Journal of Visualized Experiments

Stress-enhanced fear learning, a robust rodent model of post-traumatic stress disorder

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58306R2
Full Title:	Stress-enhanced fear learning, a robust rodent model of post-traumatic stress disorder
Keywords:	Post-traumatic stress disorder; Fear; Stress; Fear Memory; fear conditioning; Animal model
Corresponding Author:	Michael Fanselow University of California Los Angeles Los Angeles, CA UNITED STATES
Corresponding Author's Institution:	University of California Los Angeles
Corresponding Author E-Mail:	mfanselow@gmail.com
Order of Authors:	Abha K Rajbhandari
	Sarah T Gonzalez
	Michael Fanselow
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	502 Portola Plaza, Los Angeles, CA, 90095

Cover Letter

University Of California, Los Angeles
UCLA Integrative Center For Learning & Memory (ICLM)
Department Of Psychology
Department of Psychiatry & Biobehavioral Sciences
405 Hilgard Ave
Los Angeles, CA 90095-1563
•voice (310) 206-3891 • fax (310) 206-5895
http://fanselowlab.psych.ucla.edu
• email fanselow@ucla.edu

April 18, 2018

Ronald Myers, PhD.
Science Editor
JoVE
1 Alewife Center, Suite 200, Cambridge, MA 02140

Dear Ronald

We would like to submit our manuscript entitled' Stress-enhanced fear learning, a robust rodent model of post-traumatic stress disorder" for publication as an open access in JoVE.

This manuscript outlines detailed methodology for conducting rodent behavioral model of post-traumatic stress disorder termed stress enhanced fear learning (SEFL). SEFL is a robust behavioral model that can be conducted in both rats and mice. Hence it allows researchers an ability to probe and delineate brain mechanisms that govern PTSD-like fear behaviors.

This manuscript has not been published elsewhere and is not under consideration by another journal. The study was supported by a grant from the National Institute of Mental Health RO1MH62122 (MSF), Staglin Center for Brain and Behavioral Health (MSF), NRSA-F32 MH10721201A1 and NARSAD 26612 (AKR), NSF DGE-1650604 (SG). All authors have approved the manuscript and agree with submission to JoVE and the authors have no conflicts of interest to declare.

We would like to recommend the following researchers as potential reviewers for this paper

1. Jennifer Quinn
2. Jeansok Kim
3. Mat Lattal
4. Marie Monfils
5. Don Lysle
6. Courtney Miller

Please address all correspondence to:

Michael S Fanselow,
502 Portola Plaza, Los Angeles, CA, 90095
email address: mfanselow@gmail.com or fanselow@ucla.edu

We look forward to hearing from you soon.

Sincerely,

Michael S Fanselow,
Distinguished Professor
Staglin Family Chair in Psychology,
Director Staglin Music Festival Center for Brain & Behavioral Health
Department of Psychology
Department of Psychiatry and Biobehavioral Sciences
UCLA Integrative Center for Learning & Memory (ICLM)
email address: mfanselow@gmail.com or fanselow@ucla.edu

TITLE:

Stress-Enhanced Fear Learning, a Robust Rodent Model of Post-Traumatic Stress Disorder

AUTHORS & AFFILIATIONS:

Abha K. Rajbhandari[1,2], Sarah T. Gonzalez[1,2], Michael S. Fanselow[1,2,3]

[1]Department of Psychology, University of California, Los Angeles, CA, USA
[2]Staglin Center for Brain and Behavioral Health, University of California, Los Angeles, CA, USA
[3]Department of Psychiatry and Biobehavioral Sciences, University of California, Los Angeles, CA, USA

Email Address of Corresponding Author:
Michael S. Fanselow (mfanselow@gmail.com)

Email Addresses of Co-Author:
Abha K. Rajbhandari (arajbhandari@ucla.edu)
Sarah T. Gonzalez (sgonla@ucla.edu)

KEYWORDS:

Post-traumatic stress disorder, fear, stress, fear memory, fear conditioning, animal model

SUMMARY:

Here we describe the detailed methodology required to conduct stress-enhanced fear learning (SEFL) experiments, a preclinical model of post-traumatic stress disorder, in both rats and mice. The model utilizes aspects of Pavlovian fear conditioning and freezing as an index of enhanced fear in rodents.

ABSTRACT:

Fear behaviors are important for survival, but disproportionately high levels of fear can increase the vulnerability for developing psychiatric disorders such as post-traumatic stress disorder (PTSD). To understand the biological mechanisms of fear dysregulation in PTSD, it is important to start with a valid animal model of the disorder. This protocol describes the methodology required to conduct stress-enhanced fear learning (SEFL) experiments, a preclinical model of PTSD, in both rats and mice. SEFL was developed to recapitulate critical aspects of PTSD, including long-term sensitization of fear learning caused by an acute stressor. SEFL uses aspects of Pavlovian fear conditioning but produces a distinct and robust sensitized fear response far greater than normal conditional fear responses. The trauma procedure involves placing a rodent in a conditioning chamber and administering 15 unsignaled shocks randomly distributed over 90 minutes (for rat experiments; for mouse experiments, 10 unsignaled shocks randomly distributed over 60 minutes are used). On day 2, rodents are placed in a novel conditioning context where they receive a single shock; then, on day 3 they are placed back in the same context as on day 2 and tested for changes in freezing levels. Rodents that previously received the trauma display enhanced levels of freezing on the test day compared to those that received no shocks on the

first day. Thus, with this model, a single highly stressful experience (the trauma) produces extreme fear of the stimuli associated with the traumatic event.

INTRODUCTION:

Fear is a critical behavior for survival, enabling individuals to recognize and respond to threats. However, exaggerated fear responses can contribute to the development of psychiatric disorders such as post-traumatic stress disorder (PTSD). One characteristic of PTSD is an exaggerated response to mild stressors, particularly those reminiscent of the original trauma, and a tendency to develop new fears[1,2]. In the laboratory, fear is often measured through freezing behavior, which is a reliable and ethologically valid index of fear in humans and rodents[3,4]. While it is known that PTSD involves dysregulation of fear and enhanced fear expression, there is a lack of robust animal models of PTSD that reliably capture this augmented fear response to a relatively innocuous stimulus.

