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Tulane University
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SCIENCE AND ENGINEERING

June 16, 2020

Re: JoVE manuscript JoVE61536

Dear Dr. DSouza,

Please find attached the revised version of our manuscript entitled, "A novel Pavlovian fear conditioning paradigm to study freezing and flight behavior". We have revised the manuscript according to the editorial comments as well as the suggestions of all three reviewers and we address all concerns point-by-point in the attached rebuttal.

In response to Reviewers 1 and 3, we added additional details to the Discussion related to control experiments used to establish the parameters of the protocol. In response to Reviewer 1, we modified Figure 1 to include illustrations of the different behavioral contexts. We also modified this Figure to better illustrate the use of auditory pips, as suggested by Reviewer 3. Finally, we retitled the protocol in light of Reviewer 3's comments.

We kindly thank you for your time and consideration.

Sincerely,

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TITLE:

A Novel Pavlovian Fear Conditioning Paradigm to Study Freezing and Flight Behavior

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KEYWORDS:
Fear conditioning, freezing, flight, anxiety, fear, panic, defensive behavior

SUMMARY:
Defensive behavioral responses are contingent upon threat intensity, proximity, and context of exposure. Based on these factors, we developed a classical conditioning paradigm that elicits clear transitions between conditioned freezing and flight behavior within individual subjects. This model is crucial for the understanding the pathologies involved in anxiety, panic, and post-traumatic stress disorders.

ABSTRACT:
Fear- and anxiety-related behaviors significantly contribute to an organism's survival. However, exaggerated defensive responses to perceived threat are characteristic of various anxiety disorders, which are the most prevalent form of mental illness in the United States. Discovering the neurobiological mechanisms responsible for defensive behaviors will aid in the development of novel therapeutic interventions. Pavlovian fear conditioning is a widely used laboratory paradigm to study fear-related learning and memory. A major limitation of traditional Pavlovian fear conditioning paradigms is that freezing is the only defensive behavior monitored. We recently developed a modified Pavlovian fear conditioning paradigm that allows us to study both conditioned freezing and flight (also known as escape) behavior within individual subjects. This model employs higher intensity footshocks and a greater number of pairings between the conditioned stimulus and unconditioned stimulus. Additionally, this conditioned flight paradigm utilizes serial presentation of pure tone and white noise auditory stimuli as the conditioned stimulus. Following conditioning in this paradigm, mice exhibit freezing behavior in response to the tone stimulus, and flight responses during the white noise. This conditioning model can be applied to the study of rapid and flexible transitions between behavioral responses necessary for survival.

INTRODUCTION:

Fear is an evolutionarily conserved adaptive response to an immediate threat[1,2]. While organisms possess innate defensive responses to a threat, learned associations are crucial to elicit appropriate defensive responses to stimuli predictive of danger[3]. Dysregulation in brain circuits controlling defensive responses is likely to contribute to maladaptive reactions associated with multiple debilitating anxiety disorders, such as post-traumatic stress disorder (PTSD), panic disorder[4], and specific phobias[5,6]. The prevalence rate in the United States for anxiety disorders is 19.1% for adults and 31.9% in adolescents[7,8]. The burden of these illnesses is extremely high on the daily routine of individuals and negatively impacts their quality of life.

Over the last several decades, Pavlovian fear conditioning has served as a powerful model system to gain tremendous insight into the neural mechanisms underlying fear-related learning and memory[9–11]. Pavlovian fear conditioning entails pairing a conditioned stimulus (CS, such as an auditory stimulus) with an aversive unconditioned stimulus (US; for example, an electrical footshock)[12]. Because freezing is the dominant behavior evoked and measured in standard Pavlovian conditioning paradigms, the neural control mechanisms of active forms of defensive behavior such as escape/flight responses remain largely unexplored. Previous studies show that different forms of defensive behavior, such as flight, are evoked depending upon the threat intensity, proximity and context[13,14]. Studying how the brain controls different types of defensive behavior may significantly contribute to the understanding of the neuronal processes that are dysregulated in fear and anxiety disorders.

To address this critical need, we developed a modified Pavlovian conditioning paradigm that elicits flight and escape jumps, in addition to freezing[15]. In this paradigm, mice are conditioned with a serial compound stimulus (SCS) consisting of a pure tone followed by white noise. Following two days of pairing the SCS with a strong electrical footshock, mice exhibit freezing in response to the tone component and flight during the white noise. Behavioral switches between conditioned freezing and flight behavior are rapid and consistent. Interestingly, mice exhibit flight behavior only when the white noise CS is presented in the same context as a previously delivered footshock (the conditioning context) but not in a neutral context. Instead, freezing responses dominate in this the neutral context, with significantly greater levels of freezing in response to the white noise compared to the tone. This is consistent with the role of context in modulating defensive response intensity and with the regulatory role of contextual information in fear-related learning and memory found in traditional threat conditioning paradigms[16,17]. This model allows for direct, within-subject comparisons of multiple defensive behaviors in a context-specific manner.

PROTOCOL:

The following steps/procedures were conducted in accordance with institutional guidelines after approval from the Institutional Animal Care & Use Committee of Tulane University.

1. Preparation of mice

1.1. Use male and/or female adult mice aged between 3-5 months. In the present study, we used male C57BL/6J mice obtained from Jackson Laboratory, but any mouse strain from a reputable supplier can be used.

1.2. At least one week before the experiment, house all the mice individually on a 12:12 h light/dark cycle throughout the study. Provide the mice *ad libitum* access to food and water.

1.3. Perform all behavioral experiments during the light cycle. Perform all sessions at the same time of day within an individual cohort. For example, if starting the experiment at 9 AM on Day 1, continue starting at that time until the experiment is completed.

2. Preparation of study materials

2.1. Study contexts

2.1.1. Choose two different contexts to perform the experiments in.

2.1.2. Use Context A for a cylindrical chamber composed of clear Plexiglas (diameter 30 cm), with a smooth Plexiglas floor. The height of the chamber should be sufficient to prevent escape (at least 30 cm high).

2.1.3. Use Context B for a rectangular enclosure (25 cm x 30 cm) with an electrical grid floor used to deliver alternating current footshocks. The height of this chamber is very important and should be at least 35 cm. high. Alternatively, use a transparent roof (ensure that video can be recoreded through this material).

NOTE: Use a chamber with smooth wall surfaces that can be easily cleaned.

2.1.4. Use a different cleaning solution to clean the contexts. For example, clean context A with 1% acetic acid and context B with 70% ethanol. Clean the contexts before beginning the first session, between testing individual mice, and after completion of the day's sessions. This is vital to remove the olfactory cues from previous mice. Thorough cleaning will also help prevent urine scaling on the shock grid, which will compromise conditioning sessions.

NOTE: The cleaning solutions also serve as an olfactory cue, therefore use the same cleaning liquid for a particular context.

2.1.5. Place context A or context B in a sound-attenuating box during respective study sessions.

2.2. Audio generator

2.2.1. Mount an overhead speaker above the contexts to deliver auditory stimuli at 75 dB.

2.2.2. Use a programmable audio generator to generate auditory stimuli on a pre-defined schedule. The 7.5 kHz pure tone is a sound with a sinusoidal waveform, whereas the white noise is a random signal having equal intensity at different frequencies, ranging from 1-20,000 Hz.

2.2.3. Use TTL pulses to deliver auditory stimuli and shock signals with temporal precision.

NOTE: Before starting the experiments, measure the sound intensity output from the mounted speaker in each chamber using dB meter.

2.3. Shocker: Connect the shocker with the electrical grid floor which is used to deliver the 0.9 mA AC shock. Define the frequency, onset, and duration of shocks in a computer program. Deliver each shock stimulus at the end of each SCS for a duration of 1 s, totaling five SCS-shock pairings per conditioning session.

3. Preparation of computer program and video tracking

3.1. Generate behavioral protocols using coding in a software program.

3.2. In the program, define the serial compound stimulus (SCS). This stimulus is a serial presentation of a 10 s pure tone (each pip is presented for 500 ms, at frequency of 7.5 kHz and rate of 1 Hz) and 10 s white noise (500 ms pips at 1 Hz).

3.3. Define the inter-trial intervals (ITI) presented following each trial, pseudorandomly.

3.4. During the study, record all mouse behavior to video for subsequent analysis.

NOTE: Commercially available fear conditioning boxes may not be set up to record the behaviors through the top-mounted camera. This is very important since the recorded video is used to calculate vertical movement, speed and total distance travelled by the animal.

3.5. For setting the software tracking, place a test mouse in each relevant context, adjust the contour size, and define the center of gravity. This will ensure the acquisition of reliable data on relative position. In addition, define the whole context area accessible to the subject.

NOTE: The adjustment of contour size for both contexts is important as the change in brightness in different contexts will change the contour size.

3.6. Determine a calibration coefficient using the chambers' known sizes and the camera's pixel dimensions and calculate speed (cm/s).

3.7. Synchronize the central computer's event markers to their real-time occurrences.

4. Behavioral experiment

4.1. Turn on all the equipment: computers, fear conditioning box controller, shocker, and video recording software. Make sure all the switches of relevant instruments are properly switched on.

4.2. Check all the functions including tone, white noise, and shock delivery, and set up the system for the data acquisition.

4.3. Transport the animals from their storage room to the conditioning room. Allow them to acclimatize there for at least 10 min.

4.4. Take the animal out from the home cage, gently place it in the respective context, and then immediately activate the computer programs.

NOTE: The initialization of both fear conditioning system and data collection (timestamps, mouse tracking and video recording) software at a time can be synchronized using TTL pulse mediated activations.

4.5. Pre-conditioning/Pre-exposure

4.5.1. On Day 1, place the subject into context A (neutral context). Allow it to acclimate to the chamber for 3 min (the baseline period), and then expose it to 4 trials of a SCS of 20 s total duration (**Figure 1A-1B**).

4.5.2. Maintain an 80 s average pseudorandom intertrial interval (ITI) (range 60-100 s). The total duration of each pre-exposure session is 590 s.

4.6. Fear conditioning

4.6.1. On Day 2 and Day 3, place the subject into Context B. Following a 3 min baseline period, expose the subject to five pairings of the SCS co-terminating with a 1 s, 0.9 mA AC footshock.

4.6.2. Maintain a 120 s average pseudorandom ITI (range 90-150 s). Have each conditioning session last for 820 s in total (**Figure 1A**).

4.6.3. Depending on the goal of the experiment, subject mice on Day 4 to either a recall test (see step 4.7) or to fear extinction (see step 4.8).

4.7. Fear recall (to test context dependence)

4.7.1. On Day 4, place the subject into Context A. After the 3 min baseline period, present it with 4 trials of the SCS without footshock, over 590 s.

4.7.2. Maintain an 80 s average pseudorandom ITI (range 60-100 s).

4.8. Fear extinction

4.8.1. On Day 4, place the subject into context B. Following the 3 min baseline period, present 16 trials of the SCS without footshock, over 1910 s.

4.8.2. Maintain a 90 s average pseudorandom ITI (range 60-120 s).

4.9. Return the animal to its home cage and repeat the procedure for all the animals.

5. Quantification of behavior

5.1. Have an observer blind to the experiment score the recorded videos for freezing behavior using automatic freezing detector thresholding followed by a frame-by-frame analysis of pixel changes.

