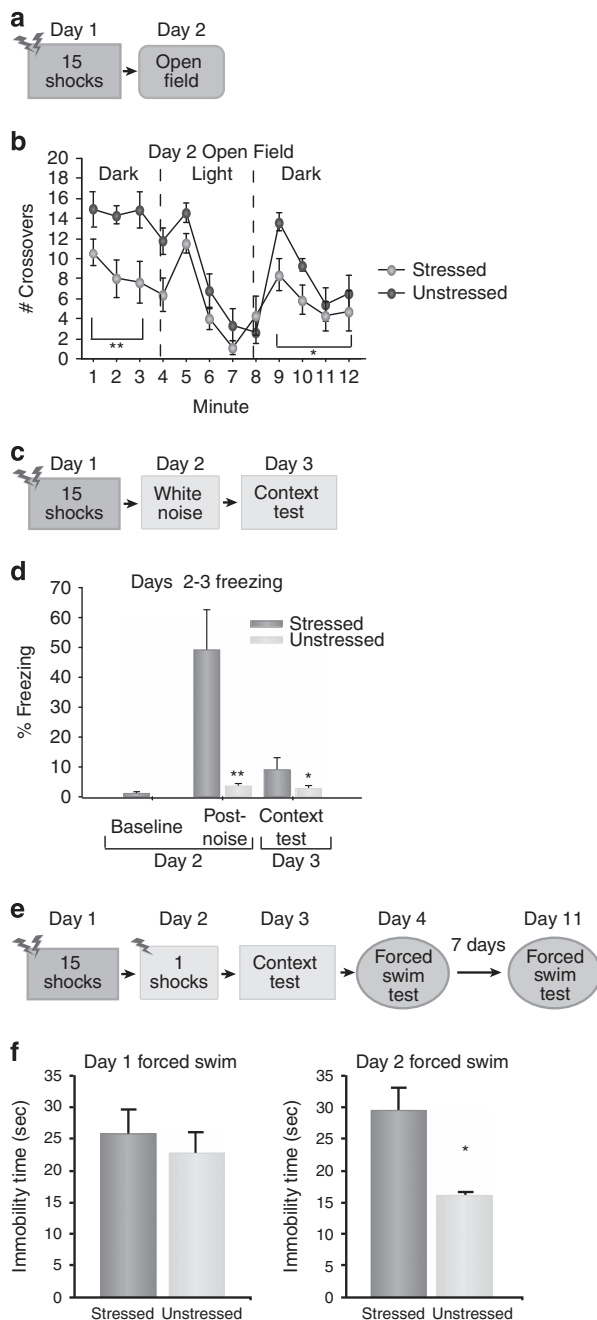


Figure 1. SEFL causes anxiety phenotype on the open field test, exaggerated startle to white noise, and a depressive-like phenotype in the forced swim test. (a) Experimental design. We developed a modified version of the open field test that has been validated for anxiety testing (Godsil and Fanselow, 2004; Godsil *et al*, 2005). The open field test consisted of three phases: (1) 4 min of dark, (2) 4 min of light and (3) 4 min of dark. Locomotion, defined as the number of crossovers, was quantified during the 12-min 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). (c) Experimental design for exaggerated startle test. Instead of 1-shock in conditioning context on Day 2, white noise (92 dB, 1 s) was given. (d) Freezing (\pm SEM) for baseline on Day 2, 5 min 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, $*p < 0.05$. (e) Experimental design. Training (Day 4) and testing (Day 11) sessions for forced swim test were 5 min. Water temperature was 77 °F, and the apparatus dimensions are 74 cm \times 36.5 cm. (f) Time spent immobilized (sec \pm SEM) on Day 4, and time spent immobilized (sec \pm SEM) on Day 11. $*p < 0.05$ (one-way ANOVA).

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 1a and b
Co-morbid alcohol and drug abuse	Increased voluntary alcohol consumption	Meyer et al, 2013
Symptoms present > 30 days post trauma	> 90 days	Rau and Fanselow, 2009
Increased startle reactivity	Hyper-reactivity to loud noise	Figure 1c and d
Co-morbid depression	Forced swim test	Figure 1e and f

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

15-shock stressor was not sufficient to produce SEFL. We then examined the role of the BLA in SEFL with intra-BLA infusions of muscimol, a GABA_A receptor (GABA_{AR}) agonist, pre- or post-stressor. The BLA must be functional during the 15 shocks but not after in order for SEFL to occur. Lastly, to determine whether CORT's action was on the BLA, we infused the 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.

Pre-Stressor Administration of Metyrapone Attenuates SEFL

A total of 102 experimentally naïve adult male Long Evans rats, approximately 300 g, were used in this experiment. Rats were randomly assigned to one of the two groups: those receiving 15 shocks randomly distributed over a 90-min 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: 1 h prior to the trauma, animals were given a 1.0 ml/kg intraperitoneal injection of either vehicle (of 60% saline and 40% propylene glycol) (Sigma Aldrich, St. Louis, MO), 50 mg/kg metyrapone, 100 mg/kg metyrapone, or 150 mg/kg metyrapone (Tocris Bioscience, Ellisville, MO). All groups had an *n* of 12, except for stressed/vehicle (*n* = 14) and unstressed/vehicle (*n* = 16). Animals receiving no stress during exposure were placed in chambers for an equivalent amount of time as the stressed animals—90 min. Immediately after the stressor, animals were placed in a restraining tube for a maximum of 5 min 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. CORT plasma levels were measured by enzyme immunoassay (AssayPro, St Charles, MO; Correlate-EIA corticosterone enzyme immunoassay kit).

Animals then underwent the rest of the 7-day SEFL procedure (see Figure 2a). Forty-five minutes after the context test on Day 7, tail blood was collected again in the same manner as was for Day 1. Freezing was recorded using

the VideoFreeze program (Med Associates, St Albans, VT).

Two-way analysis of variance (ANOVA) was used to analyze percent freezing as influenced by both drug dose and stress condition during Day 4 in the stress context and Days 5 and 7 in the conditioning context, as well as to compare plasma CORT levels. Moreover, trend analysis was performed to determine whether the data follow linear dose-dependent functions.

As expected, pre-stress administration of metyrapone dose-dependently reversed the stress-induced rise in plasma CORT levels after the 15-shock stressor on Day 1 (Figure 2b). Overall, stressed animals had higher CORT levels than did unstressed animals, $F(1, 94) = 11.509$, $p < 0.001$. Trend analysis showed that in stressed but not unstressed animals, metyrapone linearly dose-dependently decreased plasma CORT levels after the 15-shock stressor on Day 1 in a linear manner, $F(1, 94) = 5.19$, $p < 0.05$. However, CORT levels during the Day 7 context test showed no reliable group differences (data not shown; p 's > 0.05).

The Day 4 stress context test showed that metyrapone given pre-stressor dose-dependently blocked fear conditioning to this context (Figure 2c); there was 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, $F(1, 94) = 106.4$, $p < 0.0001$. The low freezing levels during the Day 5 test in the novel context showed that no groups generalized to this context (Figure 2d; p 's > 0.05).

The critical behavioral data are the freezing levels during the Day 7 conditioning context test (Figure 2e); all animals received one footshock in this novel context 24 h earlier. A significant interaction between drug dose and stress treatment was found, $F(3, 94) = 2.957$, $p < 0.05$, and trend analysis indicated that pre-stress metyrapone dose-dependently decreased freezing in stressed animals in a linear manner, $F(1, 94) = 12.796$, $p < 0.0005$. However, metyrapone had no effect on freezing for unstressed animals ($p > 0.05$).