This protocol provides the detailed methodology required to conduct stress-enhanced fear learning (SEFL) experiments, a reliable and robust preclinical model of PTSD, in both rats and mice. SEFL utilizes aspects of Pavlovian fear conditioning, yet it produces distinct responses from normal fear conditioning and recapitulates the enhanced fear following traumatic stress observed in PTSD patients[5,6]. In this model, a single highly stressful experience (referred to here as trauma) leads to lasting behavioral changes, including extreme fear of stimuli associated with the traumatic event, increased anxiety, increased startle reactivity, and altered glucocorticoid signaling[7,8]. The major feature of SEFL is that following exposure to a traumatic stressor (a series of unsignaled shocks) in a distinct context, animals show an exaggerated fear response to a mild stressor (*e.g.*, a single shock) in a different context. Importantly, the SEFL effect is not due to the generalization from the trauma context to the novel context or increased shock sensitivity[5]. In our model, we purposefully utilize procedures that reduce any generalization to a novel context such as distinct transport, odor and grid floor pattern. Therefore, unlike normal fear conditioning, SEFL is a non-associative process that leads to a new fear learning that is disproportionately related to environmental cues not directly associated with the traumatic experience. Extensive work shows that a single 90-minute session containing 15 unpredictable shocks in rats (or a single 60-minute session containing 10 unpredictable shocks in mice) induces a long-lasting sensitization of fear conditioning along with increased anxiety and dysregulation in the circadian rhythm of basal corticosterone. In contrast, pre-exposure to a single footshock does not produce SEFL[9]. Furthermore, SEFL can be utilized reliably in both rats and mice.

Hence, the SEFL model of PTSD is a powerful tool for probing the biological mechanisms involved in PTSD pathophysiology. Using SEFL, researchers can examine how exposure to a trauma can affect future fear learning. In addition, this model can be useful for investigating specific cellular and molecular mechanisms that may be involved in regulating enhanced fear expression as observed in PTSD.

PROTOCOL:

1. Subjects

1.1. Rats

1.1.1. Order rats to arrive when they are approximately 90 days old and single-housed in standard rat cages.

Note: Single housing is advised, as group housing produces variability due to interactions between animals in the home cage, particularly following stress exposure. SEFL has been demonstrated in male and female rats, in Long-Evans and Sprague Dawley rats, and in rats as young as 19 days old[7,10].

1.1.2. Randomly assign animals to at least two conditions: trauma (n = 8) and no trauma (n = 8) (see Rau *et al.*[5] for additional control conditions).

1.2. Mice

1.2.1. Order mice to arrive when they are approximately 60 days old and single-housed in standard mouse cages. Single-house the mice for at least 4 weeks prior to trauma as well as throughout the experiment duration.

1.2.2. Randomly assign animals to at least two conditions: trauma (n = 8) and no trauma (n = 8).

2. Equipment Setup

2.1. Set up one set of fear-conditioning chambers to serve as Context A and a second set of fear-conditioning chambers to serve as Context B (see **Materials Table**). Place each fear-conditioning chamber inside a sound-attenuating cubicle to prevent intrusion of outside noise (see **Materials Table**).

2.1.1. Ensure that a method of illuminating the chambers with visible light (such as a white overhead house light) is present in the chambers serving as Context A (see **Materials Table**).

2.1.2. Ensure that different solutions are available to clean the chambers between each animal and provide different odors for each chamber (*e.g.*, diluted cleaning solution and 1% acetic acid).

Note: It is critical that animal-generated odors be eliminated[11].

2.1.3. Place plastic inserts in Context B to differentiate the internal layout of the two contexts. A black Plexiglas triangular insert is recommended. Alternatively, use a white plastic sheet to create a curved back wall (see **Materials Table**).

2.1.4. Place grid floors in each fear conditioning chamber for footshock delivery, using a different grid pattern for each context to differentiate floor texture between contexts (see **Materials Table**).

Note: Sufficiently distinct grid patterns include flat grids (all bars arranged in a single horizontal plane), staggered grids (bars arranged in two offset horizontal planes), and alternating grids (bars arranged in a single horizontal plane but of varying diameters).

2.1.5. Place clean metal pans beneath each grid floor to collect droppings. Scent pans with the cleaning solution (see **Materials Table**).

2.2. Provide accurate timing and amplitude of footshock delivery for each context.

2.2.1. Connect a shock generator and scrambler capable of delivering 1 mA or lower amplitude shocks to each grid floor for footshock delivery (see **Materials Table**).

Note: The shock generators and scramblers should be located outside of the sound attenuating chamber, with cables connecting the generators and scrambler to the grid floors via openings in the sound attenuating chamber. This will prevent damage due to chewing, cleaning solution, *etc*.

2.2.2. Use a multimeter to test the current being delivered by the shock generator by placing each probe on a different bar of the grid floor and confirming that the desired shock amplitude is produced (see **Materials Table**).

2.2.3. Ensure that a method for controlling the timing and amplitude of shock delivery (*e.g.,* computer software) is available (see **Materials Table**).

2.3. Ensure that a method for video-recording each animal during each experimental session is available (see **Materials Table**).

Note: It will be necessary to record both 1) when the chambers are illuminated by visible light and 2) when the chambers are dark. The latter can be accomplished by either using a night vision camera or illuminating and recording the darkened chambers using infrared or near-infrared light.

2.4. Ensure that a distinctive method of transporting animals from the vivarium to Context B is available to further differentiate the two contexts.

Note: While methods such as a black plastic tub (38 x 30 x 24 cm) divided into four compartments or clean empty cages have been successfully used, any other transport box that is distinctly different from the home cage can be used.

3. SEFL Procedure for Rats and Mice

3.1. Handle all rodents daily by gently removing them from the homecage and holding each for 60-90 seconds for at least 7 days before beginning the SEFL procedure.

3.2. On Day 1 of the SEFL procedure, place subjects in Context A, where they will receive the traumatic stressor.

3.2.1. Set up Context A with one set of grid floors (*e.g.*, flat grids) and illuminate the chambers with visible light.

3.2.2. Use a multimeter to test the current being delivered by the shock generator by placing each probe on a different bar of the grid floor and confirming that the desired shock amplitude is produced.

Note: Damage or corrosion of the bars can result in weak or uneven shock delivery. Liquids including urine touching the grid along the wall can also adversely affect shock delivery.

3.2.3. Wipe down the chamber walls and doors and spray the pans beneath the grid floors with one solution (*e.g.*, diluted cleaning solution).

Note: This is necessary to eliminate odors from the previous animals.

3.2.4. Transport animals from the vivarium to the experimental room in their home cages placed on a cart and place individually into the fear conditioning chambers. Only bring one round's worth of animals (determined by the number of fear conditioning chambers) to the experiment room at a time.

Note: To avoid confounds due to order or timing, each round should contain animals in both the trauma and no trauma conditions.

3.2.5. For rat experiments, use the shock generator and scramblers to deliver 15 1-s, 1-mA footshocks randomly presented over 90 minutes (average ISI = 6 min) through the grid bars of the chambers containing trauma condition subjects. Expose the no trauma controls to the same context for 90 minutes without shock delivery.