NOTE: Other software packages can also be used to calculate freezing automatically by using 2 camera system. It is also possible for an observer to manually score freezing behavior.

5.2. Define freezing as a complete cessation of bodily movements, except for those required for respiration, for a minimum of 1 s.

5.3. Score jumps when all 4 of the paws leave the floor, resulting in a vertical and/or horizontal movement.

5.4. Export the marked file with freezing, jump and event markers.

5.5. Extract relevant events (freezing and jumps) from defined time periods (e.g., 10 s duration of pre-SCS, tone and white noise, for each trial).

5.6. Using the extracted start-stop durations of events in a spreadsheet file, calculate the duration of freezing (in s) by subtracting start time from end time, from the respective trial periods.

5.7. Represent this data trial-wise or day-wise by summing up freezing duration from all trials.

NOTE: Depending on the purpose of the study, the flight or freezing behaviors can be scored and calculated from any trial/duration from the study session.

5.8. Sum the total number of jumps from a particular trial duration.

5.9. Extract the file generated by mouse tracking coordinates from frame by frame X-Y axis movement of the center of gravity of mouse and calculate the speed of the mouse (cm/s).

NOTE: The speed data may be present either in the cm/s or pixel/s format. Convert the pixel/s unit to cm/s by using pixel/inch or cm value defined in the video for that testing context (please see section 3.6).

5.10. After extracting speed data for frame by frame movement of the animal, based on frame rate of the video (preferably 30 frames/s), calculate the average speed of the animal in a specific frame number bracket (multiply start and end times in s by 30 to get the start and end frame number).

5.11. Calculate the flight scores by dividing the average speed during each SCS by the average speed during the 10 s pre-SCS (baseline, BL) and then adding 1 point for each escape jump (speedCS/speedBL + # of jumps). A flight score of 1 therefore indicates no change in flight behavior from the pre-SCS period.

5.12. Optionally, score videos manually for other behaviors such as rearing and grooming.

6. Statistical analysis

6.1. Analyze data for statistical significance using statistical analysis software. For all tests, the definition of statistical significance is $p<0.05$.

6.2. Check the data for normal distribution using the Shapiro-Wilk normality test ($\alpha=0.05$).

6.3. To test the effect of cues, carry out the pairwise comparisons using the appropriate parametric (paired t-test) or non-parametric (Wilcoxon signed-rank test) test.

6.4. To assess the 2-way interaction of factors (cue X trial), perform a 2-way ANOVA followed by post-hoc tests (e.g., Bonferroni's multiple comparison test/Tukey's test).

REPRESENTATIVE RESULTS:

As described in the diagram (**Figure 1A**), the session starts with pre-exposure (Day 1), followed by fear conditioning (Days 2 and 3), and then either extinction or retrieval (Day 4).

Presentations of the SCS in the pre-exposure (Day 1) session did not elicit flight or freezing response in the mice (**Figure 2A-2B**). Behavioral analysis during conditioning (Days 2 and 3) revealed that the tone component of the SCS significantly enhanced freezing compared to freezing during the pre-SCS (**Figure 2B,2E**). Flight scores changed significantly across sessions (Day 1 to Day 3, n = 20; **Figure 2A**). Mice showed higher speed and more jumps, and thus greater flight scores, to the white noise cue compared to tone (**Figure 2C-2D**). Mice showed a clear transition of defensive behavior--exhibiting lower flight scores during the tone followed by higher flight scores during white noise (**Figure 2F**) and vice-versa for freezing responses (**Figure 2G**).

To test for the effect of threat proximity and context on conditioned flight, mice were split into two groups: one group underwent extinction training in the conditioning context (**Figure 3A-3B**), and another group was tested for fear memory recall by exposing them to the SCS in a neutral context (**Figure 3C-3D**). Mice subjected to the 16 trials of extinction training showed rapid extinction of conditioned flight (n = 12). Flight scores during the first block of four trials were higher during white noise as compared to the tone (**Figure 3A**). Flight behavior was no longer elicited by either cue at the end of the extinction session. There was an overall decrease in tone-induced freezing and an increase in white noise-mediated freezing during the extinction session. Freezing for the first block of four trials was significantly higher to the tone compared to the white noise (**Figure 3B**). This suggests imminence of the threat is vital for the flight response.

The flight response was diminished in a context-dependent manner. Exposure to the white noise in the neutral context did not elicit flight ($n = 8$). Instead, white noise presentations in the neutral context elicited freezing responses which were higher than those elicited by the tone (**Figure 3C-3D**). This demonstrates the importance of context in modulating defensive responding.

Figure 1: Study design for flight paradigm. **A**) Diagram of the sessions of the behavioral conditioned flight paradigm. **B**) Diagram detailing the composition of the serial compound stimulus (SCS), as well as the timing of the US. **C**) Context A - served as a neutral context, and used during pre-exposure and recall sessions. **D**) Context B – used for fear conditioning. This figure has been modified from Fadok et al. 2017.

