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Video Article

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

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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.

Video Link

The video component of this article can be found at https://www.jove.com/video/58306/

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. Rats
   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].
   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).

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

2. Equipment Setup

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

2. Provide accurate timing and amplitude of footshock delivery for each context.
   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. 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**).
   3. Ensure that a method for controlling the timing and amplitude of shock delivery (*e.g.,* computer software) is available (see **Materials Table**).

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.
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

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.
2. On Day 1 of the SEFL procedure, place subjects in Context A, where they will receive the traumatic stressor.
   1. Set up Context A with one set of grid floors (*e.g.*, flat grids) and illuminate the chambers with visible light.
   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. 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.
    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.
    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.
    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.
    7. After 90 minutes (rat experiments) or 60 minutes (mouse experiments), return all animals to their homecages and promptly return to the vivarium.

3. On Day 2 of the SEFL procedure, assess the fear to the trauma context if desired.
    1. Set up Context A as done on Day 1.
    2. Transport animals to the experiment room in their home cages as on Day 1.
    3. Place animals in Context A for 8 minutes without shock delivery and video record the behavior during the entire session.
    4. After 8 minutes, return all animals to their homecages and promptly return to the vivarium.

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].
    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.
    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. 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).
    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).
    5. Expose all animals to the mild stressor (described below) and video record freezing and activity during the session.
        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].
        2. Remove all animals 30 seconds after shock delivery and promptly return to vivarium.

5. On Day 4 of the SEFL procedure, test fear to the mild stressor context.
    1. Set up Context B as done on Day 3.
    2. Transport animals from the vivarium to the experimental room in the same transport as done on Day 3.
    3. Place animals in Context B for 8 minutes without shock delivery and video record freezing throughout the session.
    4. Remove all animals after 8 minutes and promptly return to the vivarium.

4. Data Analysis

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].
    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.
    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

2. Use the methods described above to measure fear during the time periods of interest (described below).
    1. Measure fear to the trauma context as the percent time spent freezing across the entire 8-min test session on Day 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.

    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. Measure fear to the mild stressor context as the percent spent time across the entire 8-min test session on Day 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: $F(1,14) = 12.05$, $p < 0.01$].

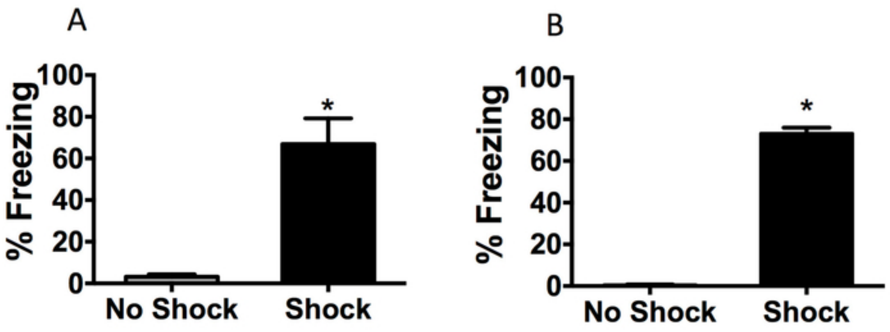

Figure 1: Freezing in Context A on Day 2. (A) Rats in the trauma condition showed higher freezing than rats in the no trauma condition ($p < 0.01$). (B) Mice in the trauma condition showed higher freezing than mice in the no trauma condition ($p < 0.0001$). Error bars represent standard errors. Please click here to view a larger version of this figure.

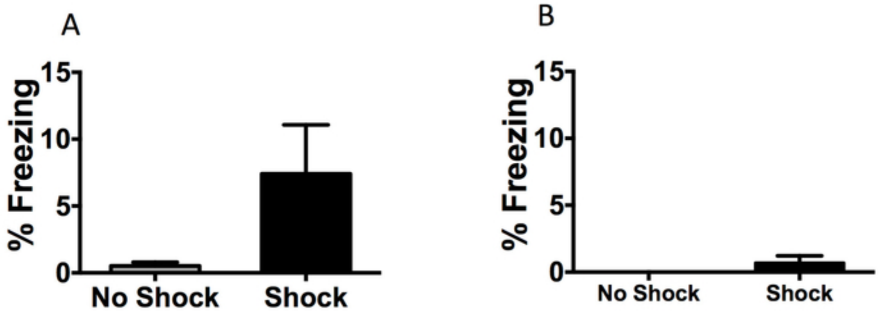

Figure 2: Baseline freezing in Context B on Day 3. (A) Rats in both the trauma and no trauma conditions exhibited low freezing and were not significantly different from each other during the baseline period before 1 shock ($p > 0.05$). (B) Mice in both the trauma and no trauma conditions exhibited low freezing and were not significantly different from each other during the baseline period before 1 shock ($p > 0.05$). Error bars represent standard errors. Please click here to view a larger version of this figure.

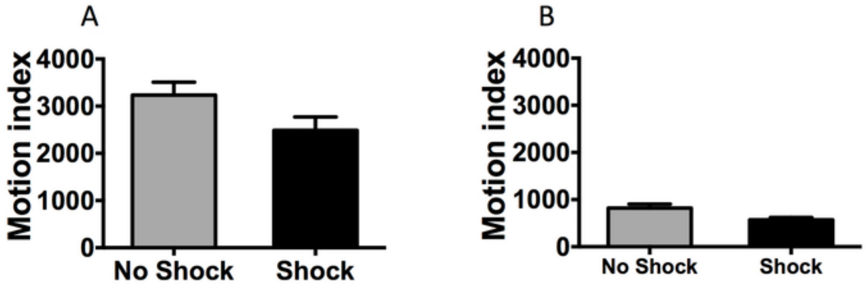

Figure 3: Trauma decreases shock reactivity on Day 3. (A) Rats in the trauma condition showed a trend towards decreased movement during and immediately following the single shock compared to rats in the no trauma condition ($p = 0.07$). (B) Mice in the trauma condition showed decreased movement during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

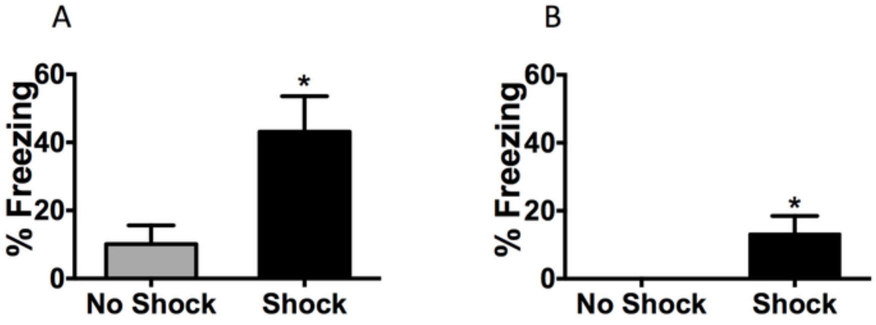

Figure 4: Trauma produces enhanced freezing immediately following the single shock on Day 3. (A) Rats in the trauma condition showed significantly enhanced freezing compared to the no trauma groups ($p < 0.05$). (B) Mice in the trauma condition showed significantly enhanced freezing compared to the no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

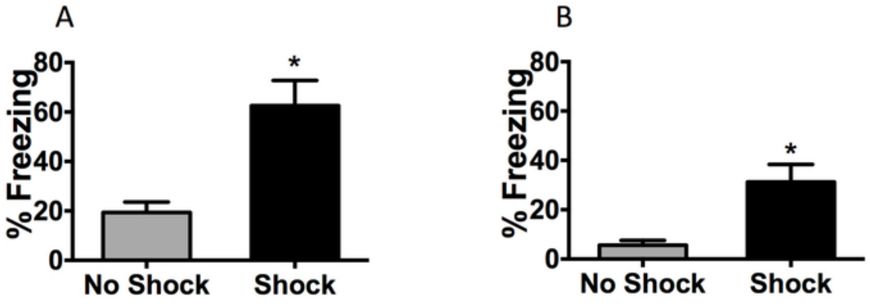

Figure 5: Trauma produces enhanced freezing in Context B on Day 4. (A) Rats in the trauma condition showed significantly enhanced freezing compared to the no trauma groups ($p < 0.01$). (B) Mice in the trauma condition showed significantly enhanced freezing compared to the no trauma groups ($p < 0.01$). Error bars represent standard errors. Please click here to view a larger version of this figure.

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.

Disclosures

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

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