3.2.6. For mouse experiments, use the shock generator and scramblers to deliver 10 1-s, 1-mA footshocks randomly presented over 60 minutes (average ISI = 6 min) through the grid floors of the chambers containing trauma condition subjects. Expose the no trauma controls to the same context for 60 minutes without shock delivery.

3.2.7. After 90 minutes (rat experiments) or 60 minutes (mouse experiments), return all animals to their homecages and promptly return to the vivarium.

3.3. On Day 2 of the SEFL procedure, assess the fear to the trauma context if desired.

3.3.1. Set up Context A as done on Day 1.

3.3.2. Transport animals to the experiment room in their home cages as on Day 1.

3.3.3. Place animals in Context A for 8 minutes without shock delivery and video record the behavior during the entire session.

3.3.4. After 8 minutes, return all animals to their homecages and promptly return to the vivarium.

3.4. On Day 3 of the SEFL procedure, expose all subjects to the mild stressor in Context B.

Note: This procedure can occur anywhere from 24 hours to 90 days after the traumatic stressor[9].

3.4.1. Set up Context B with a different set of grid floors from the ones used in Context A (*e.g.,* alternating or staggered grid floors) and black triangular or white curved Plexiglas inserts. Do not illuminate the chambers with visible light; although, infrared or near-infrared light can be used as necessary.

3.4.2. Use a multimeter to test the current being delivered by the shock generator by placing each probe on a different bar of the grid floor and confirming that the desired shock amplitude is produced. Use a multimeter to test the current being delivered by the shock generator by placing each probe on a different bar of the grid floor and confirming that the desired shock amplitude is produced.

Note: Damage or corrosion of the bars can result in weak or uneven shock delivery. Liquids including urine touching the grid along the wall can also adversely affect shock delivery.

3.4.3. Wipe down the chambers and spray the pans beneath the grid floors with the solution not used in Context A (*e.g.*, 1% acetic acid).

3.4.4. Transport animals from the vivarium to the experimental room in a method distinct from the method used for Context A (*e.g.,* a black plastic tub) and place them individually into fear conditioning chambers. Only bring one round's worth of animals to the experiment room at a time (determined by the number of fear conditioning chambers).

3.4.5. Expose all animals to the mild stressor (described below) and video record freezing and activity during the session.

3.4.5.1. After a 180-s baseline period, deliver either a single 1-s, 1-mA footshock (rats) or a single 2-s, 1-mA footshock (mice) to all animals.

Note: Ensure that during the 180-s baseline period freezing should not exceed 5%[12].

3.4.5.2. Remove all animals 30 seconds after shock delivery and promptly return to vivarium.

3.5. On Day 4 of the SEFL procedure, test fear to the mild stressor context.

3.5.1. Set up Context B as done on Day 3.

3.5.2. Transport animals from the vivarium to the experimental room in the same transport as done on Day 3.

3.5.3. Place animals in Context B for 8 minutes without shock delivery and video record freezing throughout the session.

3.5.4. Remove all animals after 8 minutes and promptly return to the vivarium.

4. Data Analysis

4.1. Measure fear during the recorded experimental sessions using freezing, defined as the lack of all movement except that which is needed for respiration.

Note: Freezing is scored most accurately by a blind human scorer, but there are several automated programs that perform well. However, all automated systems must be calibrated to a human observer to be accurate[13].

4.1.1. To score freezing by hand, have an experimenter blind to experimental conditions observes the subject every 4 seconds throughout the time period of interest[3]. At each observation, classify the subject as "freezing" or "not freezing". Compare the number of freezing observations to the total number of observations to determine the percent time spent freezing.

4.1.2. To use automated video analysis to score freezing, first verify that the results from automated video analysis match the results obtained from hand-scoring, as a substantially different freezing score from automated analysis may produce inaccurate results.

Note: A rat or a mouse that has never been shocked should show freezing between 0 and 5%, while higher values suggest poor calibration of the equipment.

4.2. Use the methods described above to measure fear during the time periods of interest (described below).

4.2.1. Measure fear to the trauma context as the percent time spent freezing across the entire 8-min test session on Day 2.

4.2.2. Measure generalization of fear from the trauma context to the mild stressor context as the percent time spent freezing during the 3-min baseline period in Context B on Day 3 prior to shock delivery.

Note: For SEFL, it is important to differentiate the contexts well enough so that there is not substantial generalization.

4.2.3. Measure fear immediately following the shock on Day 3 as the percent time spent freezing during the 30-s period that follows the shock.

4.2.4. Measure fear to the mild stressor context as the percent spent time across the entire 8-min test session on Day 4.

4.3. Measure shock reactivity by the amount, or velocity, of movement during the 3-s period during and immediately following the shock on Day 3.

Note: ANOVAs are recommended for all data analysis, as additional groups (*e.g.*, drug treatment) can be added as necessary.

REPRESENTATIVE RESULTS:

Results of the trauma context test on Day 2 are shown in **Figure 1**. Animals in the trauma condition showed significantly higher levels of freezing in Context A compared to the no trauma controls, indicating acquisition of fear to the trauma context [rats: $F(1,17) = 23.58$, $p < 0.01$; mice: $F(1,14) = 666.50$, $p < 0.0001$]. Freezing during the baseline period before the single shock in the novel context on Day 3 is shown in **Figure 2**. Both the trauma and no trauma animals showed minimal freezing levels that did not differ from each other [rats: $F(1,17) = 3.14$, $p > 0.05$; mice: $F(1,14) = 1.70$, $p > 0.05$]. This demonstrates that Contexts A and B were sufficiently distinct such that the trauma animals did not generalize from the trauma context to the novel context. Reactivity to the single shock on Day 3 is shown in **Figure 3**. The trauma animals showed lower shock reactivity compared to the no trauma controls [rats: $F(1,17) = 3.59$, $p = 0.07$; mice: $F(1,14) = 6.53$, $p < 0.05$]. This indicates that the enhanced fear learning observed in the trauma animals is not due to increased responsiveness to the shock. Freezing during the 30-s period immediately following the single shock on Day 3 is shown in **Figure 4**. The trauma animals showed greater freezing compared to the no trauma controls, indicating that exposure to the traumatic stressor increased fear immediately following the mild stressor [rats: $F(1,17) = 7.29$, $p < 0.05$; mice: $F(1,14) = 6.10$, $p < 0.05$]. The critical test of the SEFL model is the context test on Day 4 (**Figure 5**). During this test, the trauma animals showed significantly higher freezing compared to the no trauma controls, indicating that exposure to the traumatic stressor enhanced fear learning to a subsequent mild stressor [rats: $F(1,17) = 14.06$, $p < 0.01$; mice: $F(1,14) = 12.05$, $p < 0.01$].