Figure 2: Conditioned flight response. **A**) Comparison of average trial-wise flight scores ($n = 20$) following presentation of the tone and white noise across Days 1-3. A significant change in the flight scores across sessions have been noted (Day 1 to Day 3; two-way repeated measures ANOVA, cue × trial interaction, $F (13, 266) = 5.795$; $P<0.0001$). Post-hoc Bonferroni's multiple comparison test reveals a significant difference between tone and white noise induced flight scores at fear conditioning Day 1 (trial 4, $P < 0.05$) and Day 2 (trials 2-5, $P < 0.001$). **B**) Comparison of average trial-wise % freezing during the tone and white noise periods across Days 1-3. Note a statistically significant changes in % freezing across the sessions (Day 1 to Day 3, $n = 20$; two-way repeated-measures ANOVA, cue × trial interaction, $F (13, 266) = 20.81$; $P < 0.001$; **Figure 2B**). Post-hoc Bonferroni's multiple comparison test reveals a significant difference between tone and white noise induced freezing at fear conditioning Day 1 (trial 4 and 5, $P < 0.001$) and Day 2 (all trials, $P < 0.001$). **C**) Comparison of number of jump escape responses in during the pre-SCS, tone, white noise, and shock periods on Day 3. One-way ANOVA followed by Bonferroni's multiple comparisons test showed that escape jumps were significantly higher during white noise and shock as compared to tone period ($P < 0.01$ and $P < 0.001$, respectively). **D**) Comparison of flight scores during the presentation of tone and white noise on Day 3. Note a significantly higher flight scores on Day 3 during white noise period ($P < 0.001$, Wilcoxon matched-pairs signed-rank test). **E**) Comparison of % freezing during the pre-SCS, tone, and white noise on Day 3. Moreover, freezing behavior on Day 3 reveals significant effect of tone and white noise (one-way repeated-measures ANOVA, $F = 56.82$, $P<0.01$). Bonferroni's multiple comparisons test showed that presentation of tone significantly increases % freezing vs pre-SCS duration ($P < 0.01$), whereas % freezing was significantly reduced as compared pre-SCS and tone durations (both $P < 0.001$). The representative trial-wise data shows transitions of flight (**F**) and freezing (**G**) behavior following the presentation of tone and white noise in the mouse on Day 3. The represented values are means ± SEM. *$p<0.05$, **$p<0.01$, ***$p <0.001$. Pre-exp, Pre-exposure. Panels A-E are modified from Fadok et al., 2017.

Figure 3: Extinction and recall following flight conditioning (Day 4). A) Comparison of flight scores during extinction training showed rapid extinction of conditioned flight (n = 12; 16 trials, two-way repeated-measures ANOVA, cue × trial interaction, $F(15,165) = 3.05$, $P < 0.01$). Flight scores from first block of four trials (trial 1-4) of extinction observed significantly higher for white noise as compared to the tone ($P < 0.05$, Wilcoxon matched-pairs signed-rank test). **B**) Comparison of freezing showed a statically significant effect on freezing (%) following white noise (n = 12; 16 trials, two-way repeated-measures ANOVA, cue × trial interaction, $F(15,165) = 3.55$, $P < 0.01$). The freezing for the first block of four trials (trial 1-4) during extinction found to be significantly lower during white noise period as compared to the tone (Paired t-test, $P < 0.01$). **C**) Change in the context significantly affect the flight scores (n = 8; 4 trials, two-way repeated-measures ANOVA, cue × trial interaction, $F(1,7) = 27.44$, $P < 0.01$). Flight scores significantly reduced during white noise as compared to the tone period in the neutral context (two-tailed paired t-test, $P < 0.01$) D). Freezing responses across trials during retrieval were also significant (n = 8, 4 trials, two-way repeated-measures ANOVA, effect of cue $F(1,7) = 27.67$, $P < 0.01$). Exposure of WN in neutral context significantly increased the freezing responses as compared to the tone (two-tailed paired t-test, $P < 0.001$). The represented values are means ± SEM. *p<0.05, **p<0.01, ***p <0.001. Panels A-D are modified from Fadok et al. 2017.

DISCUSSION:

The described sound and shock parameters are important elements of this protocol. It is critical, therefore, to test the shock amplitude and sound pressure level before starting the experiments. Fear conditioning studies typically use 70-80 dB sound pressure levels and 0.1-1 mA shock intensity[18]; thus, the described parameters are within the bounds of traditional fear conditioning paradigms. In a previous CS-only (no footshock) control experiment, we did not observe flight or freezing responses in the mice, indicating that the auditory stimuli are not aversive when presented as described[15]. Increasing the dB level of the white noise above 80 dB may induce innate aversion. However, noise stimuli presented at 75 dB do not elicit stress in the form of suppressed behavioral activity in mice[19].

The auditory stimuli that comprise the SCS must be carefully selected. In our previous study, we determined that single-CS conditioning with white noise induces higher flight scores than conditioning with a pure tone[15]. This illustrates the importance of stimulus salience in this protocol[20]. However, a recent study showed that conditioning with a reversal of the SCS sequence (white noise-tone) results in flight to the tone and freezing to the white noise[21]. These data endorse that the learned temporal relationship of the cues is also an important factor.

Because cage changes are a potential source of stress, it is recommended to start conditioning at least 2 days after the last most recent cage change. To further minimize the impact of stress in the mice undergoing study, appropriate care should be taken to reduce the olfactory cues remaining from previous subjects, including the smell of feces and urine. Therefore, cleaning the chamber before and after each mouse is crucial. To avoid other potential sources of disturbance, it is best to conduct this protocol in a room separated from any other ongoing experiments. Mice should exhibit very low baseline freezing[15]. To test the experimental conditions, each laboratory should conduct a pilot experiment to test baseline freezing in each context.

Other than the C57BL/6J and other transgenic lines used by Fadok et al. (2017)[15], this method should be suitable for adaptation to other strains of mice and rats[20,21]. Recent data (Borkar et al. 2020)[22] suggest that both male and female mice show comparable flight responses, therefore the paradigm is suitable for both sexes. As mentioned in step 2.1.2, in response to high intensity shocks, mice jump very high, thus carefully select the height of the chamber to prevent the mice from escaping the context. It is also important to ensure the consistent and accurate timing of cues and shock stimuli. Both AC and DC shocks are effective; however, when using DC shocks, it may be necessary to increase footshock intensity to reach similar flight scores as that of AC shocks. Because DC shocks have a less detrimental effect on electrophysiological recordings, use of DC shock is recommended for studies that require electrophysiology data. It is important to note that decreasing the intensity of the footshock may decrease the intensity of the flight response.