These data demonstrate that the 15-shock stressor enhanced later fear conditioning to the single shock context (ie, SEFL occurred), and, importantly, that blocking CORT increases during the stressor subsequently blocked SEFL

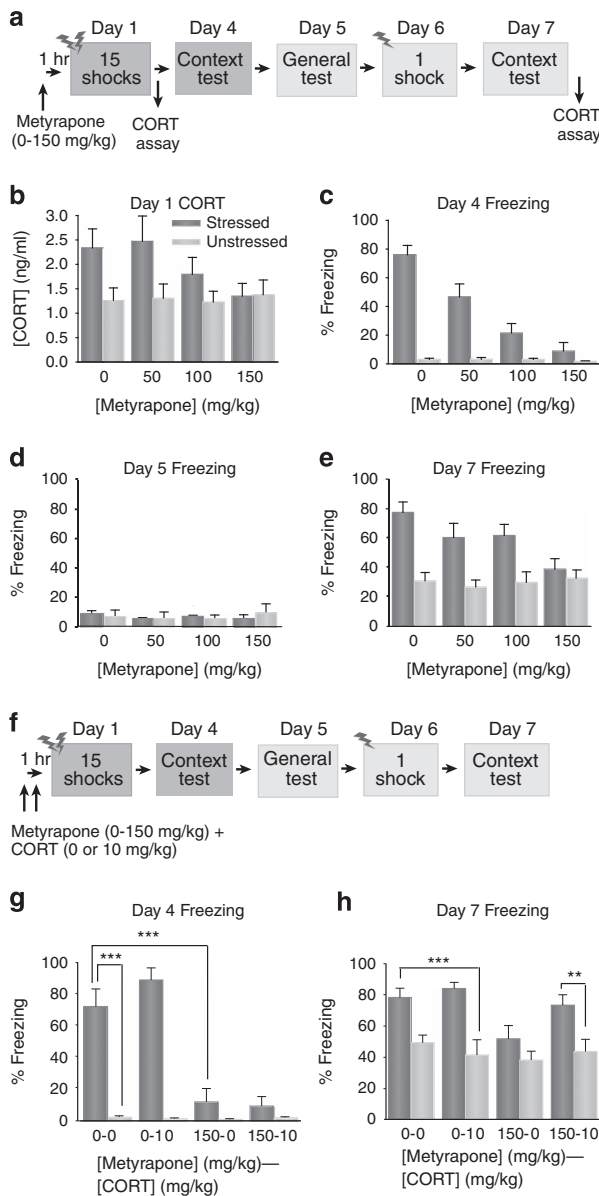


Figure 2. Pre-stressor administration of metyrapone attenuates SEFL, and co-administration of metyrapone and CORT restores SEFL. (a) Experimental design for metyrapone administration. (b) Plasma CORT levels (ng/ml; mean+SEM) after the 15-shock trauma on Day 1. Main effect of stress, $p < 0.001$; linear trend analysis for stressed animals, $p < 0.05$ (two-way ANOVA, followed by planned contrasts). (c) The mean+SEM of freezing percentage in stress context test on Day 4. Drug \times stress, $p < 0.0001$ (two-way ANOVA), linear trend analysis for stressed animals, $p < 0.0001$. (d) The mean+SEM of freezing percentage during the generalization test in the conditioning context on Day 5. No significant differences were found for any main effect or interaction (two-way ANOVA). (e) Freezing (mean+SEM) in conditioning context on Day 7. Drug \times stress interaction, $p < 0.05$, linear trend analysis for stressed animals, $p < 0.0005$ (two-way ANOVA, followed by planned contrasts). (f) Experimental design for metyrapone and CORT co-administration. (g) 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). $***p < 0.0001$ (overall one-way ANOVA, followed by planned contrasts). (h) 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). $**p < 0.01$; $***p < 0.0001$ (overall one-way ANOVA, followed by planned contrasts).

(Figure 2e). We also administered the 150 mg/kg dose of metyrapone immediately after the 15-shock stressor, before the single shock, or before the test and did not see an effect at any of those time points (data not shown), which supports our finding that CORT changes 7 days post-stressor were not reliably different. Moreover, CORT measurement in PTSD patients is typically taken long after the trauma, not immediately after, which may explain the inconsistencies in reported CORT levels in PTSD patients (Yehuda *et al*, 1990; Maes *et al*, 1998). This is not to say that CORT is not affected long-term by trauma, as we do see a disturbance in the circadian rhythm of basal CORT (Poulos *et al*, 2013). Therefore, increases in CORT specifically at the time of stress are critical for SEFL.

