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

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Author Questionnaire

1. Microscopy: Does your protocol demonstrate the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **38**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Jonathan Fadok**: This protocol is significant because it facilitates investigations into the transitions between defensive behaviors, making it ideal for researchers interested in studying complex adaptive responses to threat **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Chandu Borkar**: This protocol uses temporally precise, conditioned stimuli to elicit transitions between defensive responses, allowing us to study both conditioned freezing and flight behaviors within individual subjects **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.3. **Jonathan Fadok**: Using this model to uncover the mechanisms of defensive behaviors may provide insights into PTSD-associated dysfunction and panic disorders and could aid in the development of novel therapeutics **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Ethics Title Card

1.4. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at Tulane University.

Protocol

2. Behavioral Protocol and Video Tracking Setup

2.1. To set up the behavioral protocols, in the program, define the SCS (S-C-S) **[1-TXT]**, setting the stimuli to be delivered as either a 10-second pure tone or a 10-second white noise and defining the inter-trial intervals to be presented pseudorandomly following each trial **[2]**.

2.1.1. WIDE: Talent defining SCS, with monitor visible in frame **TEXT: SCS: serial compound stimulus**

2.1.2. SCREEN: screenshot_1: 00:08-00:43 *Video Editor: please speed up*

2.2. To set up the software tracking, place a test mouse in each relevant context **[1]**, define the center of gravity, and adjust the contour size **[2]**.

2.2.1. Talent placing mouse into context *Videographer: Important step*

2.2.2. SCREEN: screenshot_2a: 00:05-00:30 *Video Editor: please speed up* OR SCREEN: screenshot_2b: 00:05-00:09

2.3. Use the size of chambers and the pixel dimensions of the camera to determine the calibration coefficient **[1]**.

2.3.1. SCREEN: screenshot_3: 00:02-00:45 *Video Editor: please speed up*

2.4. Then generate the TTL (T-T-L) pulse code for synchronizing the event markers of the central computer to their real-time occurrences **[1-TXT]**.

2.4.1. SCREEN: screenshot_4a: 00:04-01:32 *Video Editor: please speed up* OR SCREEN: screenshot_4b: 00:02-00:15 **TEXT: TTL: time to live**

3. Behavioral Experiment Setup

3.1. Before beginning an experiment, turn on the fear conditioning box controller, shocker, and video recording software **[1]**.

3.1.1. WIDE: Talent turning on instrument

3.2. Mount an overhead speaker above the contexts for delivery of the auditory stimuli at 75 decibels **[1]** and set a programmable audio generator to generate auditory stimuli on a pre-defined schedule **[2-TXT]**.

3.2.1. Talent mounting/adjusting/checking speaker over context(s) *Videographer: Important step*

3.2.2. Talent setting generator *Videographer: Important step* **TEXT: *e.g.*, 7.5 kHZ pure tone with sinusoidal wave; 1-20,000 Hz random white noise signal with equal intensity at different frequencies**

3.3. After confirming that the tone and white noise are functional **[1-TXT]**, set the system for data acquisition **[2]** and transport 3-5-month old male or female mice to the conditioning room **[3]**.

3.3.1. Talent checking tone and/or noise *Videographer: Important step; please include tone and white noise; Video Editor: please include tone and white noise* **TEXT: Measure sound in dB**

3.3.2. Talent setting system to acquire data, with monitor visible in frame *Videographer: Important step*

3.3.3. Talent bringing animals into room

3.4. After 10 minutes of acclimation, transfer one mouse into the context A chamber **[1]** and immediately activate the fear conditioning system and data collection programs **[2]**. NOTE: Move step 3.4 after 4.2

3.4.1. Talent placing mouse into context

3.4.2. Talent activating programs, with monitor visible in frame

4. Pre-Conditioning/Pre-Exposure and Fear Conditioning

4.1. Before beginning the pre-conditioning trial, clean a 30-centimeter-diameter, 30-centimeter-high, clear, cylindrical, plexiglass chamber with 1% acetic acid to be used as the context A chamber **[1-TXT]**.

4.1.1. WIDE: Talent cleaning chamber, with acetic acid container visible in frame *Videographer: Important step* **TEXT: Clean before and after testing individual mice**

4.2. For pre-conditioning, allow the mouse to acclimate for 3 minutes in the chamber **[1]** before exposing the mouse to four, 20-second SCS trials with a 90-second-average pseudorandom intertrial interval **[2-TXT]**.

4.2.1. Talent coding total session, with monitor and mouse visible in frame

4.2.2. SCREEN: screenshot_5: 00:05-00:12 **TEXT: Total pre-exposure duration: 590 s**

4.3. Before beginning a fear conditioning experiment, clean an at least 35-centimeter-high, 25- x 30-centimeter rectangular enclosure with an electrical grid floor with 70% ethanol to be used as the context B chamber **[1-TXT]**.