As denoted in the protocol, flight scores are calculated by normalizing speed data during tone and white noise by dividing them with individual trial pre-SCS speed values. However, if a mouse exhibits extremely high levels of freezing during the pre-SCS, the resultant flight scores may be very high, thus increasing data variability. This can be circumvented by using a different baseline measurement, such as average speed data from the 3 min baseline period at the beginning of the session or using the average overall pre-SCS (average of 5 trials).

Flexible and rapid behavioral adaptation to threat is crucial for survival. Most classical fear conditioning protocols use conditions that induce freezing as a sole determinant of fear learning. The benefit of this protocol is that it allows for study of complex defensive state transitions within subjects. Previously, this model was used to discover that behavioral transitions are processed by local recurrent inhibitory circuits in the central amygdala[15,23]. This paradigm also enabled researchers to elucidate cortico-thalamic circuits for the selection of defensive behavior[21]. These studies demonstrate that this method will facilitate studies investigating neural circuit control of rapid transitions between defensive behaviors within a subject. This has potential applications for developing a better understanding of the neurobiological underpinnings of anxiety, panic disorder, or PTSD[24,25].

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

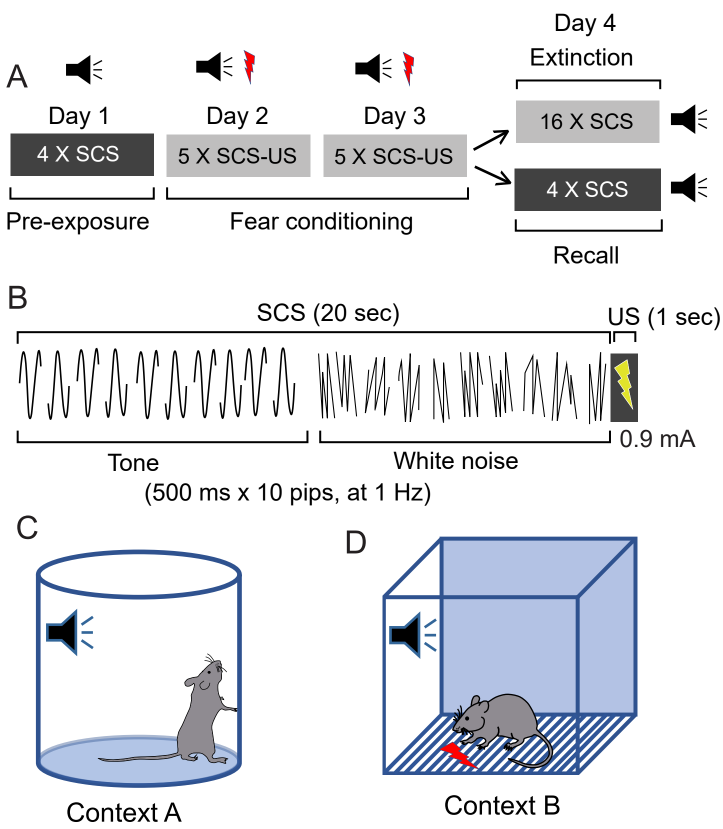
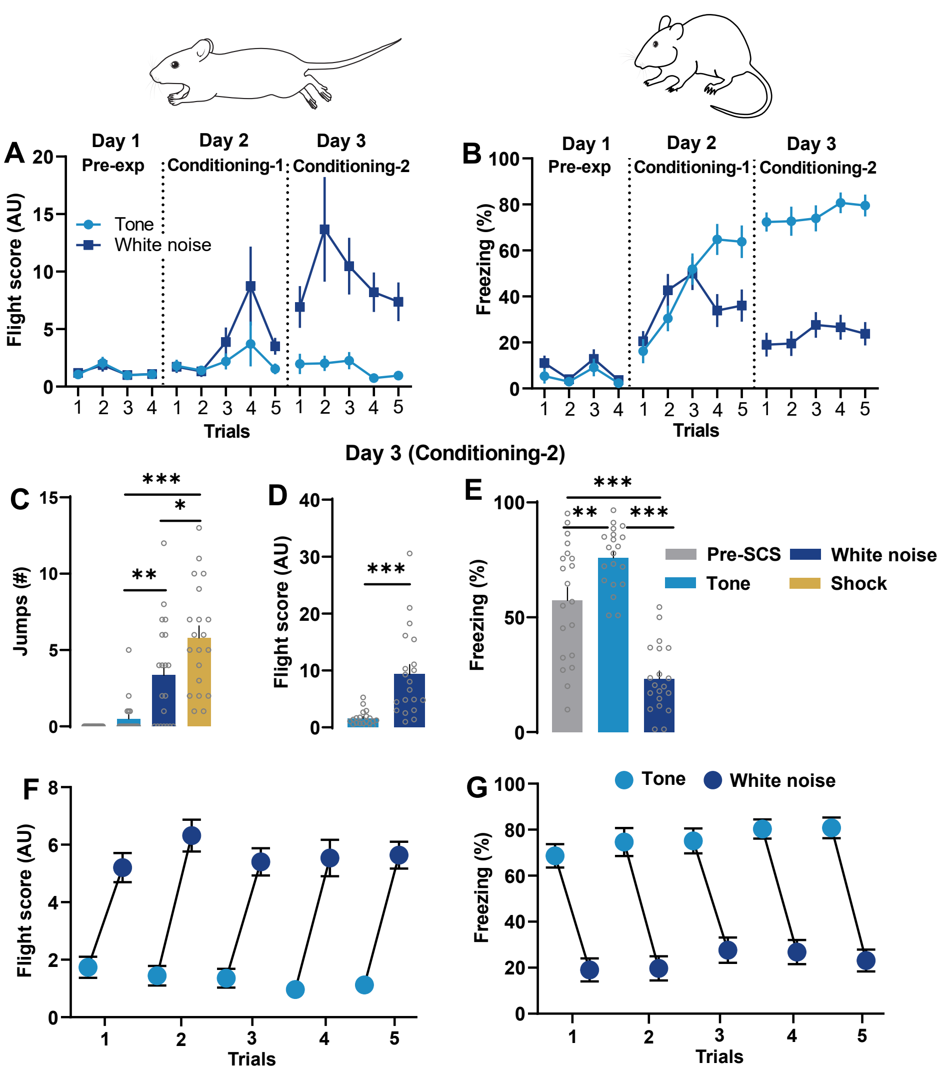

A
Day 1
4 X SCS
Pre-exposure
Day 2
5 X SCS-US
Day 3
5 X SCS-US
Fear conditioning
Day 4
Extinction
16 X SCS
4 X SCS
Recall
B
SCS (20 sec)
US (1 sec)
0.9 mA
Tone
(500 ms x 10 pips, at 1 Hz)
White noise
C
Context A
D
Context B

Figure 2



A
Day 1
Pre-exp
Day 2
Conditioning-1
Day 3
Conditioning-2
Flight score (AU)
Tone
White noise
Trials
B
Freezing (%)
Day 3 (Conditioning-2)
C
Jumps (#)
D
E
Pre-SCS
White noise
Tone
Shock
F
G

Figure 3

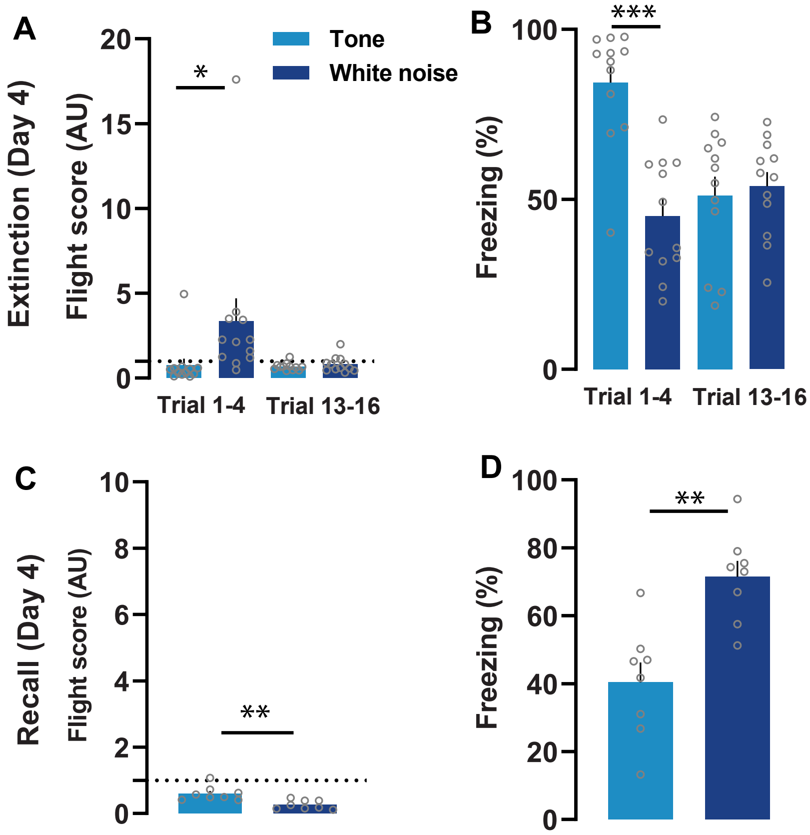

A
Extinction (Day 4)
Flight score (AU)
20
15
10
5
0
*
Tone
White noise
Trial 1-4
Trial 13-16
B
Freezing (%)
100
50
0
***
Trial 1-4
Trial 13-16
C
Recall (Day 4)
Flight score (AU)
10
8
6
4
2
0
**
D
Freezing (%)
100
80
60
40
20
0
**

Figure 1

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Figure 2

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Figure-2.ai

Figure 3

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Figure-3.ai

Name of Material/ Equipment	Company	Catalog Number
Audio generator	Med Associates, Inc.	ANL-926
C57/BL6J mice	Jackson laboratory, USA	664
Cineplex software (Editor/ studio)	Plexon	
Fear conditioning box	Med Associates, Inc.	VFC-008
MedPC software V	Med Associates, Inc.	SOF-736
Neuroexplorer	Plexon	
Neutral context		
Prism 8-statistical analysis software	GraphPad Software, Inc.	
Shocker	Med Associates Inc.	ENV-414S
Speaker	Med Associates, Inc.	ENV-224AM

Comments/Description

Aged 3-5 month
For video tracking and behavioral scoring analysis
25 X 30 X 35 cm dimentions
CinePlex Studio v3.8.0
Used for extract the scored data in PlexonEditor
Plexiglass cylinder 30 X 30 cm

Stainless steel grid
Suitable for pure tone tone and white noise

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Response: We have proofread the manuscript.

• **Textual Overlap:** Significant portions show significant overlap with previously published work. Please re-write the text on lines 19-22 to avoid this overlap.

Response: We have corrected the text on these lines.

• Please include an ethics statement before your numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.

Response: The ethics statement now occurs before the numbered protocol.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Response: We have added additional details about the protocol steps in the revised manuscript.

• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.

Response: We have adjusted the numbering and alignment of the protocol section to follow JoVE's instructions for authors.

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

Response: We highlighted the relevant portions of text.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Response: We have rewritten portions of the discussion to ensure that it covers the required topics in paragraph form. The revised discussion also addresses reviewer comments regarding control experiments.

• **References:** Please spell out journal names.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Response: All our figures either are new representations of data or comply with the re-use policies of the publisher. We confirmed this with the publisher and we have uploaded the text of the reprint permission as a Word document to the Editorial Manager site.

Comments from Peer-Reviewers:

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This manuscript describes a novel way to study different responses to classical fear conditioning in rodents, aiming at utilizing a within-subjects design. The authors designed a modified Pavlovian fear conditioning paradigm that allows for the study of both freezing and flight behaviors in response to the unconditioned stimulus. In addition, this paradigm uses higher-than-average shock intensities, a greater number of pairings between conditioned and unconditioned stimulus, and the added presentation of a white noise stimuli following the tone stimuli. Pavlovian fear conditioning is used to model and study fear and anxiety-related behaviors in rodents, but too often focuses on one response behavior: freezing. This new paradigm is intended to allow the researchers to study a different response behavior: flight or any escape like behavior. The authors conducted a pilot study with the novel paradigm, and briefly discuss the results: Mice exhibit freezing in response to the tone stimulus, but flight in response to the white noise stimulus. Expanding the behaviors that are studied in fear conditioning will allow for a broader understanding of the multitude of behavioral outcomes that result from fear- and anxiety-related disorders in humans. This novel paradigm takes important steps in broadening our understanding of the complex behavioral responses to Pavlovian fear conditioning and this manuscript is extremely helpful in outlining how to properly set-up, conduct

and analyze the data for the new paradigm. There are a few points and questions that should be addressed prior to publication.

Response: We are thankful to the reviewer for their comments.

Major Concerns:
1. A figure depicting what the two different contexts look like would be helpful for those trying to re-create the paradigm (lines 107-109, section 2.1.2).

Response: We have modified Figure 1 to include illustrations of the two different contexts.

2. How did the authors select the specific parameters that they did for fear conditioning (i.e. how long the average ITI should be, how long the session is in total, shock intensity, tone frequency)?

Response: During the development of this paradigm, we tested the effects of several experimental variables to determine which conditions would elicit the most robust flight behavior. For example, we compared behavioral responses following conditioning with a pure tone or white noise and found that conditioning with white noise led to higher flight scores (Fadok et al. 2017). The duration of the ITI was determined by a combination of empirical evidence and literature demonstrating that longer, pseudorandom ITIs are better for Pavlovian learning (Lavond and Steinmetz, 1952; Barnet et al., 1995; Lattal, 1999). The total session length is a product of having a baseline period that allows assessment of contextual learning, the time it takes for the trials and ITIs, and a one-minute post-conditioning period. Finally, we have empirically determined that conditioning with lower shock intensities results in lower flight scores (unpublished data).

3. It would be helpful if the authors summarized some of the findings and control experiments done for previous manuscripts (e.g. reversal of compound stimulus order, extinction results), to help new readers appreciate the scope of what is already understood about this novel paradigm.

Response: We have added a paragraph to the discussion that summarizes the results of previously published experiments using this paradigm. These results include the single-cue conditioning experiments (see response to Point 2, above) and no-shock control experiments published in Fadok et al. 2017. We also reference the findings of Hersman et al. 2020 who explored the role of stimulus salience in the intensity of the conditioned flight response. That paper contains experiments testing the reversal of compound stimulus order, but it should be noted that Dong et al. 2019 obtained opposite results with a reversed SCS.

Finally, our recent data show induction of robust freezing and flight responses to the tone and white noise, respectively, in both male and female mice (Borkar et al. 2020). We did not find significant sex differences for the flight response, although generalized/contextual freezing was higher in female mice (Borkar et al., 2020).

4. More discussion of the extinction results would be useful as well; it is interesting that the presentation of the white noise in the neutral context elicits more freezing than the tone does. Any thoughts on the reason for this switch in white noise responding?

Response: We agree that the behavioral changes observed in the extinction and context transfer experiments are very interesting. During extinction training within the conditioning context, we observe that flight responses during the white noise are rapidly abolished and are subsequently replaced by freezing (Fadok et al. 2017). We conclude from these experiments that the imminence of the threat is vital for the flight response. If mice are conditioned in this paradigm and then tested in a neutral context, freezing values are significantly higher during the white noise compared to the tone (Fadok et al. 2017). We conclude from these data that this is a demonstration of the importance of context in modulating defensive responding. Only in the conditioning context is the perceived threat imminence strong enough to elicit flight responses.

Unfortunately, the author instructions for JoVE do not allow us to add an extensive discussion of these issues to the manuscript as all discussion must be centered on the protocol itself.

Minor Concerns:
1. Line 274, there might need to be a space between "lastmost"

Response: This has been corrected.

2. Line 301, there is an extra "s" in "selection"

Response: This has been corrected.

3. Line 417, there is a missing hyphen between "pre" and "SC"

Response: This has been corrected throughout the manuscript.

Reviewer #2:
This technical report details procedures for performing serial compound stimulus conditioning, an important new procedure useful for studying threat imminence perception and defensive behavior action selection. The report is thorough and comes from the person who developed the protocol, making it a valuable addition to the field. Based on our experience running these experiments, I have just a few minor additions that might be helpful for those who plan to set up SCS conditioning for the first time:

Response: We are thankful to the reviewer for their suggestions, which were useful for improving the quality of the paper.

1. One thing that might be emphasize more is the importance of placing one camera at the top of the chamber to acquire speed data. This is implied in "2. Preparation" section, but could be explicitly stated in "3. Video tracking" section, since over-head cameras are not standard equipment in all commercial fear conditioning packages (e.g. this is not part of Med Associates package, and so would need to be added).

Response: This point has been added to the relevant section.

2. In "5. Quantification of behavior" section, it states that freezing behavior can be manually annotated by frame-by-frame analysis, but this can also be done automatically by thresholding frame-by-frame pixel changes… something commonly done using a side view camera recording with plain background. Med Associates offers a software package for this (VideoFreeze), though other custom programs could also be used.

Response: We have clarified our analysis method and we have expanded this section to better demonstrate how we quantify freezing behavior. We use an automatic freezing detector that is part of the Cineplex software package (Plexon). This software uses contour tracing to identify the subject against the background and then provides a frame-by-frame analysis of pixel changes. An observer blinded to the experiments performs the initial thresholding and then the algorithm automatically detects freezing events. We have added a note addressing the fact that other systems, or manual scoring, can also be used.

Reviewer #3:
Manuscript Summary:
This concisely written manuscripts introduces a behavioral paradigm, which allows to study the switch between passive (freezing) and active (flight) fear responses. It is based on classical conditioning to a sine wave tone and white noise, with subsequent re-exposure to the acoustic stimuli in a neutral or the original conditioning context. This experimental procedures holds the promise of becoming a standard routine in threat research. I have a few minor points which should be addressed during the revision process:

Response: We appreciate the reviewer's efforts to improve the manuscript.

Minor Concerns:
(1) Title: I cannot see the "selection" aspect in the behavioral response. To me, the switch between passive and active fear is rather reflexive and just a matter of threat intensity (accumulation of contextual fear + the stronger auditory-cued fear in case of white noise). In the same line of reasoning - I consider it slightly overstated that the protocol employed results in "conditioned flight" (otherwise, this response should have been prevalent also in the neutral test context). Therefore, I would reconsider this term (and change the title, e.g., "to study the switching between passive and active fear"). In general, it would be helpful to present data of control experiments, which show that conditioning with sine wave tones ~ foot shock alone (i.e., without white noise) do not result in increased flight behavior, if the animals are exposed to white noise in the conditioning chamber after conditioning.

Response: We agree with the reviewer that our paradigm elicits transitions between different, innately programmed, defensive behaviors as a product of threat intensity. This paradigm is useful for understanding how brain circuits operate to determine selection of defensive behavioral output; however, we agree that selection as used in the title is not necessary. We have removed this word from the title. We have also removed "conditioned flight" from the title. In general, we use this term in the same way that many in the field use the term "conditioned freezing"—an adaptive defensive response that occurs during the CS only after conditioning. We have removed this term from the title because the paradigm elicits both flight and freezing within subjects.

The results of control experiments and the published results of other experiments using this paradigm (Dong et al. 2019; Fadok et al. 2017; Hersman et al. 2020), are discussed in response to Reviewer 1 and are now part of the discussion section of the protocol. Unfortunately, we do not have results from the control experiments that the reviewer mentions. One could hypothesize that a nonassociative process could induce a behavioral response to white noise in such an experiment (i.e. sensitization induced by the shock), as has been described previously in traditional fear conditioning (e.g. Kamprath and Wotjak 2004).

(2) Point 1.1. (p. 2): The statement about "power analysis" is not very meaningful without specifications of the effect size, the beta error and a priori knowledge about the variance of the behavior.

Response: We have removed this statement.

(3) The correct nomenclature would be "C57BL/6J".

Response: This oversight has been corrected.

(4) Other than stated at p. 7, l. 308/309, it is not evident why this model should follow the "predatory imminence theory in terms of threat intensity, proximity and context". This requires further explanation. To me, the switch from passive to active fear primarily relates to the strength of activation of the fear matrix (as simulated by increasing electrical or chemical stimulation of the PAG and related brain structures).

Response: We have removed this statement from the discussion. We developed this paradigm using the principles of the predatory imminence theory as a model, but a detailed discussion of why we believe the results follow this theory is not germane to the implementation of this protocol in another laboratory and is therefore beyond the scope of a JoVE protocol.

(5) Figure 1: Too schematic - the protocol described at p. 3/ section 3.2. mentions repeated pips, which are not evident from the sketch (which indicates a continuous presentation of the sine wave tone and white noise). Please revise!

Response: We have corrected Figure 1 to illustrate the use of pips in this paradigm.

(6) Figure 2: Please add individual data to the bar graphs (to get an impression about the distribution of the data).

Response: We have added individual data to the bar graphs in the revised manuscript.

(7) Legend to Fig. 2: Here and in the text, there is an apparent discrepancy in the nomenclature of the experimental days compared to the figure (e.g., "d-1" vs. "Day 1). I would suggest to stick to the nomenclature chosen in the figure (in particular, since "d-1" could be read as "day minus 1"). There is some confusion when referring to the experimental days in the legend to Fig. 2A and 2B ("d-1" and "d-2" have to be replaced by "Day 2" and "Day 3", respectively). Please mention the experimental day for the data shown in Fig. 2F and G.

Response: We have corrected this throughout the manuscript.

References:

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Fadok, Jonathan

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To: Fadok, Jonathan
Subject: RE: Reuse of data in methods paper

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From: Fadok, Jonathan [mailto:jfadok@tulane.edu]
Sent: 03 June 2020 21:03
To: Journalpermissions
Subject: Reuse of data in methods paper

To Whom It May Concern,

I am writing a methods paper to be published in the online journal, JoVE. In this paper, I would like to show some of the results displayed in Figure 1 of my first-author publication in Nature (DOI: 10.1038/nature21047). Would this be permissible under the Author reuse permissions, provided that the figure is referenced?

Sincerely,

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