Co-Administration of Metyrapone and CORT Rescues SEFL

In order to confirm that metyrapone's effect was mediated by its action on CORT, we determined whether co-administration of CORT restores sensitized fear after metyrapone injection. Additionally, we determined whether CORT administration in the absence of shock was sufficient to produce SEFL. A total of 116 experimentally naïve adult male Long Evans rats, approximately 300 g, were used in this study. Animals were given either a pre-training injection of 150 mg/kg metyrapone or vehicle in the rats' housing area. Either 10 mg/kg CORT (Sigma-Aldrich) or vehicle (15% alcohol/85% saline) was injected 10 min prior to the 15-shock stressor. The rats then underwent the 7-day SEFL procedure. There were a total of eight groups, and n per group ranged between 12 and 17 (see Figure 2f).

A one-way ANOVA was performed to determine significant freezing differences during both context tests. *A priori* planned comparisons were also made to determine whether CORT alone could induce SEFL in unstressed animals, and if CORT can rescue SEFL from stressed animals that received metyrapone.

During the Day 4 stress context test, there was an overall difference between groups, $F(7, 108) = 36.355$, $p < 0.0001$ (Figure 2g). The freezing levels for stressed/vehicle animals (Group 0-0) were significantly higher than for unstressed/vehicle animals (Group 0-0), $F(1, 108) = 60.54$, $p < 0.0001$. The latter group showed similar freezing levels to unstressed controls, $p > 0.05$. Stressed/vehicle rats froze significantly more than did stressed/metyrapone/vehicle animals (Group 150-0), $F(1, 108) = 47.43$, $p < 0.0001$, which showed similar freezing levels to both stressed/metyrapone/CORT animals (Group 150-10) and unstressed controls, p 's > 0.05 .

Like the first metyrapone experiment, no groups generalized to the second conditioning context on Day 5 (data not shown, p 's > 0.05). On Day 7, there were reliable freezing differences between groups as analyzed by an overall one-way ANOVA, $F(7, 108) = 6.619$, $p < 0.0001$ (Figure 2h). *A priori* planned comparisons indicated that the freezing levels of the stressed/vehicle animals were significantly higher than those of unstressed/vehicle animals receiving

CORT (Group 0-10), $F(1, 108) = 11.891$, $p < 0.001$; this means that CORT alone does not induce sensitized fear. Although CORT without stress did not generate SEFL, CORT did restore SEFL in stressed rats that received metyrapone (Group 150-10). These animals froze significantly more than did their unstressed counterparts, $F(1, 108) = 7.297$, $p < 0.01$.

Whereas metyrapone prevented SEFL, CORT administration alone did not mimic the effect of stress (Figure 2h). However, SEFL was restored with CORT after metyrapone injection. Thus, changes in CORT are necessary but not sufficient for producing SEFL. This differs from the results from the Day 4 stress context test, 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 have a permissive role critical to SEFL-inducing changes elsewhere. Additionally, CORT's restoration of SEFL indicates that metyrapone's effect is likely caused by its ability to block CORT synthesis.

BLA Inactivation Reduces SEFL

We next sought to find important brain regions for SEFL and targeted the BLA, a brain region crucial for fear conditioning and emotional learning in animals (Miserendino *et al*, 1990; Fanselow and Kim, 1994; Rogan *et al*, 1997; McKernan and Shinnick-Gallagher, 1997; Muller *et al*, 1997; Walker and Davis, 2002). Indeed, PTSD patients show enhanced amygdala activity during encoding and exposure to negative stimuli (Shin *et al*, 2006; Brohawn *et al*, 2010). Furthermore, the BLA has an important role in stress-induced fear responses (Kim *et al*, 2001; Adamec *et al*, 2005; Rodriguez Manzanares *et al*, 2005) and strikingly, 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 the BLA in SEFL, we inactivated the BLA using the GABA_AR agonist muscimol (Sigma-Aldrich) either prior to or after the 15-shock stressor (see Figure 3a). All animals underwent surgery to implant guide cannulae into the BLA 10–14 days before the start of experiments. Briefly, rats were anesthetized (isoflurane: induction at 5%, maintenance 2.5%) before stereotaxic mounting (Kopf Instruments, Tujunga, CA). 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.

Animals were randomly assigned to one of three groups: pre-trauma muscimol animals received micro-infusions of

muscimol 20 min before the 15 shocks, vehicle controls received ACSF 20 min before being placed in the stressor context without shocks, and post-trauma muscimol animals underwent the 15 shocks first, receiving micro-infusions of muscimol 45 min after the trauma. For infusions, 33-gauge injector cannulae that extend 1 mm below the guides were inserted. The drug 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 $0.1 \mu\text{l}/\text{min}$ to reach a volume of $0.25 \mu\text{l}/\text{side}$, delivered via a Harvard #22 syringe pump (Harvard Apparatus, South Natick, MA, USA). The animals then underwent a shortened 3-day SEFL procedure. Freezing in the conditioning context was recorded on Day 3. After exclusion of animals with misplaced cannulae groups consisted of: vehicle controls ($n = 4$), pre-stressor muscimol ($n = 4$), and post-stressor muscimol ($n = 4$). A one-way ANOVA was performed to determine between-group differences for freezing during the context test.

Figure 3b depicts verification of correct bilateral cannulae placement on a schematic diagram. Figure 3c illustrates clear group differences during the context test in the conditioning context, 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 from unstressed controls, $p > 0.05$.

Pre-Stressor Intra-Amygdala Mifepristone Prevents SEFL

To determine whether CORT's action was on the BLA, we infused the GR antagonist mifepristone directly into the BLA immediately prior to the 15-shock stressor (Figure 3d). Experimentally naïve adult male Long-Evans rats were used in this experiment. All animals underwent surgery to implant guide cannulae into the BLA 10–14 days before the start of experiments. Animals either received micro-infusions of mifepristone (Sigma-Aldrich) or vehicle (20% DMSO and 80% ACSF) 10 min before the 15 shocks, or were unstressed vehicle controls. The animals then underwent the shortened 3-day SEFL procedure. 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/ACSF ($n = 7$), and unstressed/ACSF ($n = 5$). A one-way ANOVA was performed to determine between-group differences for freezing during the context test.

Figure 3e shows verification of correct bilateral cannulae placement on a schematic diagram. Figure 3f 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,

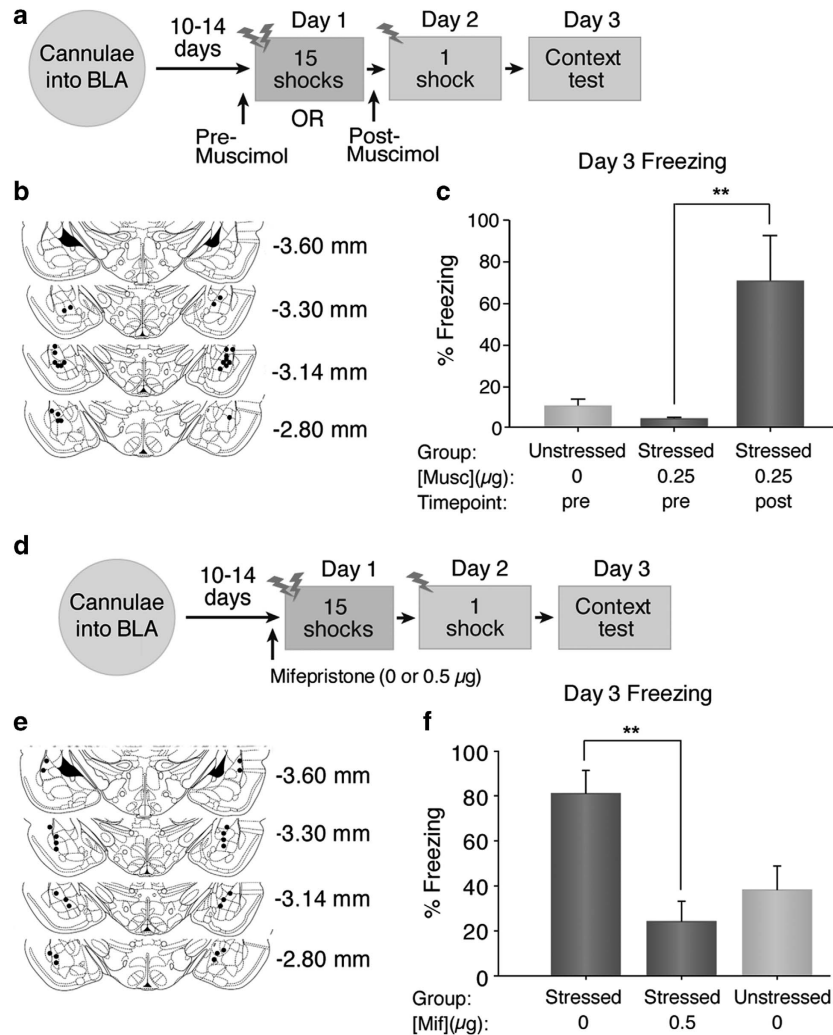


Figure 3. Basolateral amygdala inactivation or intra-basolateral infusions of mifepristone reduces SEFL. (a) Experimental design for intra-amygdalar muscimol infusions. (b) Cannulae placement for amygdalar inactivation. (c) Freezing (mean+SEM) in the conditioning context on Day 3. ** $p < 0.003$ (one-way ANOVA, followed by planned contrasts). (d) Experimental design for mifepristone infusions. (e) Cannulae placement for mifepristone infusions. (f) Freezing (mean+SEM) in the conditioning context on Day 3. ** $p < 0.001$ (one-way ANOVA, followed by planned contrasts).

$F(1, 14) = 16.42$, $p < 0.001$; however, stressed/mifepristone animals did not show significantly different freezing levels from unstressed/vehicle controls, $p > 0.05$. This indicates that CORT activation of GRs in the BLA is necessary for SEFL induction.

Summary

The experiments in this section 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 blocked 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 section will address the underlying mechanisms of expression of enhanced fear.

STRESS-ENHANCED FEAR EXPRESSION

Next, to explore the potential mechanisms of SEFL expression, we looked for changes in glutamatergic receptor subunits in the BLA. Glutamatergic modifications in excitatory neurotransmission—in particular, NMDAR and AMPAR—within the amygdala participate in different components of fear learning, including acquisition, expression, and extinction (Miserendino *et al*, 1990; Fanselow and Kim, 1994; Walker and Davis, 2002). Increased excitatory neurotransmission in the BLA could also enhance fear conditioning (Fanselow *et al*, 1993).

Metyrapone Prevents a Post-Stressor GluA1 Increase

Rats received either 150 mg/kg metyrapone or vehicle 1 h before the 15-shock stressor (see Figure 4a). Two weeks after the test in the conditioning context, western blot

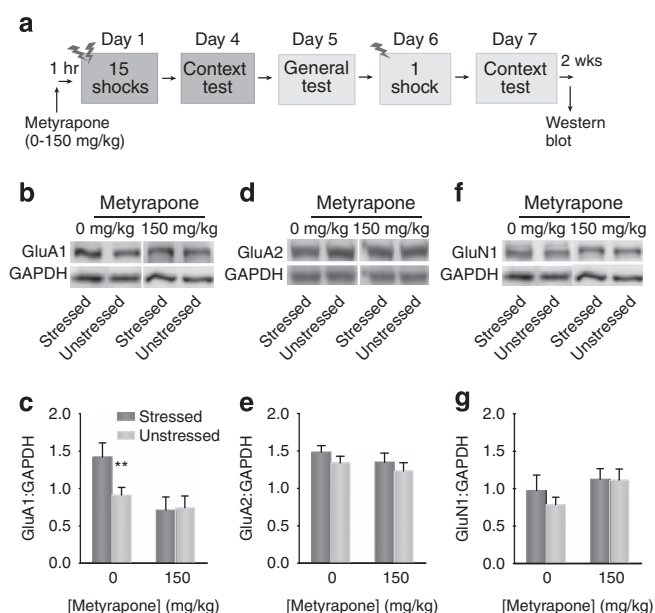


Figure 4. Increases in GluA1 after SEFL treatment are prevented by metyrapone. (a) Experimental design. (b, d, f) Representative western blot images of GluA1, GluA2, GluN1, and GAPDH from the BLA of stressed and unstressed rats receiving vehicle or 150 mg/kg metyrapone. (c) Mean GluA1: GAPDH OD ratios (+SEM). Main effect of drug, $p < 0.005$, $**p < 0.01$ (one-way ANOVA, followed by *a priori* planned comparisons). (e) Mean GluA2: GAPDH OD ratios (+SEM). (g) Mean GluN1: GAPDH OD ratios (+SEM).