4.3.1. Talent cleaning chamber and/or enclosure, with 70% ethanol container visible in frame **TEXT: Clean before and after testing individual mice**

4.4. Then connect the shocker with the electrical grid floor of the context B chamber **[1]** and define the frequency, onset, and duration of the shocks in the appropriate computer program **[2]**.

4.4.1. Talent connecting shocker with grid floor *Videographer: Difficult step*
4.4.2. Talent at computer, defining parameters, with monitor visible in frame

4.5. When all of the parameters have been set, check that the shock intensity is delivered properly **[1]**, from both shocker and grid floor **[2-added]**.

4.5.1. Talent checking parameters from shocker
4.5.2. Added shot: Talent checking the actual shock intensity from the grid floor

4.6. On days 2 and 3, place the mouse into the context B chamber for 3 minutes **[1]** before exposing the animal to five pairings of the SCS co-terminating with a 1-second, 0.9 milliamp AC footshock and a 120-second average intertrial interval **[2-TXT]**. At the send of session, put the mouse back in the home cage **[3-added]**.

4.6.1. Talent placing mouse into context B on day 2 and 3 *Videographer: Important/difficult step*
4.6.2. SCREEN: screenshot_5: 00:32-00:40 OR SCREEN: screenshot_5: 00:52-01:01 **TEXT: Total fear conditioning duration: 820 s**
4.6.3. Added shot: Talent placing back the mouse in home cage.

5. Fear Recall and Fear Extinction

5.1. Depending on the goal of the experiment, to subject the animals to a recall session, on day 4, place the mouse in the context A chamber for 3 minutes **[1]** before presenting the animal with four SCS trials without footshock with an 90-second-average pseudorandom intertrial interval over 590 seconds to test the animal's fear recall response **[2]**.

5.1.1. WIDE: Talent placing mouse into chamber *Videographer: Important step*
5.1.2. SCREEN: screenshot_5: 01:37-01:44

5.2. Or, to test for fear extinction, on day 4, place the mouse into the context B chamber for 3 minutes **[1]** before subjecting the animal to 16 trials of SCS without footshock with a 90-second-average pseudorandom intertrial interval over a period of 1910 seconds **[2]**.

5.2.1. Talent placing mouse into chamber B *Videographer: Important step*
5.2.2. SCREEN: screenshot_5: 01:13-01:23

6. Behavior Quantification

6.1. To quantify the mouse's behavior, at the end of the experiment, have an observer blind to the analyses score the recorded videos for freezing behavior **[1]** using automatic freezing detector thresholding followed by a frame-by-frame analysis of the pixel changes **[2]**.

6.1.1. WIDE: Talent at computer, scoring videos, with monitor visible in frame
6.1.2. SCREEN: screenshot_6: 00:50-01:40 *Video Editor: please speed up*

6.2. Define freezing as a complete cessation of bodily movements, except for those required for respiration, for a minimum of 1 second **[1]**.

6.2.1. SCREEN: screenshot_6: 02:08-02:10

6.3. Score a "jump" as an instance when all 4 of paws leave the floor, resulting in a vertical and/or horizontal movement **[1]**.

6.3.1. SCREEN: screenshot_6: 05:17-05:27 *Video Editor: please speed up*

6.4. When the entire segment has been analyzed, export the marked file with freezing, jump, and event markers **[1]** and extract the relevant events from the defined time periods of interest into a spreadsheet **[2]**.

6.4.1. SCREEN: screenshot_7: 00:01-00:27 *Video Editor: please speed up*
6.4.2. SCREEN: screenshot_7: 00:30-00:49 *Video Editor: please speed up*

6.5. To calculate the duration of freezing, subtract the start time from the end time for each respective trial period **[1]**.

6.5.1. SCREEN: screenshot_7: 00:50-01:08 *Video Editor: please speed up*

6.6. Sum the total number of jumps from a particular trial duration **[1]**.

6.6.1. SCREEN: screenshot_7: 01:09-01:22 *Video Editor: please speed up*

6.7. To calculate the speed of the mouse, track the coordinates from the frame-by-frame X-Y axis movement of the center of gravity of the mouse **[1]**.

6.7.1. SCREEN: screenshot_8: 00:28-00:44 *Video Editor: please speed up*

6.8. To calculate the flight scores, divide the average speed during each SCS by the average speed during the 10-second pre-SCS and add 1 point for each escape jump. A flight score of 1 indicates no change in flight behavior from the pre-SCS period **[1-TXT]**.

6.8.1. SCREEN: screenshot_8: 00:45-01:31 *Video Editor: please speed up* **TEXT: Optional: Manually score for other behaviors, such as rearing and grooming**

6.9. Then analyze the data for statistical significance using an appropriate statistical analysis software program **[1-TXT]**.