FIGURE AND TABLE LEGENDS:

Figure 1: Freezing in Context A on Day 2. (A) Rats in the trauma condition showed higher freezing than rats in the no trauma condition ($p < 0.01$). (B) Mice in the trauma condition showed higher freezing than mice in the no trauma condition ($p < 0.0001$). Error bars represent standard errors.

Figure 2: Baseline freezing in Context B on Day 3. (A) Rats in both the trauma and no trauma conditions exhibited low freezing and were not significantly different from each other during the baseline period before 1 shock ($p > 0.05$). (B) Mice in both the trauma and no trauma conditions

exhibited low freezing and were not significantly different from each other during the baseline period before 1 shock ($p > 0.05$). Error bars represent standard errors.

Figure 3: Trauma decreases shock reactivity on Day 3. (A) Rats in the trauma condition showed a trend towards decreased movement during and immediately following the single shock compared to rats in the no trauma condition ($p = 0.07$). (B) Mice in the trauma condition showed decreased movement during and immediately following the single shock compared to mice in the no trauma condition ($p < 0.05$). Error bars represent standard errors.

Figure 4: Trauma produces enhanced freezing immediately following the single shock on Day 3. (A) Rats in the trauma condition showed significantly enhanced freezing compared to the no trauma groups ($p < 0.05$). (B) Mice in the trauma condition showed significantly enhanced freezing compared to the no trauma groups ($p < 0.05$). Error bars represent standard errors.

Figure 5: Trauma produces enhanced freezing in Context B on Day 4. (A) Rats in the trauma condition showed significantly enhanced freezing compared to the no trauma groups ($p < 0.01$). (B) Mice in the trauma condition showed significantly enhanced freezing compared to the no trauma groups ($p < 0.01$). Error bars represent standard errors.

DISCUSSION:

SEFL is a robust behavioral model of PTSD that can be recapitulated in both rats and mice and can be used to study the sensitized fear responses that characterize PTSD. Following traumatic stress, rodents show an increased fear response in a distinctly different context only after that context is paired with a mild stressor that serves as a reminder of a previous traumatic experience. Following the traumatic stress rodents unsurprisingly show high levels of fear when returned to the traumatic stress context on Day 2, indicating that memory for the traumatic stress is intact (**Figure 1**). However, they show minimal fear generalization from the traumatic stress context to a novel context, as indicated by minimal freezing during the 3-min baseline period on Day 3 (**Figure 2**). This indicates that any learning enhancement to this novel context is not simply due to generalization from the trauma context. Furthermore, animals exposed to the traumatic stressor do not show increased reactivity to the single shock on Day 3 (**Figure 3**), indicating that the learning enhancement is not due to the single shock being perceived as more painful following previous shock exposure. Critically, animals exposed to the traumatic stressor show increased freezing both immediately following the single shock on Day 3 (**Figure 4**) and when returned to the single shock context on Day 4 (**Figure 5**), indicating an enhanced fear response.

Prior experiments have also shown that SEFL produces an enhanced anxiety-like phenotype, as indicated by decreased exploration during the open field test[8]. The effects of the SEFL procedure have been shown to be long-lasting, persisting for at least 90 days after trauma, further establishing the robustness of the model[5]. Hence, SEFL is a valuable tool for probing biological mechanisms of PTSD.

It is important to note that SEFL is not merely due to fear generalization or increased fear expression, since the traumatic experience must come before the mild stressor to increase fear of the context paired with the mild stressor[5]. This precludes the interpretation that SEFL derives from enhanced fear expression. In addition, SEFL cannot be interpreted as generalization of fear from the trauma context to a novel context because previous results show that extinction of fear of the traumatic memory does not mitigate SEFL[5,14]. As a hallmark of PTSD is resistance to extinction (in the form of exposure therapy), this further strengthens the link between SEFL and PTSD[15]. Also, manipulations that produce amnesia of the fear conditioning to the trauma context leave SEFL unaffected, further indicating that SEFL is not due to fear generalization[5,10]. Finally, while we typically examine enhanced learning of contextual fear, the unsignaled shock stress also enhances auditory fear conditioning. These findings indicate that SEFL is a form of stable sensitization in the fear learning circuitry.

While SEFL model is simple in design, aspects of the protocol need to be carefully adhered to for consistent results. For instance, researchers should take caution to use very different methods of transport for Context A and Context B to reduce baseline generalization. Failure to make Contexts A and B sufficiently different can also result in high levels of generalization from Context A to Context B prior to shock, complicating interpretation of the results. Another factor that should also be taken into consideration is the time that animals remain in Context B following the single shock. Failure to remove animals from the context shortly after the single shock can produce extinction of fear to Context B, resulting in decreased freezing during the subsequent context test.

SEFL procedure can be adapted to multiple species, as demonstrated by its ability to produce the sensitized fear phenotype in both mice and rats. It is important to note the slight differences in the protocol between mice and rats; for example, mice require a slightly more intense mild stressor (a 2-s shock compared to a 1-s shock in rats). This is necessary to account for the fact that mice in general show lower freezing levels than rats (see **Figure 5**). Furthermore, it is important to note that these protocols were developed primarily for Long-Evans rats and C57Bl/6 mice. While the robustness of this procedure suggests that it can be adapted for different strains of mice and rats, it is important to consider behavioral differences between strains. For example, DBA/2 mice show decreased fear conditioning compared to C57Bl/6 mice and may therefore require a stronger training protocol[16]. In contrast, Sprague-Dawley rats tend to show higher freezing levels than Long-Evans rats and may require a weaker training protocol to prevent ceiling effects[17]. We recommend the manipulating of current between 0.5 and 1.5 mA, as it is a very effective way to titrate the strength of conditioning.

In conclusion, the SEFL procedure produces reliable and long-lasting behavioral enhancements in fear learning that captures the increased fear responses observed in PTSD patients. SEFL also alters other measures of anxiety including decreased exploratory behavior in the open field test, potentiated startle reactivity, and increased glucocorticoid receptor expression in the BLA[8]. Hence, SEFL can be powerful tool for understanding certain aspects of this PTSD phenotype.

ACKNOWLEDGMENTS:

This work was funded by National Institute of Health R01AA026530 (MSF), Staglin Center for Brain and Behavioral Health (MSF), NRSA-F32 MH10721201A1 and NARSAD 26612 (AKR), and NSF DGE-1650604 (SG).

DISCLOSURES:

Dr. Fanselow is a founding board member of Neurovation Labs.

REFERENCES:

1. Bremner, J.D., Krystal, J.H., Southwick, S.M., Charney, D. S. Functional neuroanatomical correlates of the effects of stress on memory. *Journal of Traumatic Stress.* **8** (4), 527-53 (1995).
2. Dykman, R.A., Ackerman, P.T., Newton, J.E. Posttraumatic stress disorder: a sensitization reaction. *Integrative Physiological and Behavioral Science.* **32** (1), 9-18 (1997).
3. Fanselow, M.S., Bolles, R.C. Naloxone and shock-elicited freezing in the rat. *Journal of Comparative and Physiological Psychology*. **93** (4), 736-44 (1979).
4. Fanselow, M.S. What is Conditioned Fear? *Trends in Neurosciences.* **7**, 460-462 (1984).
5. Rau, V., DeCola, J.P., Fanselow, M.S. Stress-induced enhancement of fear learning: an animal model of posttraumatic stress disorder. *Neuroscience and Biobehavioral Reviews.* **29** (8), 1207-23 (2005).
6. Perusini, J.N., Fanselow, M.S. Neurobehavioral perspectives on the distinction between fear and anxiety. *Learning and Memory*. **22** (9), 417-25 (2015).
7. Poulos, A.M., *et al.* Sensitization of fear learning to mild unconditional stimuli in male and female rats. *Behavioral Neuroscience.* **129** (1), 62-7 (2015).
8. Perusini, J.N., *et al.* Induction and Expression of Fear Sensitization Caused by Acute Traumatic Stress. *Neuropsychopharmacology.* **41** (1), 45-57 (2016).
9. Rau, V., Fanselow, M.S. Exposure to a stressor produces a long lasting enhancement of fear learning in rats. *Stress*. **12** (2), 125-33 (2009).
10. Poulos, A.M., *et al.* Amnesia for early life stress does not preclude the adult development of posttraumatic stress disorder symptoms in rats. *Biological Psychiatry.* **76** (4), 306-14 (2014).
11. Fanselow, M.S., Sigmundi, R.A. Species-specific danger signals, endogenous opioid analgesia, and defensive behavior. *Journal of Experimental Psychology: Animal Behavior Processes.* **12** (3), 301-9 (1986).
12. Jacobs, N.S., Cushman, J.D., Fanselow, M.S. The accurate measurement of fear memory in Pavlovian conditioning: Resolving the baseline issue. *Journal of Neuroscience Methods.* **190** (2), 235-9 (2010).
13. Anagnostaras, S.G., *et al*. Automated assessment of pavlovian conditioned freezing and shock reactivity in mice using the video freeze system. *Frontiers in Behavioral Neuroscience.* (2010).
14. Long, V.A., Fanselow, M.S. Stress-enhanced fear learning in rats is resistant to the effects of immediate massed extinction. *Stress.* **15** (6), 627-36 (2012).
15. Craske, M.G., *et al*. Optimizing inhibitory learning during exposure therapy. *Behaviour Research and Therapy.* **46** (1), 5-27 (2008).
16. Paylor, R., Tracy, R., Wehner, J., Rudy, J.W. DBA/2 and C57BL/6 mice differ in contextual fear but not auditory fear conditioning. *Behavioral Neuroscience.* **108** (4), 810-7 (1994).
17. Graham, L.K., *et al.* Strain and sex differences in fear conditioning: 22 kHz

ultrasonic vocalizations and freezing in rats. *Pyschology and Neuroscience*. **2** (2), 219-225 (2009).

Figure1

Click here to download Figure Fig1.png

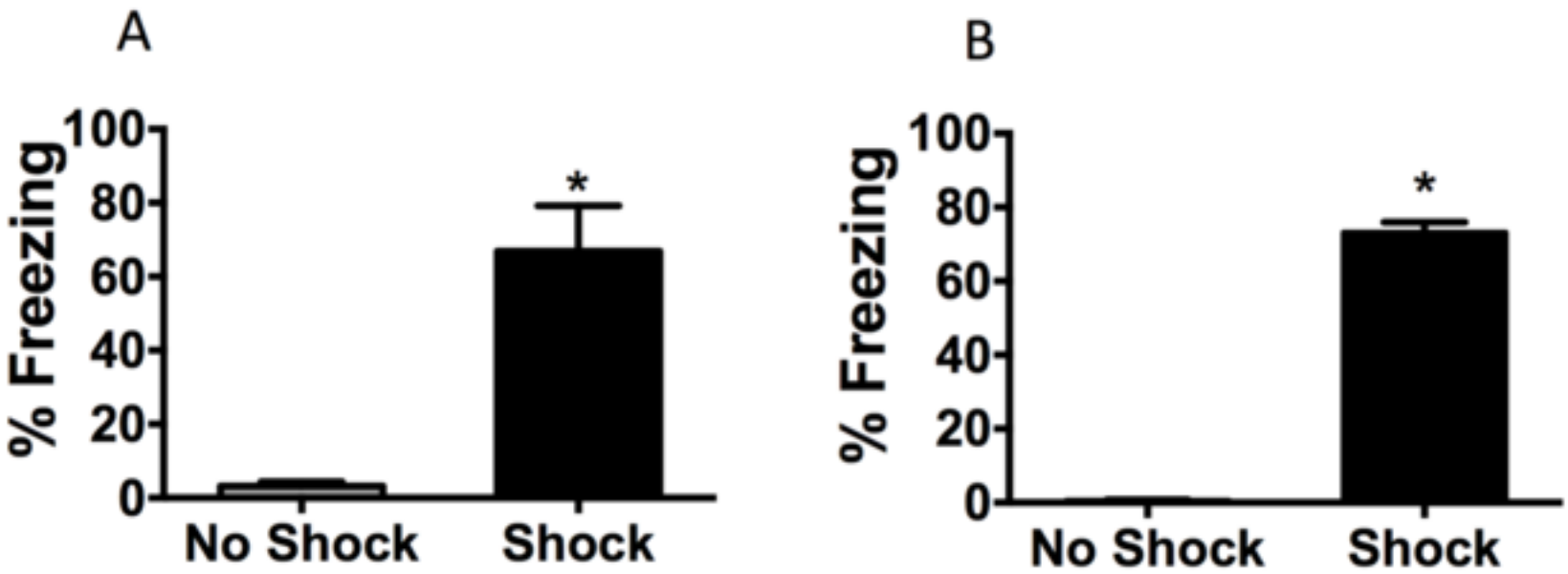


Figure2

Click here to download Figure Fig2.png

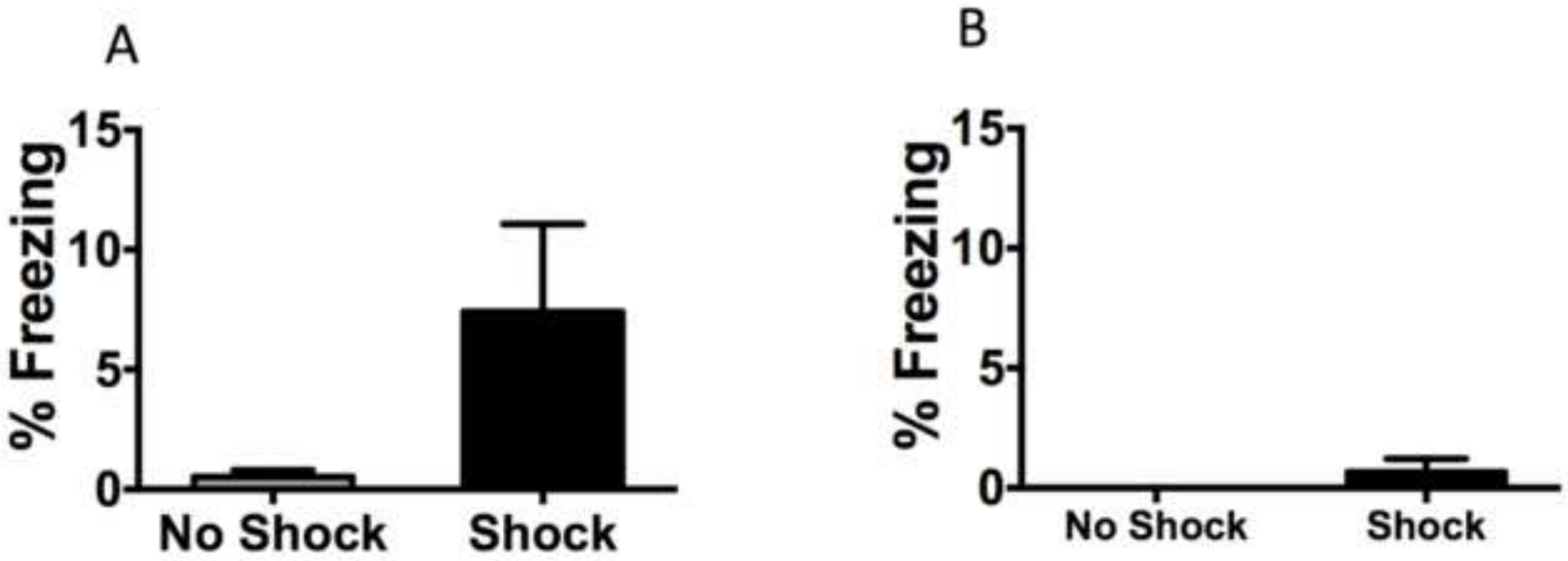


Figure3

Click here to download Figure Fig3.png

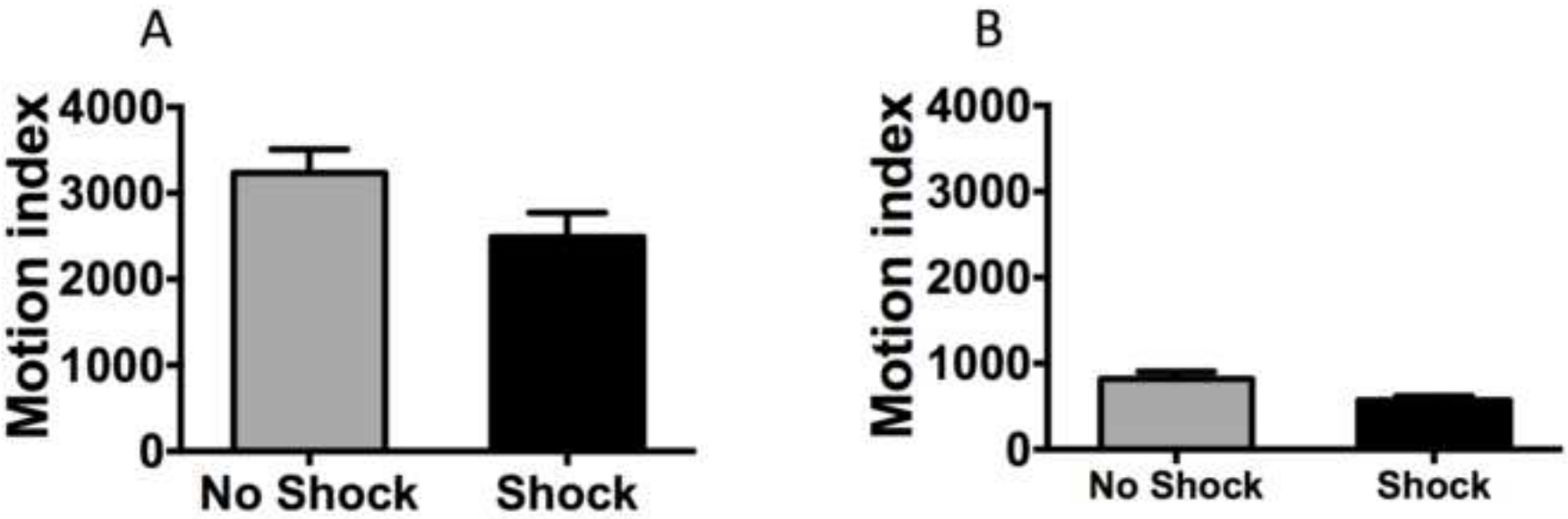


Figure4

Click here to download Figure Fig4.png

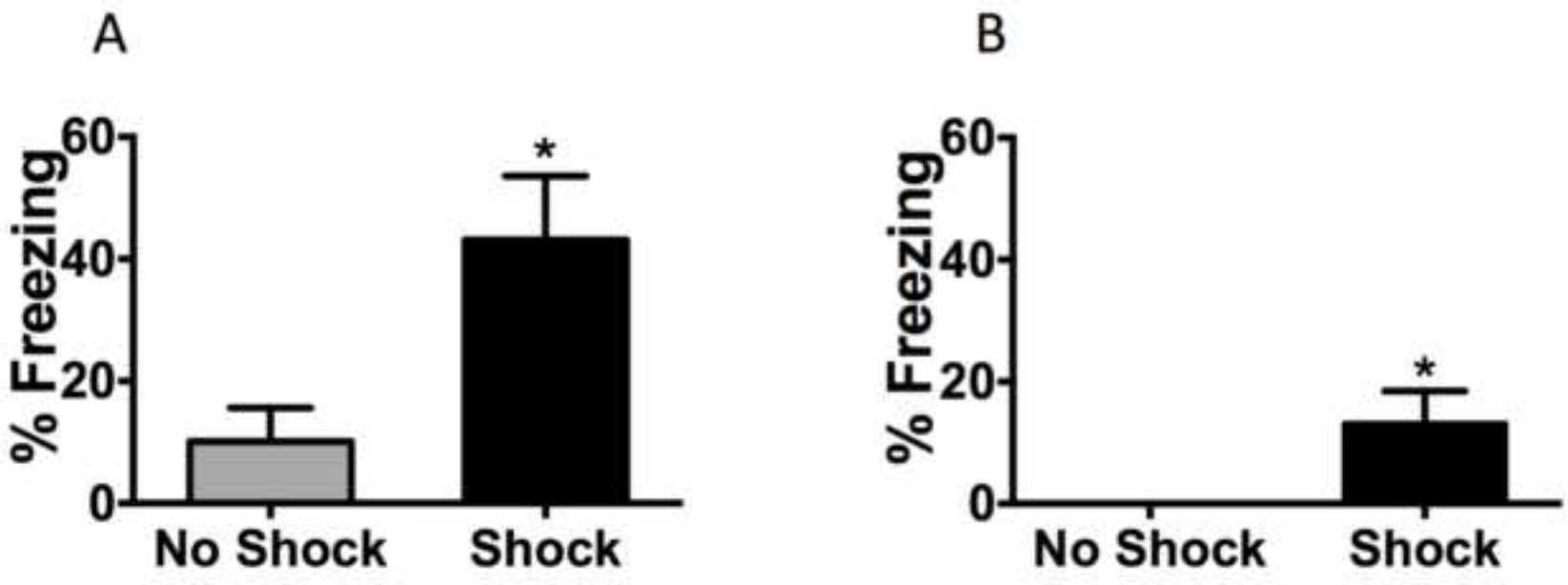
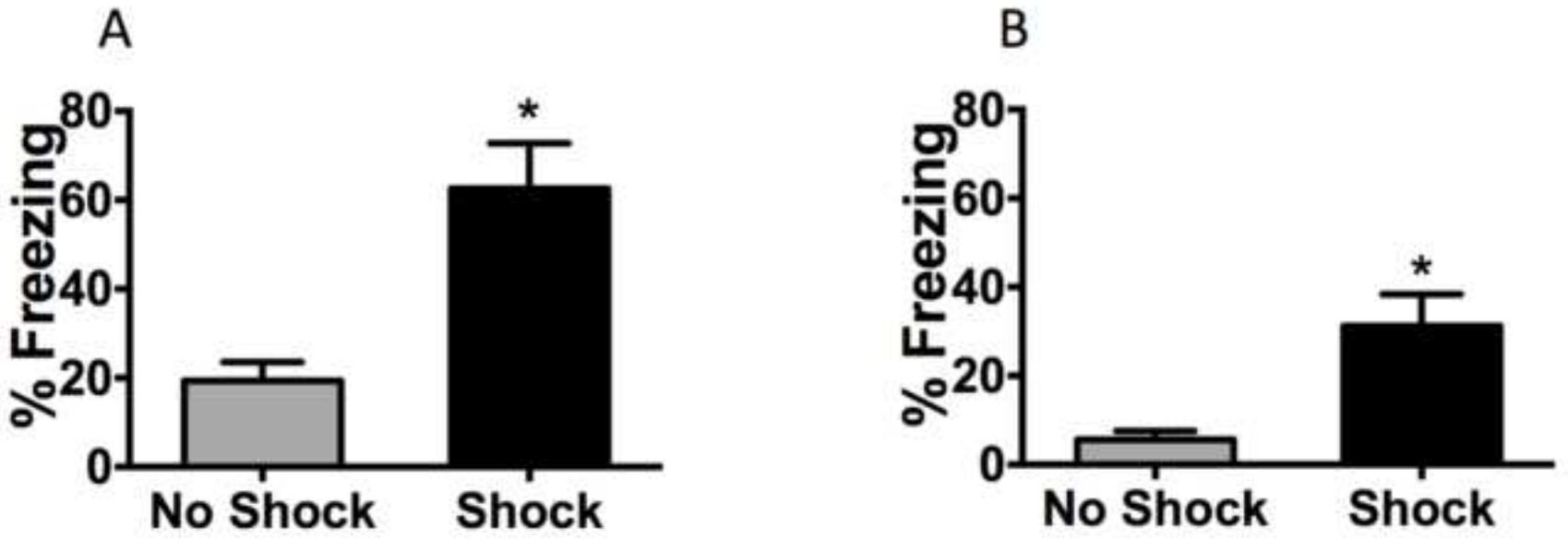


Figure5

Click here to download Figure Fig5.png



Table

Click here to download Table of Materials JoVE_Materials SG 7-3-18 (1).xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Fear Conditioning Chamber for Low Profile Floors	Med Associates Inc.	VFC-008-LP	Fear conditioning chamber
Sound Attenuating Cubicle	Med Associates Inc.	NIR-022SD	Sound-attentuaing cubicle to prevent intrusion of outside noise
NIR/White Light Control Box	Med Associates Inc.	NIR-100VR	Light control box capable of delivering white and near-infra
NIR VFC Light Box	Med Associates Inc.	NIR-100L2	White overhead houselight
Windex Original Glass Cleaner	Windex		Solution for cleaning and scenting fear conditioning chamb
Acetic acid	Fisher Scientific	A38-212	Solution for cleaning and scenting fear conditioning chamb
A-Frame Chamber Insert	Med Associates Inc.	ENV-008-IRT	Black Plexiglas triangular insert to differentiate internal lay
Curved Wall Insert	Med Associates Inc.	VFC-008-CWI	White plastic sheet to differentiate internal layout of Conte
Low Profile Contextual Grid Floor with 1/8" Grid Rods for Mouse	Med Associates Inc.	VFC-005A	Flat grid floor for mice
Low Profile Contextual Grid Floor with Alternating 1/8" & 3/16" Grid Rods Mouse	Med Associates Inc.	VFC-005-S	Staggered grid floor for mice
Low Profile Contextual Grid Floor with 1/8" Staggered Grid Rods for Mouse	Med Associates Inc.	VFC-005A-L	Alternating grid floor for mice
Low Profile Contextual Grid Floor with 3/16" Grid Rods for Rat	Med Associates Inc.	VFC-005	Flat grid floor for rats
Low Profile Contextual Grid Floor with Alternating 3/16" & 3/8" Grid Rods	Med Associates Inc.	VFC-005-L	Alternating grid floor for rats
Low Profile Contextual Grid Floor with 3/16" Staggered Grid Rods for Rat	Med Associates Inc.	VFC-005-S	Staggered grid floor for rats

Metal pans	Med Associates Inc.		Metal pans to catch droppings underneath grid floors
Standalone Aversive Stimulator/Scrambler	Med Associates Inc.	ENV-414S	Shock generator and scrambler for footshock delivery
Multimeter	Fluke	87-5	Tool for measuring footshock amplitude
VideoFreeze Software	Med Associates Inc.	SOF-843	VideoFreeze software for controlling shock delivery
High Speed Firewire Monochrome Video Camera	Med Associates Inc.	VID-CAM-MONO-4	Video camera capable of recording in near-infrared light

ared light

ers between animals
ers between animals

out of Contexts A and B

exts A and B

Compatibility Report for JoVE_Materials SG.xls
Run on 7/3/2018 11:18

The following features in this workbook are not supported by earlier versions of Excel. These features may be lost or degraded when opening this workbook in an earlier version of Excel or if you save this workbook in an earlier file format.

Minor loss of fidelity	# of occurrences
Some cells or styles in this workbook contain formatting that is not supported by the selected file format. These formats will be converted to the closest format available.	2

Version

Excel 97-2003

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: **Stress-enhanced fear learning, a robust rodent model of post-traumatic stress disorder**

Author(s): **Rajbhandari AK, Gonzalez ST, Fanselow MS**

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: ☑ Standard Access ☐ Open Access

Item 2 (check one box):

☑ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	**Michael Fanselow**
Department:	**Psychology**
Institution:	**UCLA**
Article Title:	**Stress-enhanced fear learning, a robust rodent model of post-traumatic stress disorder**
Signature:	 Date: **04/09/2018**

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

UNIVERSITY OF CALIFORNIA, LOS ANGELES UCLA

BERKELEY • DAVIS • IRVINE • LOS ANGELES • MERCED • RIVERSIDE • SAN DIEGO • SAN FRANCISCO SANTA BARBARA • SANTA CRUZ

DEPARTMENT OF PSYCHOLOGY
DEPARTMENT OF PSYCHIATRY
UNIVERSITY OF CALIFORNIA-LOS ANGELES
405 HILGARD AVENUE
LOS ANGELES, CALIFORNIA 90095-1563

Alisha DSouza, Ph.D.
Senior Review Editor
JoVE
617.674.1888

Dear Dr. DSouza

We appreciate the rapid and thorough reviews of our manuscript entitled, "Stress-enhanced fear learning, a robust rodent model of post-traumatic stress disorder". We thank you for the opportunity to submit a revised version of the paper. The comments were very helpful and we have incorporated all the suggestions into our revision; changes are indicated in red.

Sincerely,

Michael S. Fanselow, PhD
Staglin Family Chair in Psychology,
Director Staglin Music Festival Center for Brain & Behavioral Health
UCLA Distinguished Professor,
Chair of Learning & Behavior,
Director UCLA Behavioral Testing Core,
Co-Director UCLA ICLM.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The authors lucidly describe stress-enhanced fear learning (SEFL), which arguably is the most applicable rodent model of PTSD. The introduction, protocol, results, and discussion sections are all outstandingly written, and the figures convincingly support the SEFL model. This article will likely impact the preclinical PTSD research as well as the general field of fear learning and memory.

We appreciate the comment and feedback.

Reviewer #2:

In the manuscript by Rajbhandari et al, the authors detail a stress-enhanced fear learning protocol for use in rats and mice. Overall, the protocol is thorough in detail with clear description of expected outcomes. Just a few comments to help readers interested in adopting the approach and to enhance readability:
1. In the DSM-5, PTSD is no longer classified as an anxiety disorder. It is now under "Trauma and Stressor-Related Disorders".

Changed "anxiety disorder" to "psychiatric disorder" to reflect DSM classification in Long Abstract and Introduction.

2. Some justification and discussion regarding the requirement for single housing, which is a stressor in itself, would be helpful.
Added statement to Protocol: "Single housing is advised as group housing produces variability due to interactions between animals in the home cage, particularly following stress exposure."

3. Please include instruction in the Protocol for when animals should be transported to the fear conditioning room on SEFL days. For instance, is it "one round's worth of animals" or all animals, followed by an acclimation period? If it is the latter, where are the animals placed for acclimation?
Added statement to Protocol: "Only bring one round's worth of animals to the experiment room at a time."

4. Given the multiple statistical comparisons in the Representative Results section, are t-tests the most appropriate statistical choice?
We have revised our Representative Results section to use one-way ANOVAs rather than t-tests, although the statistical results are the same. ANOVAs are typically applicable for experiments using this method, which often involve addition of another independent variables (e.g. drug treatment). We argue that corrections for multiple statistical comparisons are not necessary because we are making comparisons between only 2 independent groups on each measure, and as each measure is collected at a different time point and for different durations it would be inappropriate to combine all measures into a single analysis.

5. There seems to be a small detail that is likely a simple typo. The Discussion states that mice require 2-sec foot shocks, but the Protocol (3.2.4.1) states 1-sec for mice.
For mouse experiments, the traumatic stressor consists of 10 1-sec, 1-mA footshocks while the mild stressor consists of a single 2-sec, 1-mA footshock. The portion of the protocol referenced in this comment (3.2.4.1) describes the 1-sec shocks used for the traumatic stressor, while the Discussion is referring to the 2-sec shock used as the mild stressor, as described in the Protocol (3.4.3.1).

Reviewer #3:

This is a clearly written methods paper outlining the procedure to induce stress-enhanced fear learning (SEFL), a model of post-traumatic stress disorder. Animals (rats or mice) are exposed to a traumatic event (15 unsignaled electric footshocks in Context A). The following day, they are exposed to a single unsignaled footshock in a second different context (B). On the third day, contextual fear memory is measured in Context B. Animals exposed to the trauma show enhanced levels of freezing on the test day. The senior author (Fanselow) has published multiple papers using this technique starting in 2005 (Rau, DeCola and Fanselow, 2005).
One benefit of this protocol is that it recapitulates certain aspects of PTSD, namely a long-term sensitization of fear learning caused by an acute stressor. One of the strengths of this manuscript is the authors insistence that Context A and B must be sufficiently different to reduce baseline generalization between these two contexts. Indeed, even the method of transportation to the testing chambers must be different on days 1 and 2. I have only a few minor suggestions for improvement:

1. The authors could add a sentence summarizing a few of the findings from their 2009 paper (Rau and Faneslow, 2009). Namely, that pre-exposure to 1 shock does not lead to SEFL, but 4 or 15 shocks do.
Added statement to Introduction: "In contrast, pre-exposure to a single footshock does not produce SEFL (Rau and Fanselow, 2009)."

2. The authors note that extinction in context A does not mitigate SEFL. They could also add that a hallmark of PTSD is that it is resistant to exposure therapy (extinction), thus strengthening the link between SEFL and PTSD.

Added statement to Discussion: "As a hallmark of PTSD is resistance to extinction (in the form of exposure therapy), this further strengthens the link between SEFL and PTSD (Craske et al, 2008)."