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. Rats were sacrificed, 400- μ m-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 were measured by the D_C protein assay system (Bio-Rad, Hercules, CA). Samples (15 μ g/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) by wet transfer (Bio-Rad, 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), and GluN1 (aa 834-938; 1:1000 dilution; Upstate; EMD Millipore) antibodies, followed by HRP-conjugated secondary (Goat anti-rabbit; EMD Millipore) 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 measurements were compared by densitometry using ImageJ (NIH, Bethesda, MD).

For the mean GluA1: GAPDH optical density 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 (Figure 4b and c). *A priori* planned comparisons indicated that although stressed/vehicle animals showed a significantly increased amount of GluA1 compared with stressed/metyrapone animals, $F(1, 32) = 8.03$, $p < 0.01$, there were no GluA1 protein differences between stressed and unstressed controls, as well as between stressed and unstressed animals that both received metyrapone, p 's > 0.05 . There were no significant main effects of stress, drug, or a stress by drug interaction for GluA2 (Figure 4d and e), nor for GluN1 protein levels (Figure 4f and g) in the BLA, p 's > 0.05 .

Interestingly, besides preventing SEFL, metyrapone prevented the elevation in GluA1 and kept its expression level at that of unstressed controls. By contrast, there were no differences in GluA2 or GluN1 levels in stressed or metyrapone-treated groups. Because facilitating glutamatergic activity at AMPAR enhances the rate of fear conditioning (Kim *et al*, 1993) and SEFL occurs predominantly by increasing the rate of fear conditioning (Fanselow *et al*, 1993), the long-term increase in GluA1 subunits is a highly plausible mechanism for the expression of SEFL.

Summary

The results from this experiment 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. By contrast, there were no differences in GluA2 or GluN1 levels in stressed or metyrapone-treated groups. 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 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 the expression of fear via these long-term potentiation mechanisms (Kim *et al*, 1993; Fanselow and Kim, 1994; Walker and Davis, 2002; Hubert *et al*, 2013). Therefore, an increase in AMPAR subunits but not NMDAR subunits 2 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 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 2 weeks after the stressor is inconsistent with prior results

showing that the expression of calcium-permeable AMPARs peaks 24 h 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). We postulate that this increased excitability may lead to enhanced plasticity in response to subsequent mild stressors.

FUTURE RESEARCH DIRECTIONS

The results from these studies discussed in this review 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.

The next logical step is to target this upregulation of GluA1 post-stressor. Metyrapone prevented SEFL only when given prior to stress, which severely limits its applicability as a potential treatment. Induction and maintenance of long-term potentiation requires the insertion of GluA1-containing AMPAR in the synapse. Although the process is induced by stress hormones (ie, glucocorticoids), maintenance of AMPAR at the synapse involve both protein kinase A-mediated phosphorylation and endosomal recycling (Kessels and Malinow, 2009; Hanley, 2010; Whitehead *et al*, 2013). Preventing the upregulation of GluA1 after the 15-shock stressor or perhaps blocking this synaptic AMPAR maintenance may prevent SEFL from developing following a stressor.

Moreover, it is critical to parse the nonassociative and associative components of SEFL and treat each with different methods. 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 (Davis *et al*, 2006; Monfils *et al*, 2009; Zelikowsky *et al*, 2014). Hence, the findings of these experiments suggest a potential for developing novel treatments for PTSD.

In conclusion, these findings have outlined a clear set of mechanisms that underlie the 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 associative fear expression but also for the expression of SEFL. These long-lasting GluA1 increases

may be targeted to produce an enduring reversal of this enhanced fear responding, which suggests hope for those suffering from severe PTSD.

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The authors declare no conflict of interest.

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