6.9.1. SCREEN: screenshot_9: 00:04-00:25 *Video Editor: please speed up* **TEXT: p<0.05 significant**

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
2.2., 3.2., 3.3., 4.1., 4.6., 5.1., 6.2.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
4.4., 4.6.

Results

7. Results: Representative Freezing and Flight Behavior Analyses

7.1. SCS presentations in the pre-exposure session **[1]** do not elicit flight **[2]** or freezing responses in mice **[3]**.

7.1.1. LAB MEDIA: Figures 2A and 2B
7.1.2. LAB MEDIA: Figures 2A and 2B *Video Editor: please emphasize Day 1 data line in Figure 2A graph*
7.1.3. LAB MEDIA: Figures 2A and 2B *Video Editor: please emphasize Day 1 data lines in Figure 2B graph*

7.2. Behavioral analysis during conditioning **[1]** reveals that the tone component of the SCS **[2]** significantly enhances freezing compared to freezing during the pre-SCS **[3]**.

7.2.1. LAB MEDIA: Figures 2B and 2E
7.2.2. LAB MEDIA: Figures 2B and 2E *Video Editor: please emphasize light blue data line in Figure 2B*
7.2.3. LAB MEDIA: Figures 2B and 2E *Video Editor: please emphasize light blue data bar and/or add/emphasize bracket and asterisks over grey and light blue data bars*

7.3. Flight scores change significantly across sessions **[1]** and mice exhibit higher speeds and more jumps to the white noise cue **[2]** compared to the tone cue **[3]**.

7.3.1. LAB MEDIA: Figure 2A
7.3.2. LAB MEDIA: Figures 2C and 2D *Video Editor: please emphasize dark blue data bar in both graphs*
7.3.3. LAB MEDIA: Figures 2C and 2D *Video Editor: please emphasize dark blue data bar in both graphs*

7.4. Mice show a clear defensive behavior transition **[1]**, exhibiting lower flight scores during the tone **[2]** and higher flight scores during white noise **[3]**, with the opposite observed for freezing responses **[4]**.

7.4.1. LAB MEDIA: Figures 2F and 2G
7.4.2. LAB MEDIA: Figures 2F and 2G *Video Editor: please emphasize light blue data circles in Figure 2F*
7.4.3. LAB MEDIA: Figures 2F and 2G *Video Editor: please emphasize dark blue data circles in Figure 2F*
7.4.4. LAB MEDIA: Figures 2F and 2G *Video Editor: please emphasize Figure 2G*

7.5. Mice subjected to 16 trials of extinction training demonstrate a rapid extinction of conditioned flight **[1]**, with flight scores during the first block of four trials measuring higher during white noise **[2]** compared to the tone cue **[3]**.

7.5.1. LAB MEDIA: Figure 3A
7.5.2. LAB MEDIA: Figure 3A *Video Editor: please emphasize Trial 1-4 dark blue data bar*
7.5.3. LAB MEDIA: Figure 3A *Video Editor: please emphasize Trial 1-4 light blue data bar*

7.6. At the end of the extinction session, flight behavior is no longer elicited by either cue **[1]**.

7.6.1. LAB MEDIA: Figure 3A *Video Editor: please emphasize Trial 13-16 data bars*

7.7. An overall decrease in tone-induced freezing and an increase in white noise-mediated freezing is observed during the extinction session **[1]**, with freezing for the first block of four trials significantly higher in response to the tone **[2]** than to the white noise **[3]**.

7.7.1. LAB MEDIA: Figure 3B
7.7.2. LAB MEDIA: Figure 3B *Video Editor: please emphasize Trial 1-4 light blue data bar*
7.7.3. LAB MEDIA: Figure 3B *Video Editor: please emphasize Trial 1-4- dark blue data bar*

7.8. Exposure to white noise in a neutral context does not elicit flight **[1]**.

7.8.1. LAB MEDIA: Figure 3C *Video Editor: please emphasize data bars in Figure 3C and/or add/emphasize bracket and asterisks*

7.9. Rather, white noise presentations in the neutral context **[1]** elicit freezing responses that are higher than those elicited by the tone **[2]**.

7.9.1. LAB MEDIA: Figure 3D
7.9.2. LAB MEDIA: Figure 3D *Video Editor: please emphasize data blue data bar*
7.9.3. LAB MEDIA: Figure 3D

Conclusion

8. Conclusion Interview Statements

8.1. **Chandu Borkar**: It is important to clean the contexts thoroughly and it is critical to test the shock amplitude and sound pressure level before starting an experiment **[1]**.

8.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (4.1., 4.2., 5.1.)

8.2. **Jonathan Fadok**: This paradigm is currently being used by groups who are interested in understanding the complexities of defensive behavior and can be used to expand our knowledge of defensive action selection **[1]**.

8.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera