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Contributions of associative and non-associative learning to the dynamics of defensive ethograms

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eLife Assessment

This study is deemed to be an **important** work that carefully deconstructs multi-faceted conditioned fear behavior in mice. The well-controlled experiments provide **convincing** data that will be of interest to other researchers in the field.

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Abstract

Defensive behavior changes based on threat intensity, proximity, and context of exposure, and learning about danger-predicting stimuli is critical for survival. However, most Pavlovian fear conditioning paradigms focus only on freezing behavior, obscuring the contributions of associative and non-associative mechanisms to dynamic defensive responses. To thoroughly investigate defensive ethograms, we subjected male and female adult C57BL/6J mice to a Pavlovian conditioning paradigm that paired footshock with a serial compound stimulus (SCS) consisting of distinct tone and white noise (WN) stimulus periods. To investigate how associative and non-associative mechanisms affect defensive responses, we compared this paired SCS-footshock group with four control groups that were conditioned with either pseudorandom unpaired presentations of SCS and footshock, shock only, or reversed SCS presentations with inverted tone--WN order, with paired or unpaired presentations. On day 2 of conditioning, the paired group exhibited robust freezing during the tone period with switching to explosive jumping and darting behaviors during the WN period. Comparatively, the unpaired and both reverse SCS groups expressed less tone-induced freezing and rarely showed jumping or darting during WN. Following the second day of conditioning, we observed how defensive behavior changed over two extinction sessions. During extinction, the tone-induced freezing decreased in the paired group and mice rapidly shifted from escape jumping during WN to a combination of freezing and darting. The unpaired, unpaired reverse, and shock-only groups displayed defensive tail rattling and darting during the SCS, with minimal freezing and jumping. Interestingly, the paired reverse group did not jump to WN, and tone-evoked freezing was resistant to extinction. These findings demonstrate that non-associative factors promote some defensive responsiveness,

but associative factors are required for robust cue-induced freezing and high-intensity flight expression.

Introduction

Defensive responses have evolved to maximize survival (Anderson and Adolphs, 2014), and animals rapidly switch behaviors depending on threat imminence, context of exposure, and previous experience with stimuli (Perusini and Fanselow, 2015). Understanding the mechanisms underlying adaptive defensive behavior may grant insight into the pathophysiology of post-traumatic stress and panic disorders, wherein heightened responses to external stimuli are observed, yet neuroscientists need more tractable methods with which to investigate how the nervous system controls complex experience-dependent behavior.

Pavlovian fear conditioning has been widely used as a model system to understand the neural mechanisms underlying fear-related learning and memory (Bolles, 1970; Bolles and Collier, 1976; Grewe et al., 2017; Roy et al., 2017; Bouton et al., 2021). In standard Pavlovian conditioning paradigms, freezing is the dominant defensive behavior evoked by contexts and learned cues that are paired with an aversive unconditioned stimulus (US), like footshock (Blanchard and Blanchard, 1969; Bolles and Collier, 1976). Other defensive responses like escape jumping (Chu et al., 2022) and darting (Gruene et al., 2015) are measured less often within conditioning, limiting insight into defensive response dynamics. To address this critical need, we developed a modified Pavlovian conditioning paradigm that elicits both freezing and flight behaviors in response to conditioned stimuli (Fadok et al, 2017; Borkar er al., 2020; Borkar and Fadok, 2021; Borkar et al., 2024), findings that have been replicated by others in both mice and rats (Dong et al., 2019; Totty et al., 2021). In this paradigm, mice are conditioned with a serial compound stimulus (SCS) consisting of a pure tone followed by white noise (WN), which terminates with a strong electrical footshock. After conditioning, mice exhibit contextual freezing which significantly increases in response to tone, and mice switch to robust flight responses upon WN presentation. These findings demonstrate that the magnitude and mode of defensive behavior change with the psychological distance of threat, consistent with the predatory imminence continuum theory (Perusini and Fanselow, 2015).

However, the influence of non-associative elements on this ethological profile has recently been discussed (Fanselow et al., 2019; Hersman et al, 2020; Trott et al. 2022). It has been suggested that the inherent salience of the WN stimulus contributes more to WN-evoked flight response than its predictive association with the US (Hersman et al, 2020). Others claim that the immediate transition from freezing to flight behavior is a result of the rapid change and relative increase in stimulus intensity from tone to WN that is caused by non-associative sensitization, or by inherent stimulus properties, akin to an acoustic startle response (Fanselow et al., 2019, Trott et al. 2022). In addition, sensitization and stimulus salience are known to intensify freezing responses to auditory stimuli (Kamprath and Wotjak, 2004), and mice show increased reactivity to a WN stimulus after experiencing stress (Hoffman et al., 2022). These findings highlight the need to better elucidate the associative and non-associative elements of Pavlovian fear conditioning that influence expression of defensive behavior.

To address this, we utilized four control groups for non-associative effects of conditioning. To test the importance of the SCS-shock contingency, we utilized an unpaired control procedure in which the US and the SCS were presented in a separated, pseudorandom, and non-predictive fashion (Rescorla, 1967). To test the effects of sensitization by the shock, we presented footshock alone during conditioning. To test the impact of stimulus intensity and salience, we conducted paired and unpaired conditioning using a reversed SCS where the WN preceded the tone. We compared these four control groups against a paired SCS-shock conditioning group to determine the effects of associative learning on SCS-evoked fear behavior. All groups went through two extinction

sessions with SCS presentations alone to elucidate the extent to which prior associative pairing affects de-escalating response strategies, as well as to identify defensive behaviors that are expressed in the absence of a strict threat-signaling association.

Materials & methods

Animal Subjects

We used C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine, Stock #000664), aged 3-6 months in this study. Equal numbers of males and females were used in all experiments. All mice were individually housed on a 12 h light/dark cycle throughout the study with *ad libitum* access to food and water. Behavioral experiments were performed during the light cycle. All animal procedures were performed in accordance with institutional guidelines and were approved by the Institutional Animal Care & Use Committee of Tulane University.

Apparatus

Behavioral testing was performed in two contexts. Context A consisted of a 30 cm diameter transparent acrylic cylinder with a smooth acrylic floor, cleaned with 1% acetic acid between each subject. Context B consisted of a modular fear conditioning chamber (ENV-307W, Med Associates, Inc., Fairfax, Vermont) with metal grid flooring and walls of polycarbonate, stainless steel, and polyurethane, cleaned with 70% ethanol solution between sessions. Alternating current footshocks (ENV-414S, Med Associates, Inc.) were delivered to the mice during conditioning in Context B. A programmable audio generator (ANL-926, Med Associates, Inc.) generated auditory stimuli that were delivered at 75 dB in each context via an overhead speaker (ENV-224AM, Med Associates, Inc.). A serial compound stimulus (SCS) was used as previously described (Fadok et al., 2017, Borkar and Fadok, 2021, Borkar et al., 2020). The SCS consisted of ten pips of tone (7.5 kHz, 0.5 ms at 1 Hz) followed by ten pips of white noise (0.5 ms at 1 Hz), and the reversed SCS consisted of ten pips of white noise followed by ten pips of tone. Behavioral protocols were generated using Med-PC software (Med Associates, Inc.) to control auditory stimuli and shock with high temporal precision.

Experimental Design: SCS Conditioning and Extinction Paradigm

Mice were randomly allocated to one of five groups: Paired (PA), Paired Reverse (PA-R), Unpaired (UN), Unpaired Reverse (UN-R), and Shock Only (SO). Behavioral testing took place over 5 days. For all days of the paradigm, PA-R and UN-R mice experienced the reversed SCS at identical presentation timings as their respective PA and UN counterparts. On Day 1 (Pre-Exposure), subjects were placed in Context A for a baseline period of 3 min, followed by 4 presentations of the SCS with a pseudorandom inter-stimulus interval (ISI) period of 90-100 s and a period of 40 s following the final SCS presentation, totaling 590 s per session. Day 2 and Day 3 (Conditioning) took place in Context B. On each conditioning day (CD1 and CD2), mice were subjected to one of three conditions after a 3 min baseline period. For all groups, each conditioning session lasted 820 s. PA mice (n=16 males, 16 females) and PA-R mice (n=5 males, 5 females) were presented with 5 pairings of the SCS co-terminating with a 1 s, 0.9 mA footshock, with pseudorandom ISI periods of 90-150 s and a period of 60 s following the final footshock of the session. UN mice (n=10 males, 10 females) and UN-R mice (n=5 males, 5 females) were presented with pseudorandom presentations of SCS and footshocks separate from one another with ISI periods of 40-60 s, with a period of 90 s following the final stimulus of the session. Stimuli were ordered such that the SCS could not reliably predict footshock. PA, PA-R, UN, and UN-R mice all received the same number of SCS and footshock presentations, only differing by SCS-footshock contingency. SO mice (n=10 males, 10 females) did not receive presentations of the SCS during conditioning and were given 5 footshocks with pseudorandom ISI periods of 120-160 s each session, with a period of 80 s following the final shock of the session. For all groups, stimulus timing and ISI differed between CD1 and CD2 to avoid

predictable anticipation of stimuli before presentation. Days 4 and 5 (Extinction) took place in Context B, and each session consisted of 16 presentations of the SCS with pseudorandom ISI periods of 60-120 s, with a period of 50 s following the final SCS of the session. Each Extinction session (Ext1 and Ext2) lasted 1910 s. Subjects were sacrificed after the conclusion of behavioral testing.

Behavioral Recording and Analysis

All sessions were recorded to video using a camera (Pike, Allied Vision, Stadtroda, Germany) mounted above the behavioral contexts with stimulus events encoded to the same files using TTL pulses (Omniplex, Plexon, Dallas, Texas). Contour tracking (Cineplex, Plexon) was used to automatically detect freezing based on frame-by-frame changes in pixels. Freezing behavior was defined as a complete cessation of movement for at least 1 s, and results were confirmed with an observer blinded to condition. By determining a calibration coefficient using the known size of the behavioral context and the camera's pixel dimensions, speed (cm/s) was extracted using the animal's center of gravity. An activity index was calculated for each animal using a ratio of its speed during either the tone or WN stimulus (CS) period and its average speed from the combined 10 s periods prior to each SCS presentation (pre-SCS) during the session; the number of jumps performed during that stimulus period was then added to this ratio ($Speed_{CS}/Speed_{avg\ pre\text{-}SCS}$+Jumps). Previously we calculated flight scores per trial using speed from each trial's pre-SCS period (Fadok et al., 2017; Borkar et al., 2020), but here we utilized an average from all pre-SCS periods in our calculations to avoid denominators that were close to or equal to 0, a complication noted by other groups (Hersman et al., 2020). Reflecting this change, we now refer to this measure of locomotor change as an "activity index" instead of a "flight score" as before. Escape jumps and tail rattling behaviors were manually classified by an observer blinded to condition. Jumps were defined as the period where the mouse had all four paws above the chamber floor. Tail rattling was defined as rapid back-and-forth vibrations of the tip and midsection of the tail. Darting behavior was detected and classified using machine learning software as described below and was defined as rapid bursts of movement across the floor of the chamber. Distance traveled over pre-SCS, tone, and WN periods was calculated per mouse by plotting its average speed per 0.5 s intervals and integrating the area under the curve.

When performing behavioral analyses that reported cumulative frequencies per group, 20 random subjects from the PA group were used to match the population sizes of the UN and SO control groups. Due to the lower number of subjects in the PA-R and UN-R groups, they were excluded from frequency-based comparisons.

Analysis of Darting Using Machine Learning

Darts were scored using the program Simple Behavior Analysis (SimBA, Nillson et al., 2020) to generate a machine learning algorithm capable of automatically detecting the occurrence of the behavior of interest. To generate this model, top-down footage (640×480 pixel resolution, 30 frames per second) of 16 male and female C57BL/6J mice that underwent SCS fear conditioning in Context B was collected and analyzed in DeepLabCut (DLC) (Mathis et al., 2018) to assign 8 tracking points (Nose, L Ear, R Ear, Center of Mass, L Flank, R Flank, Tail Base, Tail Tip) to subjects. The DLC markerless tracking model was generated using manually assigned points from ~2,000 frames trained using the ResNet50 Neural Network for 125,000 iterations. 2,370 frames containing darting behavior were identified and added to the training set for SimBA. The darting start point was defined as the first frame in which the mouse began accelerating from a resting position, and the end point was defined as the last frame before the mouse returned to a full stop. Once the model was generated, all videos from all subjects were analyzed using a discrimination threshold of .37 and a minimum duration of 266 ms (8 frames).

Statistical Analysis

Sample sizes for each group were justified via power analysis (α = 0.05, power = 80%). Data were analyzed for statistical significance using Prism 9 (GraphPad Software Inc., San Diego, California). For all tests, the definition of statistical significance was $p<0.05$. All data were checked for normal distribution using the Shapiro-Wilk normality test (α=0.05). For pairwise comparisons between groups, unpaired t-test with Welch's correction was used to assess behavioral differences since all relevant datasets had normal distributions. One-way analysis of variance (ANOVA) was used to assess behavioral differences between all conditioning groups. Two-way ANOVA was used to assess interactions of time point and conditioning variant between groups, as well as interactions of stimulus and conditioning variant within groups. When either ANOVA yielded significant interactions, Tukey's post-hoc multiple comparisons test was used to detect significant behavioral differences between groups.

Results

Stimulus-evoked freezing and activity are affected by SCS-shock contingency and stimulus order

Equal numbers of male and female mice were randomly assigned to either a paired (**PA**), unpaired (**UN**), shock-only (**SO**), paired reverse (**PA-R**), or unpaired reverse (**UN-R**) group for fear conditioning and fear extinction training (**Fig 1**). Data from the PA, UN, and SO groups were statistically tested for sex differences and the significant results from these analyses are listed in **Table 1**. Given that most comparisons did not yield significant differences, data from male and female mice were pooled for statistical comparisons between groups. Additionally, given the minimal sex differences observed within the PA, UN, and SO groups, we reduced the number of subjects in the PA-R and UN-R groups and therefore did not statistically test for sex differences.

Behavioral data from the PA, UN, PA-R, and UN-R groups during the second day of fear conditioning (CD2) were compared to observe how conditioned defensive behavior differs based on the associative value and stimulus order of the SCS. A two-way ANOVA was used to analyze the effect of trial and group on freezing during the tone and WN. There was no statistically significant interaction between trial and group for tone-induced freezing (**Fig. 2A**; $F_{(12, 340)} = 0.65$, $p=0.80$); however, there was a significant main effect of trial ($F_{(4, 340)} = 6.3$, $p<0.0001$) and group ($F_{(3, 340)} = 19.6$, $p<0.0001$). All groups showed little freezing to the WN (**Fig 2B**), and no significant interaction between trial and group ($F_{(12, 340)} = 0.76$, $p=0.69$) or main effect of trial ($F_{(4, 340)} = 2.07$, $p=0.08$) were found. We did find a main effect of group ($F_{(3, 340)} = 3.00$, $p=0.03$), which was attributed to greater freezing from the PA-R group during Trial 1.

An activity index was calculated for each mouse as a combined measurement of cue-induced locomotion with escape jumping (see Methods), and a two-way ANOVA was used to analyze tone- and WN-evoked activity indices (**Fig 2C, D**). The activity indices for all groups were very low during the tone period (**Fig 2C**), and there was no significant interaction between trial and group ($F_{(12, 340)} = 0.98$, $p=0.47$). The activity index during the tone decreased over trials, concomitant with the observed increase in freezing behavior (main effect of trial, $F_{(4, 340)} = 3.58$, $p=0.007$). There was a significant effect of group ($F_{(3, 340)} = 2.7$, $p=0.045$), which was attributed to the UN-R group displaying higher activity during Trial 2. While the WN-evoked activity indices in all groups showed no significant trial by group interaction ($F_{(12, 340)} = 0.15$, $p=0.99$), or main effect of trial ($F_{(4, 340)} = 0.54$, $p=0.70$), a significant main effect of group (**Fig 2D**, $F_{(3, 340)} = 9.03$, $p<0.0001$) was observed.

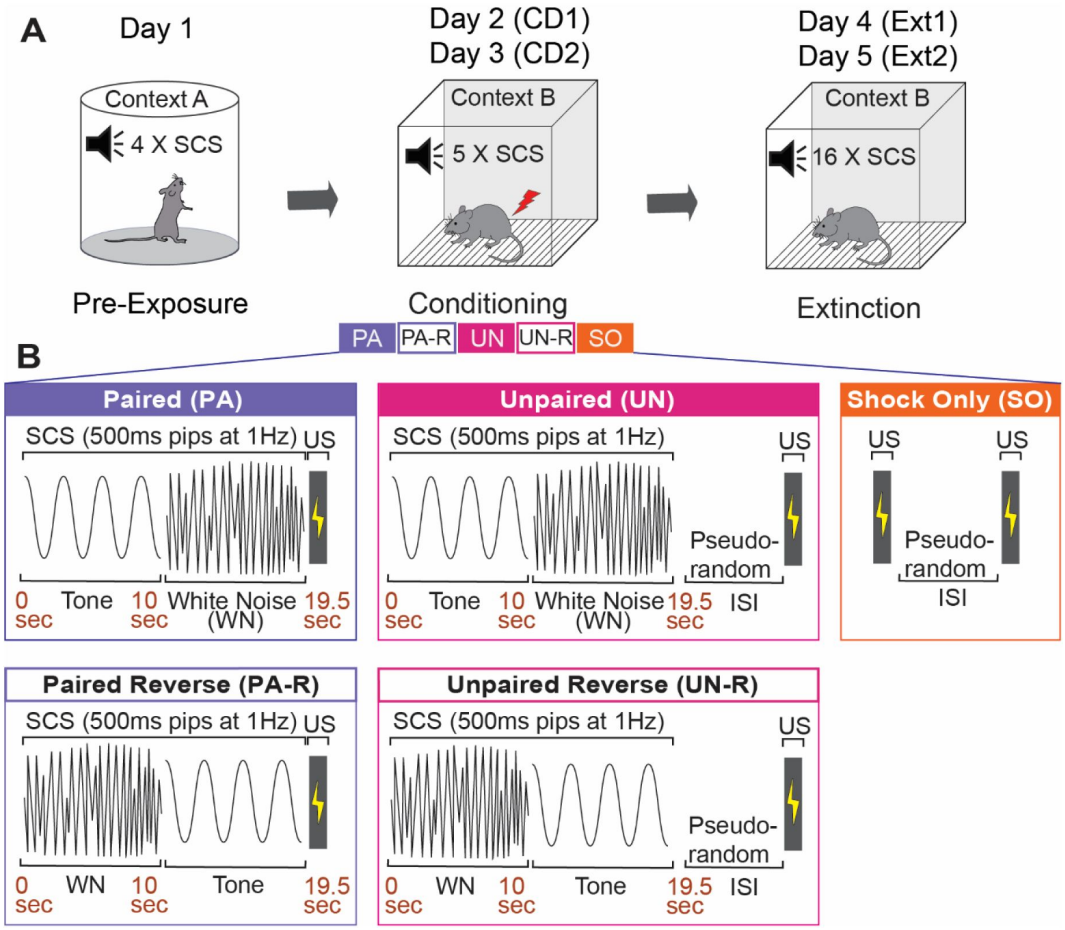

Figure 1.

Experimental Design. **A,** Graphical representation of the three stages of the SCS conditioning paradigm. **B,** Five SCS-shock association variants were used during conditioning. SCS, Serial compound stimulus; CD1, Conditioning Day 1; CD2, Conditioning Day 2; Ext1, Extinction Day 1; Ext2, Extinction Day 2; US, Unconditioned stimulus; ISI, Inter-stimulus interval.

Figure	Group	Behavioral Comparison	Statistical Test	p-value	Result
2E	UN	Average tone-evoked % freezing	Welch's unpaired t-test	p=0.0053	Males froze more to tone than females in CD2
2F	UN	Average tone-evoked activity index score	Welch's unpaired t-test	p=0.0178	Males had higher activity to tone compared to females in CD2

Table 1.

Statistical analysis of sex differences in defensive behavior.

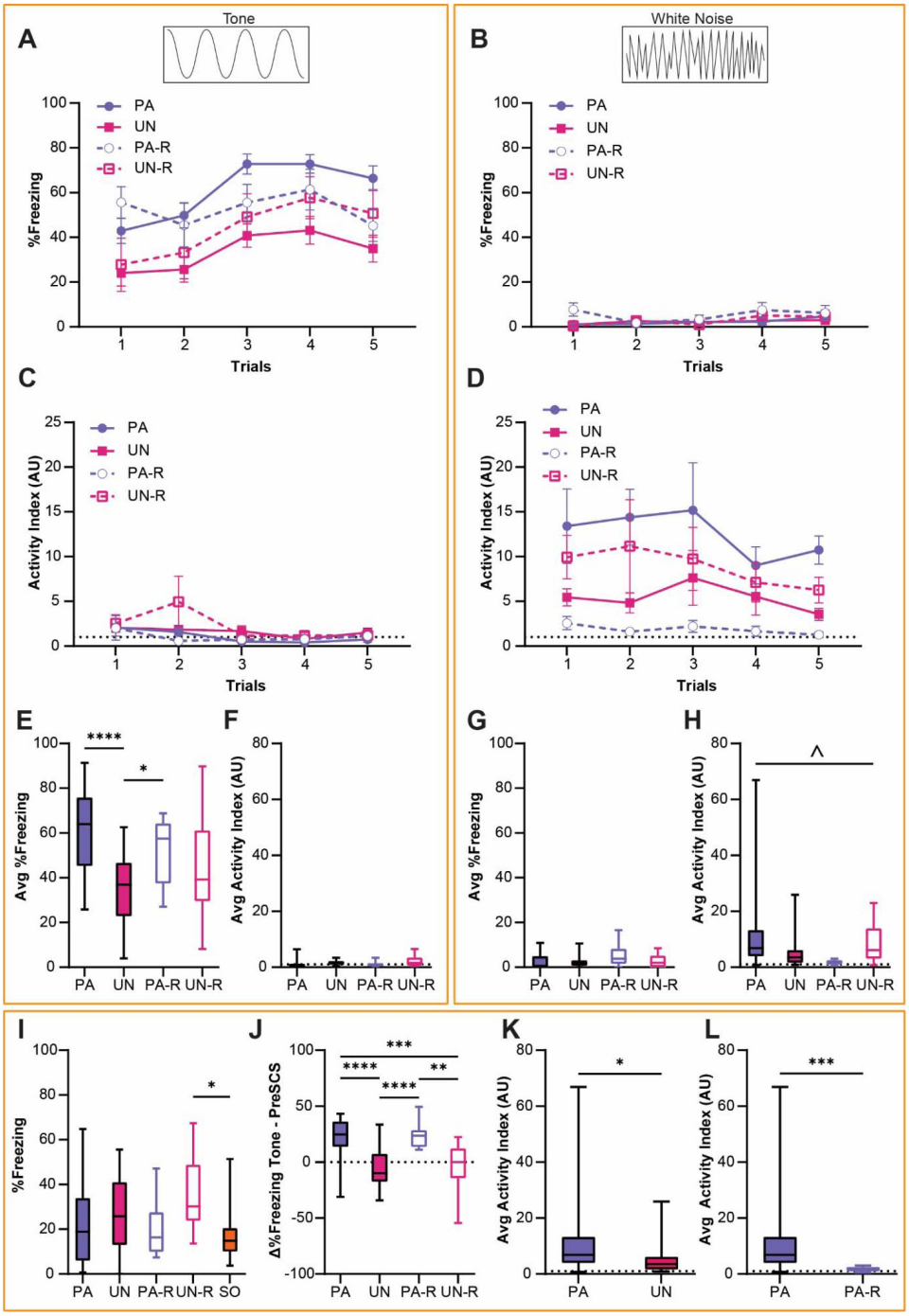

Figure 2.

Stimulus-evoked freezing and activity during CD2 are affected by SCS-shock contingency. **A,** Trial-by-trial freezing during the tone period. **B,** Trial-by-trial freezing during the WN period. **C,** Trial-by-trial activity index during the tone period. **D,** Trial-by-trial activity index during the WN period. **E,** Average freezing during the tone period from all trials of CD2. **F,** Average activity index scores during the tone period from all trials of CD2. **G,** Average freezing during the WN period from all trials of CD2. **H,** Average activity index scores during the WN period from all trials of CD2. **I,** Baseline contextual freezing levels during CD2. **J,** Differences in freezing between pre-SCS and tone periods from all trials of CD2. **K,** Average activity index scores during the WN period for the PA and UN groups from all trials of CD2. **L,** Average activity index scores during the WN for the PA and PA-R groups from all trials of CD2. Data from **Figures 2A-2D** are presented as mean ± SEM. Data from **Figures 2E-2L** are presented as box-and-whisker plots from min to max. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; ^p<0.05, effect of group.

An ordinary one-way ANOVA was used to compare average freezing and activity indices between all groups, and Tukey's multiple comparisons test was used for post-hoc comparisons. The PA and PA-R group showed significantly higher freezing during the tone than the UN group (**Fig 2E**, $F_{(3, 68)} = 9.56$, p<0.0001; PA vs UN, p<0.0001; PA-R vs UN, p=0.046), and there was no significant difference between groups in their activity indices during tone (**Fig 2F**, $F_{(3, 68)} = 2.14$, p=0.10). On the contrary, while there were no differences in WN-evoked freezing between groups (**Fig 2G**, $F_{(3, 68)} = 2.62$, p=0.058), there was a significant interaction when analyzing activity indices during WN (**Fig 2H**, $F_{(3, 68)} = 3.04$, p=0.035). However, post-hoc comparisons did not yield significant pairwise differences.

An ordinary one-way ANOVA was used to test for between-group differences in contextual freezing during the initial three minutes of the session preceding the first SCS presentation, and Tukey's multiple comparisons test was used for post-hoc comparisons. There was a main effect of group for baseline contextual freezing (**Fig 2I**, $F_{(3, 68)} = 3.18$, p=0.017), but significant differences were found only between the UN-R and SO groups (UN-R vs SO, p=0.013). Welch's unpaired t-test was used to compare the differences in freezing during the pre-SCS and tone periods to determine the extent to which the tone increased freezing. The PA and PA-R groups showed significantly greater increases in freezing from pre-SCS to tone compared to the UN and UN-R groups (**Fig 2J**, $F_{(3, 68)} = 15.92$, p<0.0001; PA vs UN, p<0.0001; PA vs UN-R, p=0.0009; PA-R vs UN, p<0.0001; PA-R vs UN-R, p=0.0055).

Given the interaction found in **Fig 2H**, we performed Welch's unpaired t-test to conduct pairwise comparisons between average activity indices during WN. WN-evoked activity was higher in the PA group compared to the UN group (**Fig 2K**, PA vs UN, $t_{(43.08)} = 2.36$, p=0.023) and the PA-R group (**Fig 2L**, PA vs PA-R, $t_{(31.62)} = 3.89$, p=0.0005). Additionally, the PA-R group displayed lower WN-evoked activity than both of the UN groups (PA-R vs UN, $t_{(20.65)} = 2.65$, p=0.015; PA-R mean ± SEM = 1.86 ± 0.28; UN mean ± SEM = 5.39 ± 1.31) and the UN-R group (PA-R vs UN-R, $t_{(9.215)} = 2.75$, p=0.022; UN-R mean ± SEM = 8.84 ± 2.52). There was no difference between the UN and UN-R groups with regards to WN-evoked activity (UN vs UN-R, $t_{(14)} = 1.214$, p=0.24). While the PA and UN groups significantly differed in average activity indices during WN, the PA and UN-R groups did not (PA vs UN-R, $t_{(30.42)} = 0.9962$, p=0.33; PA mean ± SEM = 12.54 ± 2.73).

Overall, these data show that the respective changes in defensive behavior during tone and WN were significantly affected by the explicit pairing of SCS and shock during fear conditioning, and that the order of tone and WN presentation influenced the intensity of WN-evoked responses.

Associative pairing of the SCS and shock elicits escape jumping and darting responses to WN after conditioning

Although the UN or UN-R groups did not receive an associative pairing between SCS and shock like the PA and PA-R groups, all groups still displayed increased activity indices to WN (**Fig 2D**). To determine if this behavioral response was due to defensive flight or a more basic locomotor response, we investigated the occurrence of escape jumping and darting behaviors during WN presentation on CD2. A substantial percentage of PA mice jumped during WN on every trial, and these jumps were distributed across the entire WN period (**Fig 3A, B**). In contrast, an exceedingly small percentage of the UN group jumped during WN (**Fig 3C**), and when jumps occurred, they occurred at the onset of the WN (**Fig 3D**). Both groups responded to shock with a similar number of jumps (**Fig 3B, D**). Trial-by-trial, PA mice displayed more jumping behavior across the WN period (**Fig 3E**) compared to UN mice (**Fig 3F**). Like the UN group, the reverse SCS groups also displayed lower jumping percentages to WN, with jumps in the PA-R group occurring rarely near the transition from WN to tone, (**Fig 3G**) and jumps in the UN-R group occurring near WN onset (**Fig 3H**). The PA group had the largest percentage of mice that jumped

to WN during CD2 compared to all other groups (**Fig 3I**). Lastly, all groups exhibited jumps to shock (**Fig 3J**), with greater percentages of the unpaired and reverse cohorts responding with jumps.

Similar analyses were performed for darting behavior. A high percentage of PA mice showed darting during WN (**Fig 4A**), darts were specific for the WN, and they were spread across the stimulus period (**Fig 4B**). Mice in the UN group almost never darted during the tone or WN (**Fig 4C, D**). Further, PA mice displayed darts across the WN period on every trial (**Fig 4E**), whereas UN mice did not (**Fig 4F**). The PA group also had the largest percentage of mice that darted during WN compared to all other groups (**Fig 4G**). Interestingly, we did not detect darting from the reverse groups during the SCS in CD2. The non-PA groups only rarely expressed escape jumping or darting, yet they did have elevated activity indices (**Fig 2D**). Therefore, we measured the average distance traveled over the WN period, and we used a one-way ANOVA to determine if these higher activity levels were due to a simpler locomotor response. The groups did not differ in distance traveled during preSCS (**Fig 4H**, $F_{(3, 68)} = 0.28$, p=0.84), but we did observe differences during tone (**Fig 4I**, $F_{(3, 68)} = 3.34$, p=0.024) and WN (**Fig 4J**, $F_{(3, 68)} = 9.83$, p<0.0001). During the tone, the UN group had significantly greater distance traveled than the PA group (PA vs UN, p=0.014), which is reflective of the elevated freezing during the tone in the PA group (**Fig 2E**). During the WN, the PA group had greater distance traveled than the UN (PA vs UN, p=0.0018) and the PA-R groups (PA vs PA-R, p<0.0001), but not the UN-R group (PA vs UN-R, p=0.083). Finally, all groups darted to shock in similar percentages (**Fig 4K**).

In summary, associative pairings of SCS and shock produced significant cue-induced freezing to the tone, as well as robust jumping and darting behaviors that occurred over the entirety of WN presentations. Altering the contingency between WN and shock in the unpaired or reversed SCS conditions profoundly reduced these defensive behaviors, but the increased activity in all groups during WN suggests that inherent properties of the WN interact with non-associative processes to induce locomotor responses. This is reflected in the increased distance traveled during WN in all groups compared to the tone period. Representative behavioral responses of the PA, UN, PA-R, and UN-R groups to the SCS during conditioning are provided in **Video 1** and **Video 2**.

Tone-evoked freezing in the PA group is reduced by extinction learning

We next analyzed how the defensive ethogram of each group changed over the course of two extinction sessions. A two-way ANOVA was used to analyze the effect of trial and group on freezing during the tone, and Tukey's multiple comparisons test was used for post-hoc comparisons. When analyzing tone-evoked freezing across extinction within the PA, UN, and SO groups (**Fig 5A**), a significant interaction between trial and group ($F_{(62, 2208)} = 2.3$, p<0.0001) was found. For every trial except the last, the PA group exhibited a higher level of freezing during tone compared to the control groups (p<0.05 for Trials 1-15, for both sessions). Tone-evoked freezing presented similarly between the PA-R and UN-R groups (**Fig 5B**), yielding a significant interaction between trial and group ($F_{(31, 576)} = 1.63$, p=0.018). The PA-R group maintained consistently high freezing during the tone across both extinction sessions, freezing more than the UN-R group for nearly all trials (p<0.05 for Trials 1, 3-16 on Ext1, all trials on Ext2).

To quantify the relative change in freezing over each extinction session, we calculated the difference in freezing between the first four trials and the last four trials for each session. An ordinary one-way ANOVA was used to analyze the effect of group on changes in freezing during the tone, and Tukey's multiple comparisons test was used for post-hoc comparisons. There was a significant difference between groups on the first day of extinction (**Fig 5C**, $F_{(4, 87)} = 11.9$, p<0.0001), with the PA-R group being the only one to increase freezing during tone over the session (PA-R vs PA, p<0.0001; PA-R vs UN, p<0.0001; PA-R vs UN-R, p=0.007; PA-R vs SO, p=0.0032). Freezing for the PA and UN groups decreased similarly over the session (PA vs UN, p=0.99), and the

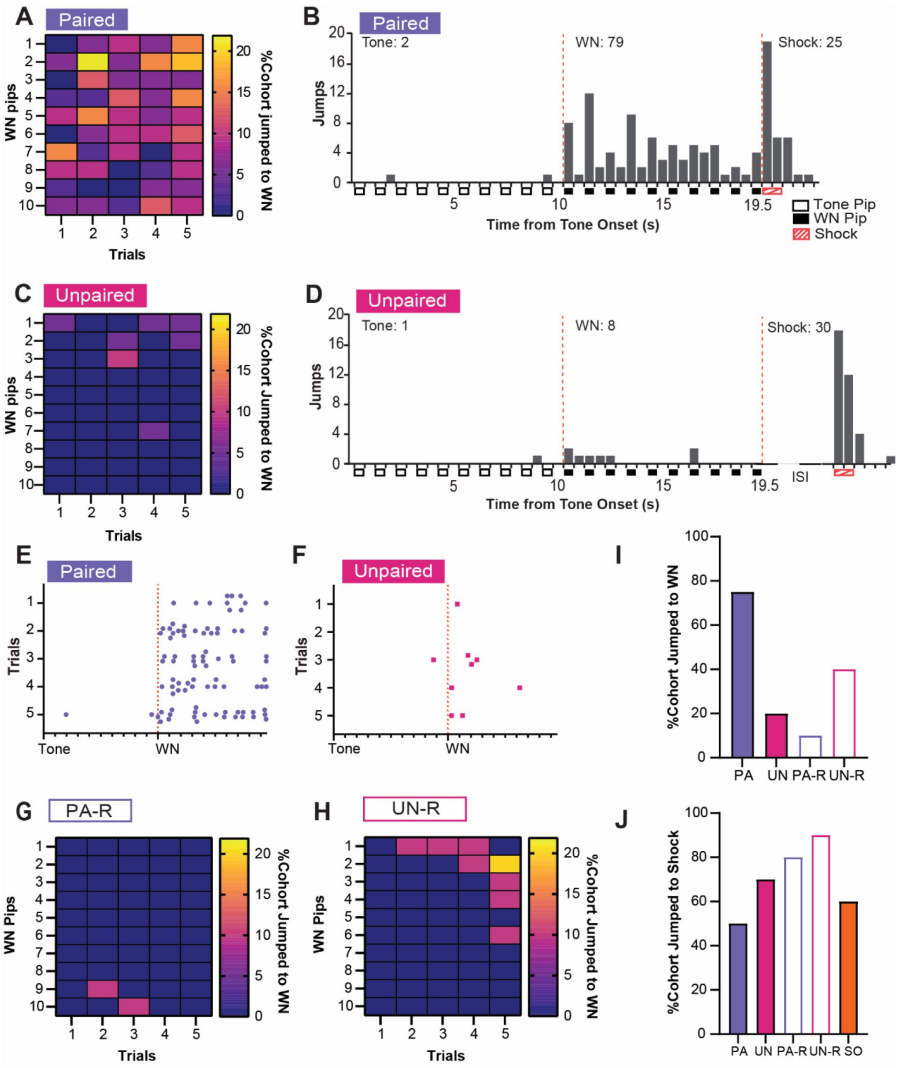

Figure 3.

Associative pairings of the SCS and shock lead to robust escape jumping during WN. **A,** The percentage of the PA group that exhibited jumping during WN on CD2. Data are distributed across 1 s bins, each coinciding with one of the ten pips of WN that occurred during each SCS presentation. **B,** The cumulative distribution of jumps from 20 randomly selected subjects of the PA group across the duration of the SCS from all 5 trials of CD2. Empty boxes represent each 0.5 s pip of tone, filled boxes represent each 0.5 s pip of WN, and striped boxes represent the 1 s shock stimulus. The vertical dotted lines depict the onset and termination of the WN period. Total jumps per stimulus are listed above histogram bars. **C,** The percentage of the UN group that exhibited jumping during WN on CD2. Data are distributed across 1 s bins, each coinciding with one of the ten pips of WN that occurred during each SCS presentation. **D,** The cumulative distribution of jumps from the UN group across the duration of the SCS from all 5 trials of CD2. Empty boxes represent each 0.5 s pip of tone, filled boxes represent each 0.5 s pip of WN, and striped boxes represent the 1 s shock stimulus. The vertical dotted lines depict the onset and termination of the WN period. ISI represents the period between SCS and shock. Total jumps per stimulus are listed above histogram bars. **E,** The distribution of jumps across the duration of the SCS from 20 randomly selected subjects of the PA group for each trial of CD2. Each dot represents a single jump event, and each tick on the x-axis represents the onset of each pip of tone or WN. The vertical dotted line depicts the onset of the WN period. **F,** The distribution of jumps across the duration of the SCS from the UN group for each trial of CD2. Each dot represents a single jump event, and each tick on the x-axis represents the onset of each pip of tone or WN. The vertical dotted line depicts the onset of the WN period. **G,** The percentage of the PA-R group that exhibited jumping during WN on CD2. Data are distributed across 1 s bins, each coinciding with one of the ten pips of WN that occurred during each SCS presentation. **H,** The percentage of the UN-R group that exhibited jumping during WN on CD2. Data are distributed across 1 s bins, each coinciding with one of the ten pips of WN that occurred during each SCS presentation. **I,** Total percentage of cohort that jumped during WN over the whole CD2 session. **J,** Total percentage of cohort that jumped to shock over the whole CD2 session.

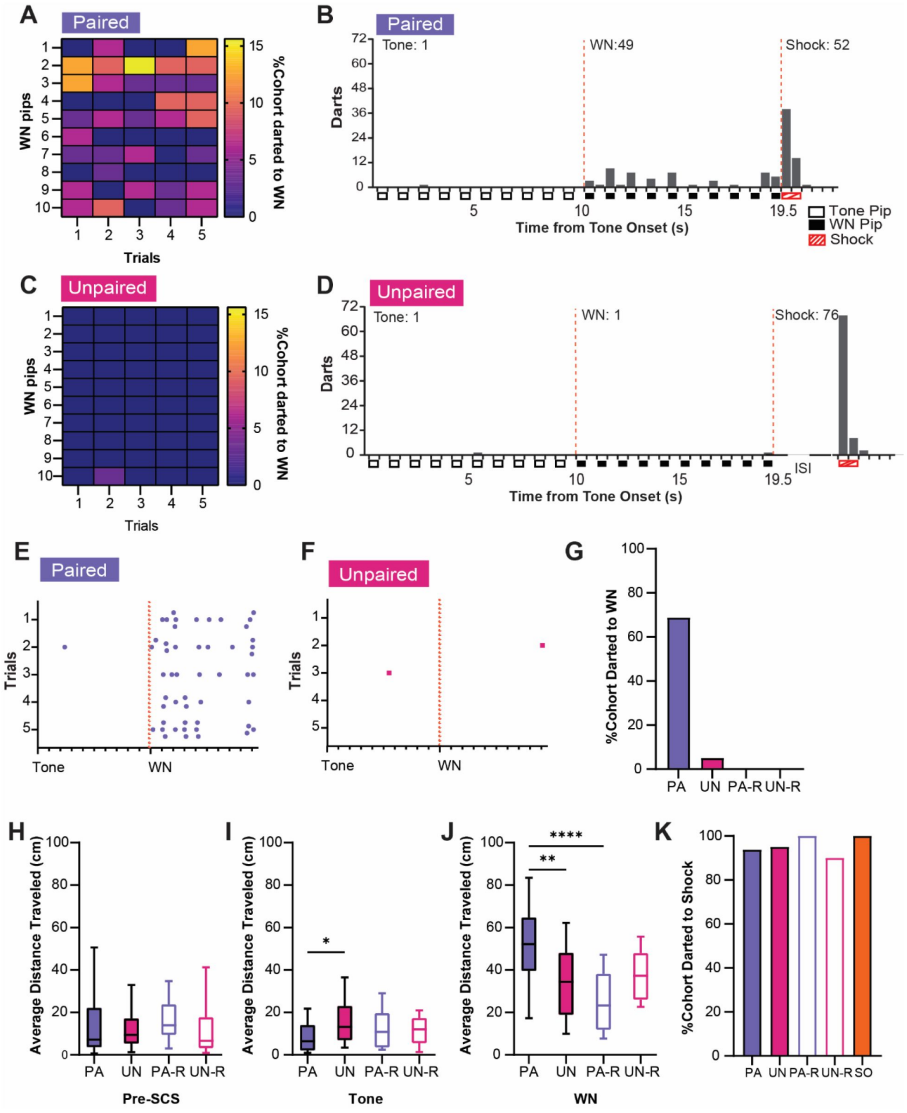

Figure 4.

Associative SCS-shock pairings elicit darting responses to WN during CD2. **A,** The percentage of the PA group that exhibited darting responses to WN. Data are distributed across 1 s bins, each coinciding with one of the ten pips of WN that occurred during each SCS presentation. **B,** The cumulative distribution of darts from 20 randomly selected subjects of the PA group across the duration of the SCS. Empty boxes represent each 0.5 s pip of tone, filled boxes represent each 0.5 s pip of WN, and striped boxes represent the 1 s shock stimulus. The vertical dotted lines depict the onset and termination of the WN period. Total darts per stimulus are listed above the histogram. **C,** The percentage of the UN group that exhibited darting responses during WN. Data are distributed across 1 s bins, each coinciding with one of the ten pips of WN that occurred during each SCS presentation. **D,** The cumulative distribution of darts from the UN group across the duration of SCS. Empty boxes represent each 0.5 s pip of tone, filled boxes represent each 0.5 s pip of WN, and striped boxes represent the 1 s shock stimulus. The vertical dotted lines depict the onset and termination of the WN period. ISI represents the period between SCS and shock. Total darts per stimulus are listed above the histogram. **E,** The distribution of darts across the duration of SCS from 20 randomly selected subjects of the PA group. Each dot represents a single dart event, and each tick on the x-axis represents the onset of each pip of tone or WN. The vertical dotted lines depict the onset of the WN period. **F,** The distribution of darts across the duration of SCS from the UN group. Each dot represents a single dart event, and each tick on the x-axis represents the onset of each pip of tone or WN. The vertical dotted lines depict the onset of the WN period. **G,** The total percentage of each group that jumped during WN over the whole session. **H**, Average distance traveled during the preSCS period. **I**, Average distance traveled during the tone period. **J**, Average distance traveled during the WN period. **K**, The total percentage of each group that jumped to shock over the whole session. Data from **Figures 4H-4J** are presented as box-and-whisker plots from min to max. *p<0.05; **p<0.01; ****p<0.0001.

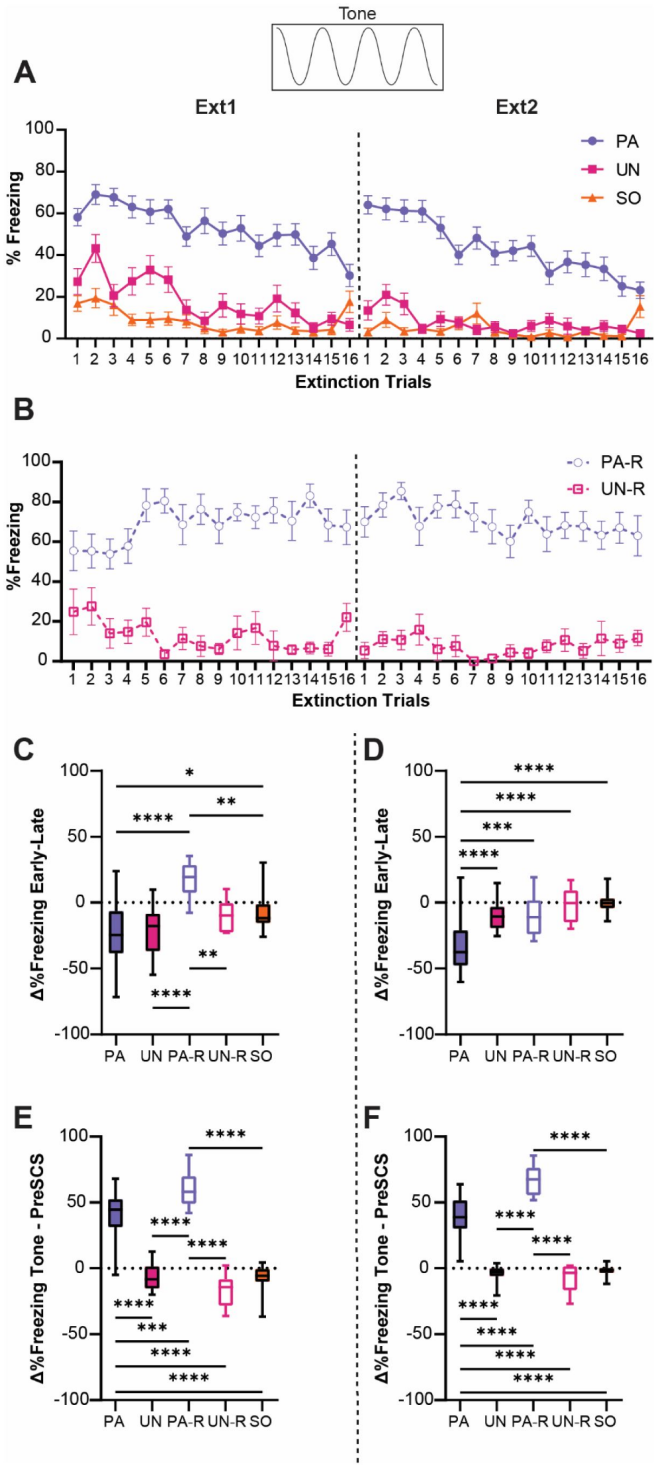

Figure 5.

Tone-evoked freezing in the PA group is reduced by extinction learning. **A,** Percent freezing during the tone period for the PA, UN, and SO groups. **B,** Percent freezing during the tone period for the PA-R and UN-R. **C**, The difference in average freezing during the tone period between the first and last 4-trial bins of Ext1. **D,** The difference in average freezing during the tone period between the first and last 4-trial bins of Ext2. **E,** The difference in average freezing between pre-SCS and tone periods during Ext1. **F,** The difference in average freezing between pre-SCS and tone periods during Ext2. Data from **Figures 5A** and **5B** are presented as Mean ± SEM. Data from **Figures 5C-5F** are presented as box-and-whisker plots from min to max. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

SO group had significantly less change in freezing compared to the PA group (PA vs SO, p=0.021), which was attributed to the low level of freezing during the tone in the SO group (**Fig 2A**). A significant difference between groups was also detected for the second extinction session (**Fig 5D**, $F_{(4,87)} = 19.2$, p<0.0001). Only the PA group exhibited a decrease in freezing during tone compared to all other groups (PA vs UN, p<0.0001; PA vs PA-R, p=0.0007; PA vs UN-R, p<0.0001; PA vs SO, p<0.0001).

To determine if freezing during the tone was cue-induced, or was simply a continuation of contextual freezing, we calculated the difference between freezing in the pre-SCS period and freezing during the tone for each extinction session (**Fig 5E, F**). An ordinary one-way ANOVA was used to analyze the effect of group, and Tukey's multiple comparisons test was used for post-hoc comparisons. For both the first (**Fig 5E**, $F_{(4, 87)} = 105.5$, p<0.0001) and second (**Fig 5F**, $F_{(4, 87)} = 137.5$, p<0.0001) sessions of extinction, only the PA and PA-R groups increased freezing levels during the tone (p<0.0001 for all PA and PA-R comparisons to other groups for both sessions). The PA-R group showed a greater change in freezing from pre-SCS to tone than the PA group (Ext1, PA vs PA-R, p=0.0003; Ext2, PA vs PA-R, p<0.0001), whereas the UN, UN-R, and SO groups had equivalent freezing during the pre-SCS and tone periods (p>0.25 for all pairwise comparisons that excluded the PA and PA-R groups for both sessions). Taken together, these data suggest that the PA and PA-R groups associated the tone with threat, while freezing in the UN, UN-R, and SO groups was more indicative of contextual fear. Interestingly, while pairing either order of SCS with shock resulted in greater freezing during tone, we observe a phenotype more resistant to extinction within the PA-R group. These data indicate that the extinction of cue-induced freezing in the conditioned flight paradigm depends on its proximity to conditioned threat.

Stimulus-induced flight is associative and is partially replaced by freezing during extinction

Activity indices were calculated to analyze the effect of extinction learning on behavioral responses during the WN. The PA, UN, and SO groups had elevated activity indices in the early trials of extinction training, yet only the PA group showed a decrease in WN-evoked activity (**Fig 6A**). A two-way ANOVA was used to analyze the effect of trial and group, and Tukey's multiple comparisons test was used for post-hoc comparisons. There was a significant interaction between trial and group ($F_{(62, 2208)} = 1.9$, p<0.0001). Starting with the fifth trial of the first extinction session, the PA group expressed significantly less activity to WN compared to the UN and SO groups (p<0.05 compared to UN and SO for Trials 5, 6, and 8-13), and PA activity index scores remained below 1 for the entirety of the second extinction session (p<0.05 compared to UN and SO for Trials 1, 2, and 4-16). For the UN and SO groups, WN-evoked activity indices remained above 1 across extinction trials, indicating levels of movement that were higher during the WN than the pre-SCS period. There was no interaction between trial and group (**Fig 6B**, $F_{(31, 576)} = 0.7$, p=0.89) for the PA-R and UN-R groups. There was an effect of group ($F_{(1, 576)} = 64.7$, p<0.0001), with activity being higher in the UN-R compared to the PA-R group. There was no significant effect of trial ($F_{(31, 576)} = 0.66$, p=0.92).

To illustrate the change in WN response over extinction, we calculated the difference in speed during WN between the first four and last four trials of each session. An ordinary one-way ANOVA was used to analyze the effect of group, and Tukey's multiple comparisons test was used for post-hoc comparisons. The PA group showed a significant decrease compared to the UN-R and SO groups (**Fig 6C**, $F_{(4, 87)} = 6.4$, p<0.0001; PA vs UN-R, p=0.0052; PA vs SO, p=0.0003) and a near-significant decrease compared to the UN group (PA vs UN, p=0.054). There were no significant differences between groups during the second extinction session (**Fig 6D**, $F_{(4, 87)} = 1.47$, p=0.22).

Interestingly, as WN-evoked activity decreased during extinction, the PA group developed and maintained a freezing response to WN, while the other groups displayed almost no freezing to WN (**Fig 6E**). A significant interaction between trial and group was detected ($F_{(124, 2784)} = 1.3$,

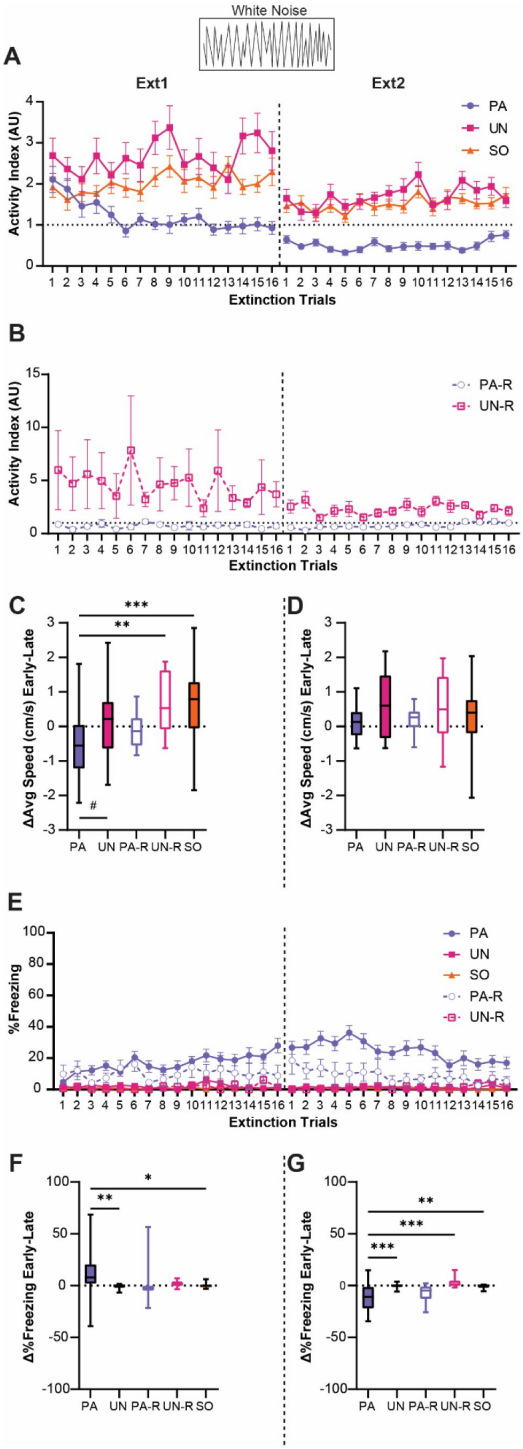

Figure 6.

Stimulus-induced flight is associative and is partially replaced by freezing during extinction. **A,** Trial-by-trial activity during the WN period for the PA, UN, and SO groups during Ext1 and Ext2. **B,** Trial-by-trial activity during the WN period for the PA-R and UN-R groups during Ext1 and Ext2. **C**, Difference in average speed during the WN period from the first and last 4-trial bins of Ext1. **D**, Difference in average speed during the WN period from the first and last 4-trial bins of Ext2. **E,** Trial-by-trial freezing during the WN period for all groups during Ext1 and Ext2. **F**, Difference in freezing during the WN period from the first and last 4-trial bins of Ext1. **G**, Difference in freezing during the WN period from early and late 4-trial bins of Ext2. Data from **Figures 6A**, **6B**, and **6E** are presented as Mean ± SEM. Data from **Figures 6C**, **6D**, **6F**, and **6G** are presented as box-and-whisker plots from min to max. #p=0.054, *p<0.05, **p<0.01, ***p<0.001.

p=0.019), and the PA group displayed greater freezing than the UN, UN-R, and SO groups for a majority of the first extinction session (p<0.05 for Trials 4, 6, and 10-16) and for most of the second session (p<0.05 for Trials 1-13). Additionally, the PA group froze more during WN than the PA-R group for several trials of the second session (p<0.05 for Trials 2-6, 8-10). When comparing changes in WN-evoked freezing between the first and last four trials for the first extinction session (**Fig 6F**, $F_{(4,87)} = 4.2$, p=0.0036), the PA group displayed significant increases compared to the UN and SO groups (PA vs UN, p=0.0086; PA vs SO, p=0.017), but did not differ from the PA-R or UN-R groups (PA vs PA-R, p=0.18; PA vs UN-R, p=0.22). For the second session (**Fig 6G**, $F_{(4,87)} = 8.1$, p<0.0001), the PA group exhibited a significant decrease in WN-evoked freezing compared to the UN, UN-R, and SO groups (PA vs UN, p=0.0005; PA vs UN-R, p=0.0006; PA vs SO, p=0.0015), but not the PA-R group (PA vs PA-R, p=0.85).Collectively, these findings show changes in the magnitudes and modes of behavior to WN within the PA group across extinction, indicating that WN-evoked flight in the PA group can be extinguished and is associative.

Stimulus evoked escape jumping and darting during extinction

To determine if the activity measured during extinction was related to defensive flight or a more basic locomotor response, we examined the expression of jumping and darting behaviors between groups. Within the first four trials of the first extinction session, PA mice displayed the most jumping behavior during WN, with the SO group displaying only two jumps occurring near the onset of WN, and the UN group displaying no jumps (**Fig 7A**). When examining darting behavior within the first four trials of extinction, minimal darting was observed during the tone period, the PA and SO groups displayed darting behavior spread across the WN period, while the UN group darted only a few times (**Fig 7B**). Jumps and darts were not present within the second extinction session for any group.

During the first extinction session, only the PA group displayed a concentration of jumping responses during WN within the first block of trials (**Fig 7C**), and jumps to WN rarely occurred in any other group (**Fig 7E**). All groups displayed variable amounts of darting to WN, with the PA, PA-R, and SO groups having the largest proportions of darters within the session (**Fig 7D, F**). Notably, the increase in darting in the PA group occurs around Trial 7, which is approximately the timepoint at which jumping is fully extinguished. Within the PA-R and SO groups, WN-evoked darting is distributed throughout the session.

These data suggest that escape jumping is largely an associative response that switches to darting as the psychological distance of threat increases; however, darting is controlled by associative and non-associative mechanisms. Representative behavioral responses of the PA, UN, and SO groups to the SCS between early and late periods of the first extinction session are provided in **Video 3** and **Video 4**.

Tail rattling is a non-associative behavioral response during extinction

We previously observed heightened tail rattling responses during the early trials of fear conditioning, which decreased with further conditioning (Borkar et al., 2020). Given that tail rattling has been shown to increase in the presence of uncertain threat (Salay et al., 2018), we measured tail rattling in all groups during extinction to determine the effects of associative and non-associative mechanisms on this defensive response. During the first extinction session, tail rattling behavior during SCS presentations was more prevalent in the UN, UN-R, and SO control groups and was most prominent during the tone period (**Fig 8A, B**). To monitor tail rattling within groups, cumulative behavioral frequencies were taken from the first and last four trials within each extinction session (reverse SCS groups were excluded due to lower N-values). During early extinction, SO and UN mice displayed more tail rattling than PA mice during the tone (**Fig 8C**). The frequency of tail rattling was lower in all three groups during the second extinction session, yet the UN and SO groups both displayed more than the PA group (**Fig 8D**). All three

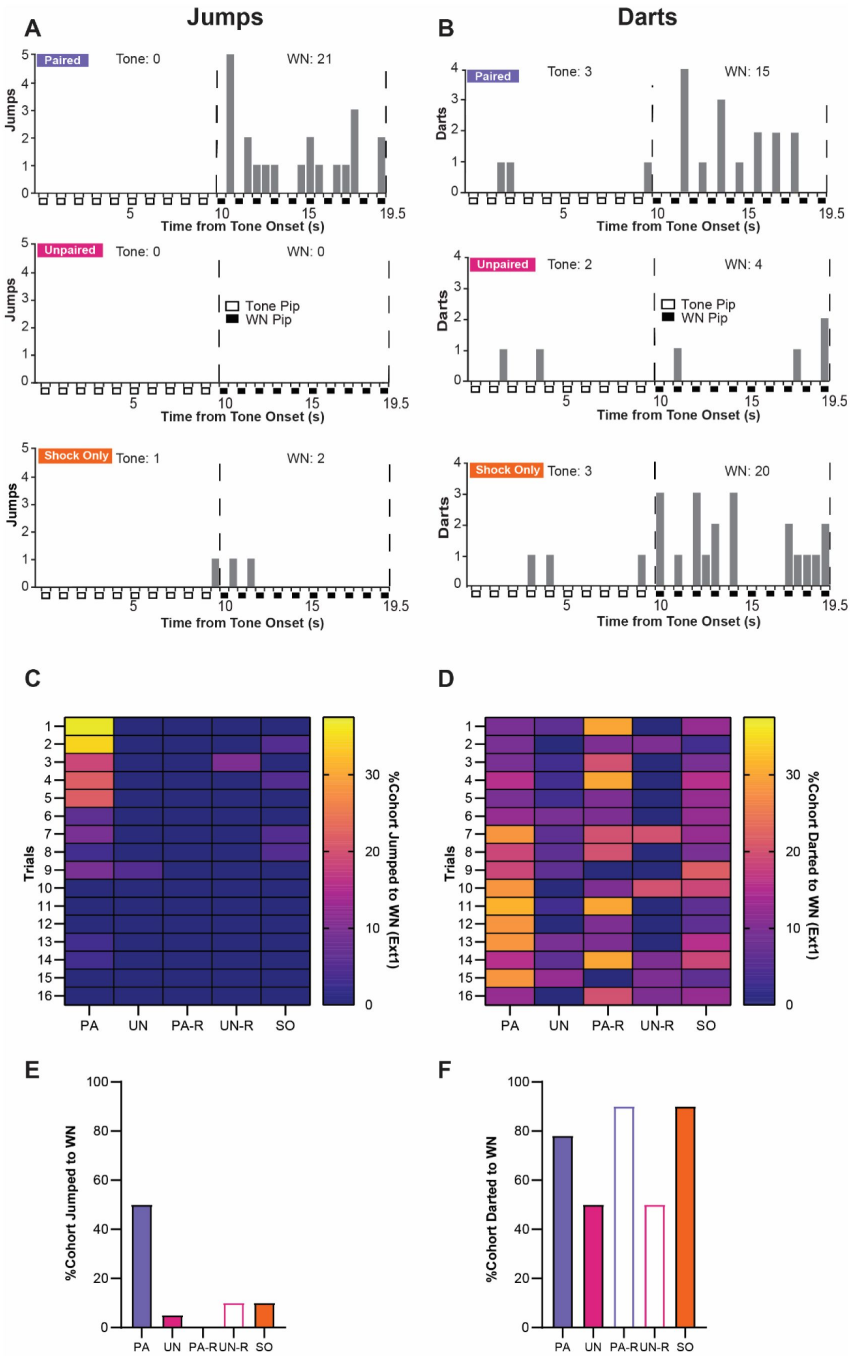

Figure 7.

Stimulus-evoked escape jumping and darting during extinction. **A,** The cumulative distribution of jumps from the first 4 trials of Ext1 for 20 randomly selected subjects from the PA group (*top*), the UN group (*middle*), and the SO group (*bottom*). Empty boxes represent each 0.5 s pip of tone, filled boxes represent each 0.5 s pip of WN, and the vertical dotted lines represent the onset and termination of the WN period. Total jumps per stimulus are listed above the histogram. **B,** The cumulative distribution of darts from the first 4 trials of Ext1 for 20 randomly selected subjects from the PA group (*top*), the UN group (*middle*), and the SO group (*bottom*). Empty boxes represent each 0.5 s pip of tone, filled boxes represent each 0.5 s pip of WN, and the vertical dotted lines represent the onset and termination of the WN period. Total darts per stimulus are listed above the histogram. **C,** The percentage of each group that exhibited jumping responses during the WN period of SCS per trial on Ext1. **D,** The percentage of each group that exhibited darting responses to the WN period of SCS per trial on Ext1. **E,** Total percentage of each cohort that jumped to WN over the whole Ext1 session. **F,** Total percentage of each cohort that darted to WN over the whole Ext1 session.

groups displayed similar levels of tail rattling by the end of the second session. Over the first extinction session, a larger proportion of the UN and SO groups displayed tail rattle during to tone (**Fig 8E**), and the UN group had the greatest proportion of tail rattle to WN (**Fig 8F**).

These data suggest that tail rattling during the SCS is mostly a non-associative defensive response that is suppressed when the SCS predictably signals threat.

Discussion

This study investigated the contributions of associative and non-associative processes to the expression of cue-induced defensive behaviors. The results signify that associative pairings and a proximal stimulus-threat association during fear conditioning produce maximal expression of cue-induced freezing and flight responses. Non-associative elements such as cue salience, change in stimulus intensity, and shock-induced sensitization elicit stress-associated behaviors like tail rattling and activity bursts, as hypothesized before (Trott et al., 2022), but the addition of the WN-threat associative pairing contributes significantly to eliciting high-intensity defensive responses like escape jumping. Therefore, these associative and non-associative factors combine to produce distinct behavioral transitions between freezing and flight.

During conditioning, we observed distinct ethograms for the PA and UN groups in response to the SCS. Freezing to tone and activity to WN were both significantly higher in the PA group compared to the UN group (**Fig 2E, K**), highlighting the impact of SCS-shock contingency on the magnitude of defensive responses. Notably, comparable results were previously reported in rats conditioned using similar parameters (Totty et al., 2021). Additionally, we found that the PA and PA-R groups showed significant increases in freezing from pre-SCS to tone, while in the UN and UN-R groups, freezing to tone was no greater than contextual freezing, suggesting that the paired groups placed associative value on the tone (**Fig 2J**). Lastly, the PA group displayed much more intense flight behavior during WN than the PA-R group (**Fig 2L**), indicating that the proximity of WN to the footshock threat affects defensive scaling.

Previous studies have factored jumping behaviors into normalized measures to gauge conditioned flight behavior (Fadok et al., 2017; Hersman et al., 2020; Borkar et al., 2020), but given the increased activity indices in the PA, UN, and UN-R groups, we examined if the escape jumping we observed was associative or non-associative (**Fig 3**). We found that the PA group exhibited consistent jumping responses to the WN stimulus during conditioning that were not reproduced in the UN, PA-R, or UN-R groups. Others have found that a salient stimulus is sufficient to induce conditioned jumping responses after multiple sessions (Furuyama et al., 2023). Given that the PA-R group did not display consistent jumping behavior to WN or tone, this suggests that a robust jumping response to WN in the SCS paradigm is not due to the non-associative salience of the WN as others have suggested (Hersman et al., 2020) but is instead due to its association with imminent threat.

Darting has been reported to be increased in rodents that undergo stress and fear conditioning, and it is more prevalent in female rats (Gruene et al., 2015, Brzozowska et al., 2017). Previous studies have measured darting to WN as a darts per minute measure (Morena et al., 2021; Hoffman et al., 2022; Trott et al., 2022) or as part of a composite escape score (Hersman et al., 2020), but our data suggests that examining darting requires a more detailed analysis. We found that the PA group exhibited darting across the entire WN period during conditioning, while the response was virtually nonexistent in the UN, PA-R, and UN-R groups (**Fig 4**). This is consistent with findings where conditioned darting has been shown to occur more often several seconds after a CS, rather than at its onset (Mitchell et al., 2023). This indicates that darting during conditioning is not caused by the salience of the tone-WN transition, but from an associative learned response. Additionally, the absence of darting in the PA-R group indicates that the

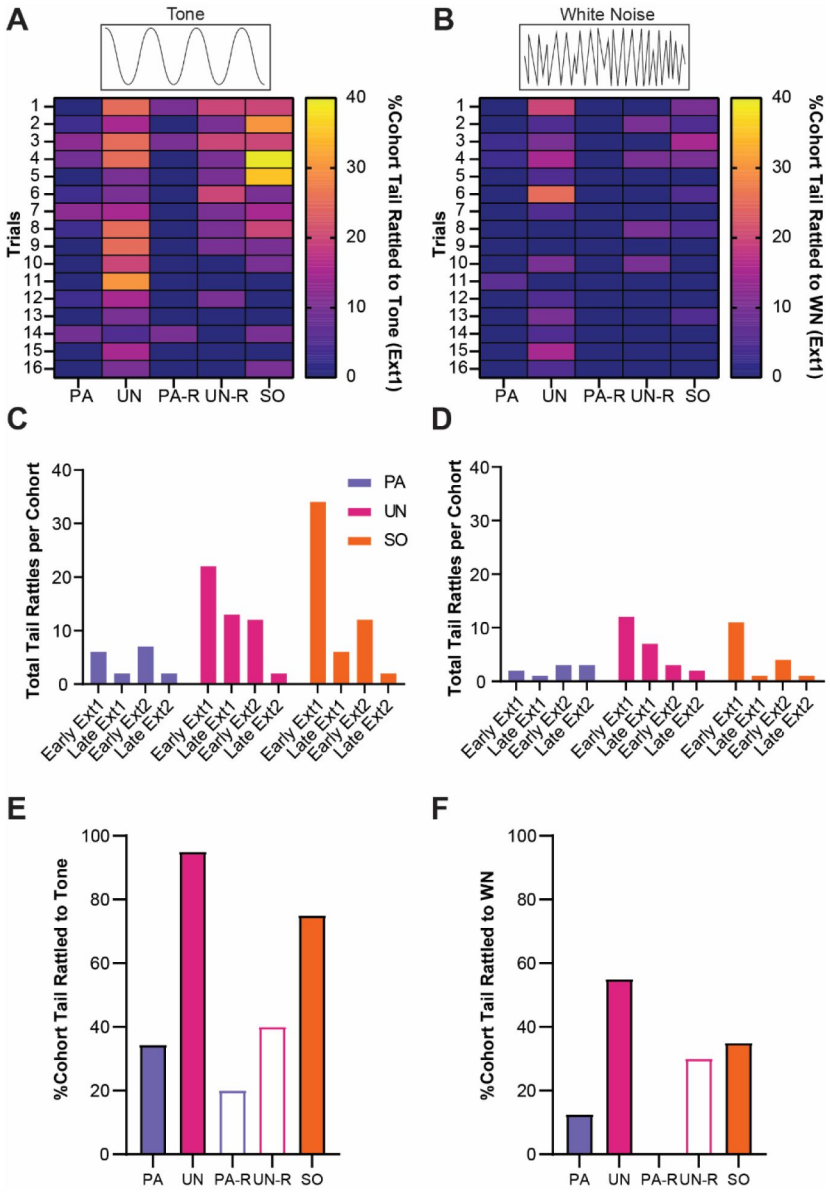

Figure 8.

Tail rattling is a non-associative behavioral response during extinction. **A,** The percentage of each group that exhibited tail rattling to the tone. **B,** The percentage of each group that exhibited tail rattling to the WN. **C,** Cumulative tail rattling during tone across early and late periods of Ext1 and Ext2. **D,** Cumulative tail rattling during WN across early and late periods of Ext1 and Ext2. **E,** Total percentage of each cohort that tail rattled to tone during Ext1. **F,** Total percentage of each cohort that tail rattled to WN during Ext1.

temporal proximity of the WN to the shock also influences darting. Our data show that the control groups engage primarily in simple locomotor behaviors induced by stimulus salience (**Fig 4J**). Overall, darting behaviors during conditioning contribute to the higher activity score observed during WN in the PA group, and our data suggests that these contributions are the result of associative learning and threat imminence, rather than non-associative stimulus salience.

During extinction, the PA and PA-R groups showed the highest level of freezing to tone compared to the non-associative control groups (**Fig 5A, B**). This indicates that the paired groups had the strongest association between the tone stimulus and threat. This is further reinforced by the larger difference between tone-evoked freezing and pre-SCS freezing in the PA and PA-R groups (**Fig 5E, F**). Freezing during tone in the non-associative control groups, on the other hand, is no greater than freezing in the interstimulus intervals. Interestingly, tone-evoked freezing in the PA group underwent within-session freezing, while that of the PA-R group was resistant to extinction (**Fig 5A-D**). The sustained tone-evoked freezing over multiple extinction sessions in the PA-R group is likely a product of the temporal proximity of the cue to the footshock, which is set at a greater intensity (0.9 mA) in the conditioned flight paradigm than is traditionally used in Pavlovian threat conditioning (typically 0.2-0.6 mA). Because freezing during the tone is not elevated over contextual freezing levels in the unpaired and SO groups, the reductions in freezing during the tone during extinction in these groups can be attributed to reduced freezing overall.

In response to WN during extinction, the PA group transitioned from explosive circa-strike flight responses containing escape jumps to a combination of anticipatory post-encounter freezing and darting (**Fig 6A**, **Fig 7**). This likely reflects a larger perceived psychological distance from threat that influences defensive strategy (Perusini and Fanselow, 2015). The control groups showed only slight changes in WN-evoked activity over extinction, similar to responses of stressed mice to an unfamiliar WN (**Fig 4**; Hoffman et al., 2022). The lack of decrease in WN-evoked activity over extinction in the PA-R group compared to the PA group, and the lack of the control groups transitioning to freezing behavior, suggests that WN-evoked flight in the PA group is associative and is dependent on the perceived threat value of the WN stimulus.

An examination of jumping and darting behaviors over the first extinction session reveals a distinct ethogram in the PA group (**Fig 7**). The presence of jumping during early extinction trials in the PA group indicates that the WN initially signaled imminent threat, resulting in an explosive circa-strike escape response. However, the change from jumping to darting may reflect a change in perceived threat imminence in the same vein as the observed change from flight to freezing. This phenomenon was only observed in the PA group, indicating that this behavioral change is associative and is consistent with the predatory imminence continuum. This jumping behavior to WN is associative and can be extinguished, making it a suitable measure for future studies interested in how the nervous system controls experience-dependent high-intensity fear reactions.

The elevated activity indices from the UN and SO groups are similar to other studies that report heightened activity to WN after multiple footshocks, or a sudden change in stimulation (Hoffman et al., 2022; Trott et al.,2022). However, given the lack of darting and jumping from the UN group during extinction (**Fig 7A, B**), their increased activity is due to other locomotor behaviors unrelated to flight. The SO group maintained a consistent level of darting throughout the first extinction session, which contributed to their overall increased activity index (**Fig 7B, D**). Given that darting can be elicited in stressed mice (Brzozowska et al., 2017), it is probable that shock sensitization can prime an animal to dart more readily to an unfamiliar, but highly salient, stimulus upon stimulus transition. Indeed, darting behavior has been shown to change based on multiple parameters, decreasing both with increased shock intensity (Mitchel et al., 2023) and with prolonged extinction training (Demars et al., 2022), suggesting that darting is both an associative conditioned response to stimuli associated with threat and a response resulting from non-associative sensitization.

Within the UN, UN-R, and SO control groups, we observed a higher degree of tail rattling responses during extinction compared to the PA and PA-R groups, and tail rattling was more prominent during the tone period (**Fig 8**). Tail rattling has been observed in mice when determining hierarchical relationships (Haber and Simmel 1976, Terranova et al., 1998, Dorofeikova et al., 2023), anticipating fighting (Miczek et al., 2001), and encountering looming threat (Salay et al., 2018). Thus, tail rattling may be a behavior elicited in stressed mice in the face of uncertain threat. Previously, we found that tail rattling occurs most often during early trials of fear conditioning, with a prominent decline later in conditioning (Borkar et al., 2020). This is consistent with our results from the UN, UN-R, and SO groups, who displayed greater tail rattling to unpaired (UN, UN-R groups) or novel (SO group) SCS presentations within the conditioning context, with reductions in tail rattling over the course of extinction exposure. Taken together, this suggests that tail rattling is not a behavior exhibited during post-encounter or circa-strike levels of threat, but rather within stressful scenarios where danger is uncertain but anticipated. Future studies that are interested in measuring defensive responses to threat signals such as context, odor, or innately aversive sensory stimuli should consider measuring tail rattling as a marker of non-associative anticipatory fear.

Current studies are investigating behaviors beyond freezing within classical Pavlovian conditioning paradigms (Tryon et al., 2021; Laine et al., 2022), but responses like jumping and darting are not always reliably elicited during CS presentation (Colon et al., 2018; Akmese et al., 2023; Biddle and Knox, 2023). Using the SCS flight conditioning paradigm, we elicit a robust continuum of consistent associative defensive responses during CS presentation that are seldom observed within classical Pavlovian conditioning. While behaviors like darting and tail ratting can occur due to non-associative stimulus-inherent properties, transitions between freezing and jumping are robustly present when associative factors signal threat imminence. Future studies can utilize this paradigm to investigate neuronal mechanisms that contribute to threat association and direct dynamic responses to threat, with important implications for developing new treatments for those that suffer from fear and anxiety disorders.

Conflict of interest statement

The authors declare no competing financial interests.

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Reviewer #1 (Public review):

Summary

The main goal of the study was to tease apart the associative and non-associative elements of cued fear conditioning that could influence which defensive behaviors are expressed. To do this, the authors compared groups conditioned with paired, unpaired, or shock only procedures followed by extinction of the cue. The cue used in the study was not typical; serial presentation of a tone followed by a white noise (or reversed) was used in order to assess switches in behavior across the transition from tone to white noise. Many defensive behaviors beyond the typical freezing assessments were measured, and both male and female mice were included throughout. The authors found changes in behavioral transitions from freezing to flight during conditioning as the tone transitioned into white noise, and a switch in freezing during extinction such that it became high during the white noise as flight behavior decreased. Overall, this was an interesting analysis of transitions in defensive behaviors to a serially presented cue consisting of two auditory stimuli during conditioning and then extinction.

Strengths

The highlights in this study were the significant switches in freezing and escape-like behaviors as the cue transitioned between the two auditory stimuli during fear conditioning, and then adjustment of those behaviors across extinction.

These main findings were a result of thorough behavioral analyses with key control groups (reversed stimulus order, unpaired conditioning, and shock only groups), assessing freezing, jumping, darting and tail rattling to try to parse out associative versus non-associative features of the behavioral profiles.

Weaknesses

While the detailed analyses of defensive behaviors in mice in a situation of signaled imminent threat adds valuable knowledge to those studying fear conditioning, the caveat is that it is unclear how broadly applicable these findings truly will be. It makes sense that similar transitions in defensive behaviors will occur across organisms, but each organism and each psychiatric disorder will have unique profiles.

https://doi.org/10.7554/eLife.90414.2.sa2

Reviewer #2 (Public review):

Summary:

The authors examined several defensive responses elicited during Pavlovian conditioning using a serial compound stimulus (SCS) as the conditioned stimulus (CS) and a shock unconditioned stimulus (US) in male and female mice. The SCS consisted of a tone pips followed by white noise. Their design included conditions in which mice were exposed to the CS and US in a paired fashion, in an unpaired fashion, or only exposed to the shock US, as well as paired and unpaired conditions that reversed the order of the SCS. They compared freezing, jumping, darting, and tail rattling across all groups during conditioning and extinction. During conditioning, strong freezing responses to the tone pips followed by strong jumping and darting responses to the white noise were present in the paired group but less robust or not present in the unpaired or shock only groups. During extinction, tone-induced freezing diminished while the jumping was replaced by freezing and darting in the paired group. Together, these findings support the idea that associative pairings are necessary for conditioned defensive responses.

Strengths:

The study has strong control groups including a group that receives the same stimuli in an unpaired fashion and another control group that only receives the shock US and no CS to test the associative value of the SCS to the US. The authors examine a wide variety of defensive behaviors that emerge during conditioning and shift throughout extinction: in addition to the standard freezing response, jumping, darting, and tail rattling were also measured.

The revised version has greatly strengthened this study by including additional control groups (e.g., reversing the order of the compound stimuli in both paired and unpaired conditions).

https://doi.org/10.7554/eLife.90414.2.sa1

Author response:

The following is the authors' response to the original reviews.

> *The reviewers found this manuscript to present convincing evidence for associative and non-associative behaviors elicited in male and female mice during a serial compound stimulus Pavlovian fear conditioning task. The work adds to ongoing efforts to identify multifaceted behaviors that reflect learning in classic paradigms and will be valuable to others in the field. The reviewers do note areas that would benefit from additional discussion and some minor gaps in data reporting that could be filled by additional analyses or experiments.*

We thank the reviewers and the editors for their thoughtful and constructive critiques of our manuscript. We have updated our manuscript with data from additional experiments as suggested by the reviewers, and we have significantly edited the text and figures to reflect these additions. Our detailed, point-by-point responses are below.

> ***Reviewer #1 (Public Review):***
>
> *The main goal of the study was to tease apart the associative and non-associative elements of cued fear conditioning that could influence which defensive behaviors are expressed. To do this, the authors compared groups conditioned with paired, unpaired, or shock only procedures followed by extinction of the cue. The cue used in the study was not typical; serial presentation of a tone followed by a white noise was used in order to assess switches in behavior across the transition from tone to white noise. Many defensive behaviors beyond the typical freezing assessments were measured, and both male and female mice were included throughout. The authors found changes in behavioral transitions from freezing to flight during conditioning as the tone transitioned into white noise, and a switch in freezing during extinction such that it became high during the white noise as flight behavior decreased. Overall, this was an interesting analysis of transitions in defensive behaviors to a serially presented cue consisting of two auditory stimuli during conditioning and then extinction.*

We thank the Reviewer for their supportive insight.

> *There are some concerns regarding the possibility that the white noise is more innately aversive than the tone, inducing more escape-like behaviors compared to a tone, especially since the shock only group also showed increased escape-like behaviors during the white noise versus tone. This issue would have been resolved by adding a control group where the order of the auditory stimuli was reversed (white noise->tone).*

We appreciate this concern, and we have added two additional groups to address this possibility. We have conducted the same experimental paradigm with 2 reverse-SCS groups (WN—tone), one with paired (new PA-R group), and one with unpaired (new UN-R group), presentations to shock during conditioning. These experiments revealed that during conditioning day 2 in both reverse order groups, WN causes reductions in freezing and increases in locomotor activity (see revised Figure 2D), an effect that is stronger in the UN-R compared to the PA-R group. This locomotor effect is neither darting nor escape jumping in the PA-R group (revised Figure 3G, I; Figure 4G). In the UN-R group, WN induces more activity than the PA-R group (Figure 2D), including some jumping at WN onset (Figure 3H), but no darting (Figure 4G). It is worth noting that WN does not elicit defensive behavior before conditioning at the sound intensity we use (75dB; see Fadok et al. 2017, Borkar et al. 2020, Borkar et al. 2024). Together, these results suggest that WN is an inherently more salient stimulus than tone, and it can elicit defensive behaviors in shock-sensitized mice through non-associative mechanisms. Indeed, stimulus salience is a key factor in this paradigm for inducing activity (see Hersman et al. 2020).

> *While the more complete assessment of defensive behaviors beyond freezing is welcomed, the main conclusions in the discussion are overly focused on the paired group and the associative elements of conditioning, which would likely not be surprising to the field. If the goal, as indicated in the title, was to tease apart the associative and non-associative elements of conditioning and defensive behaviors, there needs to be a more emphasized discussion and explicit identification of the non-associative findings of their study, as this would be more impactful to the field.*

We have rewritten the Discussion to provide a greater emphasis on the findings of the study that are more related to non-associative mechanisms. For example, we argue that cue-salience and changes in stimulus intensity can induce non-associative increases in locomotor behavior and tail rattling in shock-sensitized mice.

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *The authors examined several defensive responses elicited during Pavlovian conditioning using a serial compound stimulus (SCS) as the conditioned stimulus (CS) and a shock unconditioned stimulus (US) in male and female mice. The SCS consisted of tone pips followed by white noise. Their design included 3 treatment groups that were either exposed to the CS and US in a paired fashion, in an unpaired fashion, or only exposed to the shock US. They compared freezing, jumping, darting, and tail rattling across all groups during conditioning and extinction. During conditioning, strong freezing responses to the tone pips followed by strong jumping and darting responses to the white noise were present in the paired group but less robust or not present in the unpaired or shock only groups. During extinction, tone-induced freezing diminished while the jumping was replaced by freezing and darting in the paired group. Together, these findings support the idea that associative pairings are necessary for conditioned defensive responses.*
>
> *Strengths:*
>
> *The study has strong control groups including a group that receives the same stimuli in an unpaired fashion and another control group that only receives the shock US and no CS to test the associative value of the SCS to the US. The authors examine a wide variety of defensive behaviors that emerge during conditioning and shift throughout extinction: in addition to the standard freezing response, jumping, darting, and tail rattling were also measured.*

We thank the Reviewer for their supportive appraisal of this study's strengths.

> *Weaknesses:*
>
> *This study could have greater impact and significance if additional conditions were added (e.g., using other stimuli of differing salience during the SCS), and determining the neural correlates or brain regions that are differentially recruited during different phases of the task across the different groups.*

In the revised manuscript, we have conducted experiments with 2 reverse-SCS groups (WN—tone): one with paired (new PA-R group), and one with unpaired (new UN-R group), presentations to shock during conditioning. These experiments revealed that during conditioning day 2 in both reverse order groups, WN causes reductions in freezing and increases in locomotor activity (see revised Figure 2D), an effect that is stronger in the UN-R compared to the PA-R group. This locomotor effect is neither darting nor escape jumping in the PA-R group (revised Figure 3G, I; Figure 4G). In the UN-R group, WN induces more activity than the PA-R group (Figure 2D), including some jumping at WN onset (Figure 3H), but no darting (Figure 4G). Indeed, stimulus salience is a key factor in this paradigm for inducing activity (see Hersman et al. 2020). Together, these results suggest that WN is an inherently more salient stimulus than tone, and it can elicit defensive behaviors in shock-sensitized mice through non-associative mechanisms. It is worth noting that WN does not elicit defensive behavior before conditioning at the sound intensity we use (75dB; see Fadok et al. 2017, Borkar et al. 2020, Borkar et al. 2024).

We agree that determining the neuronal correlates and brain regions that are involved in defensive ethograms at various stages within this paradigm is of great importance, but we feel that those experiments are beyond the scope of the current study, which is focused on identifying behavioral differences based on associative and non-associative factors.

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *In LINES 72-73, authors say they used a "truly random procedure" as one of their control groups. Then in LINES 113-116, they describe this group as "unpaired" where the "SCS could not reliably predict footshock". Combined, it is unclear if this group is random or unpaired. The "truly random procedure" is defined, by the cited Rescorla paper, as "the two events are programmed entirely randomly and independently in such a way that some "pairings" of CS and US may occur by chance alone". So, truly random would indicate that the shock may occur during the cue, while unpaired indicates the shock was explicitly unpaired from the cue. If the authors used a random procedure, the groups need to be labeled as random, not unpaired, and the # of cues that happened to coincide with footshock per animal needs to be reported somewhere. If the authors used an unpaired procedure (which appears to be the case based on 40-60s ITI between SCS and footshock being reported), it needs to be clearer and consistent throughout that it was explicitly unpaired, as well as removing the claim in LINE 72-73 that they used a "truly random procedure".*

We did indeed use an explicitly unpaired procedure. We have adjusted the text and figures to better reflect this, and we removed any mentions of randomness with regards to the presentations of SCS and footshock.

> *Despite the lack of significant sex differences, it would still be helpful if data panels with individual data points (e.g. Fig 2E-J), were presented as identifiable by sex (e.g. closed vs open circles for males vs females).*

The revised manuscript now compares four or five groups per figure, making data presentation complicated. Providing the individual data points in each panel reduces figure clarity, therefore, we feel it is best to present the data as box-and-whisker plots without them. However, the source data files for each figure are available to the reader and the data are clearly labeled to be identifiable by sex.

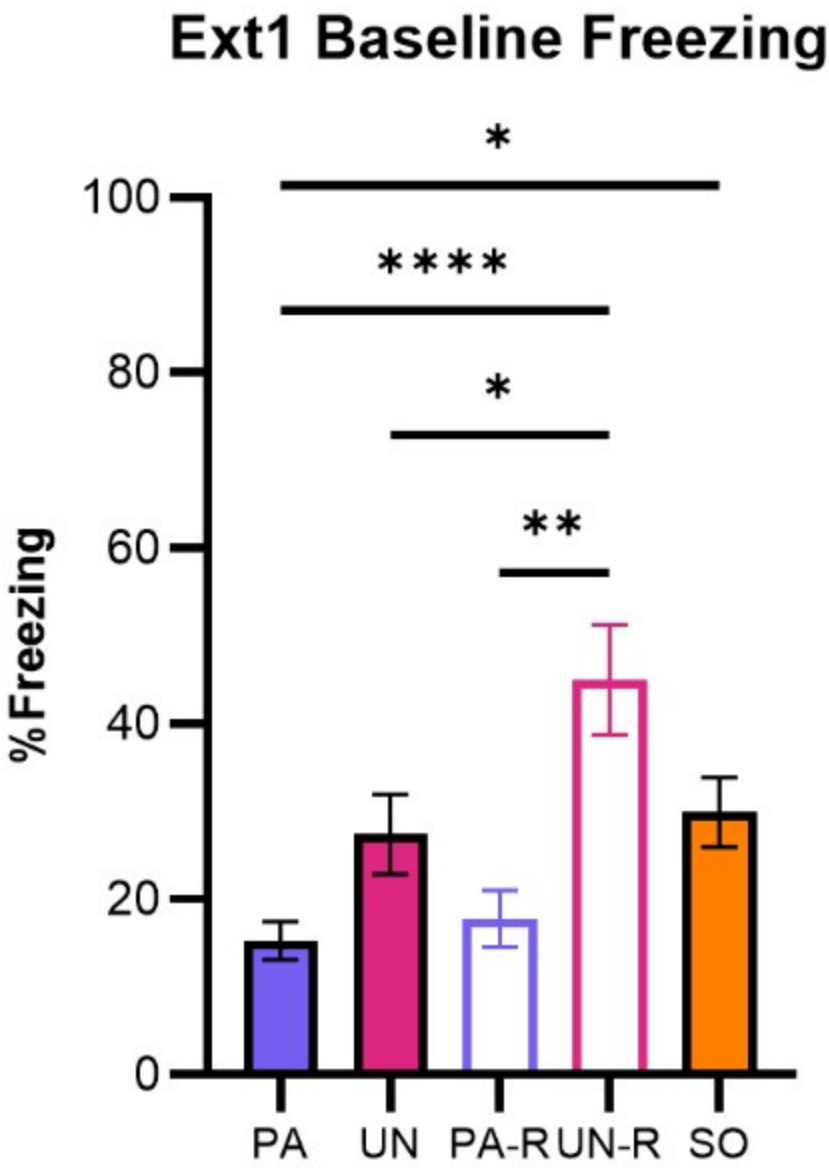
> *Is it not odd that all groups showed similar levels of contextual freezing during the 3min baseline? If shocks are unsignaled in the UN and SO groups, one would expect higher levels of contextual freezing compared to a paired group.*

We are not certain why one would expect higher levels of contextual freezing in the UN and SO groups compared to the PA group at the beginning of conditioning day 2. Another study also looked at baseline freezing in a contextual fear group (which is the same as shock only in our study) and in an auditory cued fear conditioning group within the conditioning context, and their data show that freezing during the baseline period is equivalent between groups (Sachella et al., 2022).

During baseline on Extinction Day 1, it does seem that the unpaired and SO groups tend to have higher freezing levels compared to the paired groups. Author response image 1 shows baseline freezing during the first 3 minutes of extinction day 1. After two days of conditioning in the conditioned flight paradigm, contextual freezing either is, or trends to be significantly higher in the UN, UN-R, and SO groups than the PA and PA-R groups.

Author response image 1.

Baseline Freezing levels for all groups during the first extinction session. Baseline period is defined as the first 180 seconds of the session, before any auditory stimulus was presented. PA, Paired; UN, Unpaired; SO, Shock Only; PA-R, Paired Reverse; UN-R, Unpaired Reverse. *p<0.05, **p<0.01, ****p<0.0001.



> *Do the tone and WN elicit similar levels of defensive behaviors in a naïve mouse? Or have the authors tested WN followed by tone? Is there a potential issue that the WN may be innately aversive which is then amplified with training? i.e. does a tone preferentially induce freezing while WN induces active behaviors, regardless of which sensory stimulus is temporally closer to the shock? If the change in behavior is really due to the pairing*

> *and temporal proximity to shock, then there should be increased jumps, etc to the tone if trained with WN->tone.*

WN can indeed be used as an aversive stimulus under certain conditions and at sufficiently high decibel levels. In the conditioned flight paradigm, WN is presented at 75dB, which is below the threshold for eliciting an acoustic startle response in a C57BL/6J mouse (Fadok et al. 2009). Also, during pre-exposure, when animals are naïve to the SCS, tone and WN stimuli do not elicit defensive behaviors (see Fadok et al. 2017, Borkar et al. 2020, 2024).

As suggested by the Reviewer, during revision we have included reverse-SCS paired (PA-R) and unpaired (UN-R) groups to test for the role of stimulus salience and stimulus order on defensive ethograms. During conditioning day 2, the PA-R group exhibited little freezing to the WN, with a slightly elevated activity index, and they exhibited robust freezing during tone (revised Figure 2A-H). The activity during the WN in the PA-R group was significantly lower than that of the PA group (Figure 2L). The PA-R group also did not respond to WN with escape jumps or darting (Figure 3I, 4G). The UN-R group displayed greater activity during the WN than the UN and PA-R groups, but less activity than the PA group (Figure 2D, H). The UN-R group did not dart but this group displayed some jumping at WN onset (Figure 3H), like what was observed in the UN group.

These data suggest that WN has inherent, salient properties that can induce some non-associative activity after the mouse has been sensitized by shock (see also Hersman et al. 2020 for more detailed analysis of stimulus salience in the conditioned flight paradigm). However, only in the PA group is robust flight behavior (comprised of high numbers of escape jumps and darting) observed. Therefore, both stimulus salience and temporal order are important for eliciting transitions from freezing to flight.

> *Fig 3G/4G are hard for me to understand. The figure legends say they're survival graphs but the y-axis labels "Latency to initial jump/dart (% of cohort)" confuses me. What is the purpose of these graphs? Perhaps they are not needed. Or consider presenting them similar to Fig 7C, D as those were more intuitive and faster for me to grasp.*

We had intended these plots to show that a greater proportion of the paired group jumps and darts during WN compared to the unpaired group, and that the percentage of the cohort that jumps and darts increases across conditioning trials. Because these graphs were not clear, we have removed them, and we have replaced them with graphs comparing total cohort percentages that jumped (Figure 3I) or darted (Figure 4G) over the whole CD2 session.

> *For the extinction data, I did not see within group analyses for within or between session fear extinction to the tone. So, for the paired group, were the last 4 trials of Ext 1 significantly lower than the first 4 trials? If not, then they did not show within-session extinction. Also, for the paired group, were the last 4 trials of Ext 1 significantly different than the first 4 trials of Ext 2? This would test for long-term retention and spontaneous recovery.*

In the original submission and in the revised manuscript, we calculated a delta change score for freezing during tone in the early versus late blocks of 4 trials, and then we statistically compared these differences across groups (Figure 5C, D). This allowed us to assess between-group differences in changes to tone-evoked freezing during extinction. Freezing to tone did decrease significantly over the first extinction session for the paired group (Early Ext1 vs Late Ext1, paired t-test, $t(31) = 6.23$, $p<0.0001$), and when comparing late Ext1 and early Ext2, we found that tone-evoked freezing did significantly increase (Late Ext1 vs Early Ext2, paired t-test, $t(31) = 5.26$, $p<0.0001$). This increase in cue-induced freezing between days of extinction is characteristic of C57BL/6J mice (Hefner et al., 2008). Our study did not test for more distal

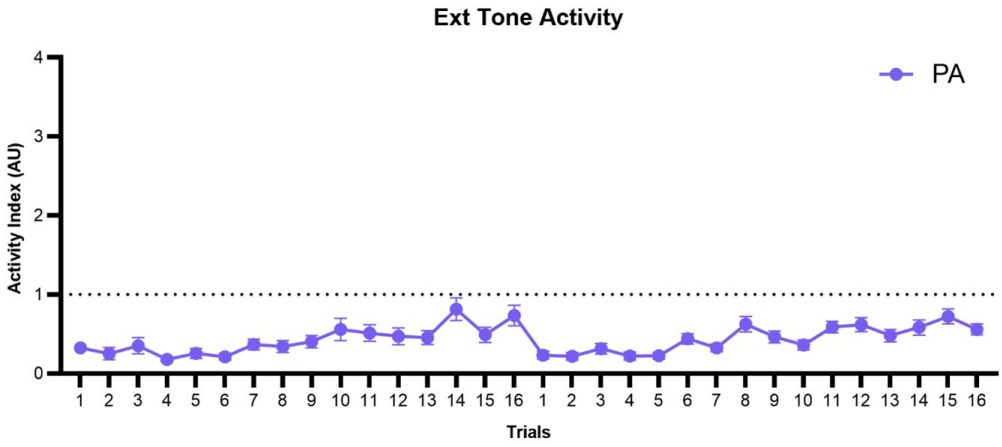
timepoints, so we cannot comment on the efficacy of long-term retention or spontaneous recovery.

> *For the conditioning and extinction data across Figs 2, 5 and 6, what I gather from them is that freezing is high to the tone and low to the WN during conditioning, and then low to the tone, and high to the WN across extinction. Then for activity levels I see they are low to the tone and high to the WN during conditioning, and then low to the WN during extinction. The piece that is missing is what are activity levels like to the tone during extinction. Are they low like in conditioning and remain low in extinction? Or do they increase across extinction as freezing decreases? As I was going through these graphs I drew myself out step function summaries of the freezing and activity levels between tone/WN for conditioning vs extinction; maybe the authors could consider a summary figure.*

We thank the Reviewer for their interest. We found that within the paired group, activity to tone remained low throughout both days of extinction (though increased within each session) and did not return to normal activity levels. We present this data in Author response image 2. We thank the Reviewer for the suggestion of a summary figure, but we feel there are too many axes of classification (between-group, within-group, multiple behaviors, tone/WN, conditioning/extinction) to coherently present our findings in a single figure.

Author response image 2.

Trial-by-trial plot of activity index during the tone period of SCS across both extinction sessions for the PA group. SCS, Serial compound stimulus; Ext, extinction; PA, Paired.



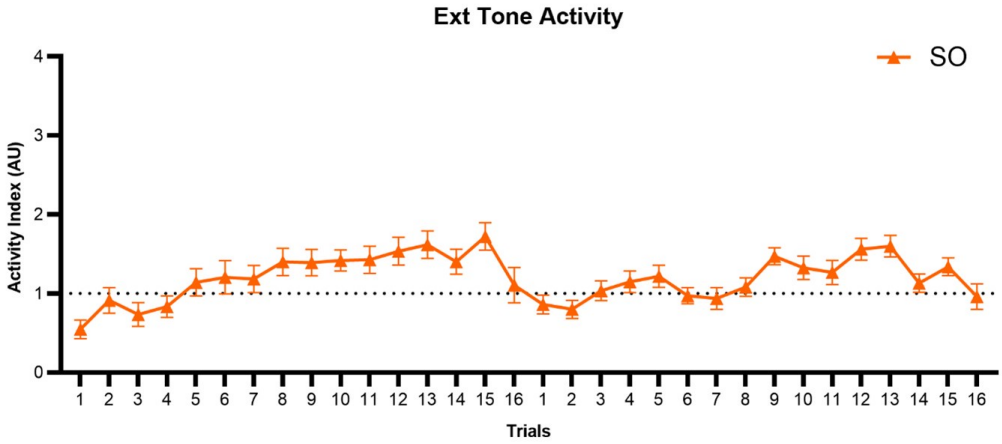
> *In the discussion (LINE 592-3), they discuss that shock sensitization in the SO group may prime a stressed animal to dart more readily to WN upon stimulus transition. Should this not also happen during the transition of silence to tone? What is special about a transition between two auditory stimuli that would result in panic like behavior in an animal that only received shock presentations? This also gets back to an earlier concern above regarding the potentially innately aversiveness of the WN.*

After 2 days of shock sensitization, we observe that mice exhibit freezing to the tone during the first three trials of extinction day 1 (Figure 5A). This non-associative freezing response is like that observed in other studies of non-associative fear processing (please see Kamprath and Wotjak, 2004). As trials progress during extinction day 1, mice do become mildly activated during the tone (Author response image 3). The transition to WN in the shock-only

group during extinction induces non-associative darting responses, but it does not induce escape jumping behavior (Figure 7). We hypothesize that the innate salience of the WN is a vital factor contributing to these escalated responses. The importance of stimulus salience in conditioned flight was also demonstrated by Hersman et al., 2020 for SCS conditioning, and by Furuyama et al., 2023 for single tone conditioning. Just as with conditional freezing responses (Kamprath and Wotjak, 2004), we believe that conditional flight is controlled by summative components, one being associative and the other non-associative.

Author response image 3.

Trial-by-trial plot of activity index during the tone period of SCS across both extinction sessions for the SO group. SCS, Serial compound stimulus; Ext, extinction; SO, Shock Only.



> *In the discussion (LINE 583), they say that the development of explosive defensive behaviors are "not achievable with traditional single-cue Pavlovian conditioning paradigms". The authors should include a caveat here that the current study did not compare their results to a group of mice that received just WN-shock pairings.*

We thank the reviewer for this comment. This statement was meant to highlight that traditional paradigms do not offer an element of signaling the temporal imminence of threat, only its inevitability. It was not our intention to state that defensive escape behaviors were unachievable in single-cue conditioning paradigms, and we regret not making this clear. Indeed, the supplement of Fadok et al. 2017 shows that WN-shock conditioning is capable of inducing flight, Furuyama et al. 2023 shows that tone-shock conditioning is capable of inducing flight under specific parameters, and Gruene et al. 2015 demonstrates that single CS-US pairings induce conditional darting behaviors in female rats. We have adjusted the text to better reflect our intent.

> *Minor comment to LINE 613-5: Speaking as someone who has done fear conditioning in both mice and rats, tail rattling may be specific to mice (I have seen this often) and likely not observable in rats (never seen it).*

We thank the Reviewer for this information. We have adjusted our text to mainly discuss mouse-specific tail rattling.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *The research questions in this study are novel and bring new insight to the field. However, there are some issues that can be addressed to improve the overall quality of the study, namely, the reader is left wanting to know more, especially about how neural circuits contribute to these different defensive behaviors during this task. Below are some recommendations for the authors that would greatly improve the impact and significance of this study.*
>
> *(1) What are the neural correlates or circuits recruited during these different defensive behaviors across the course of conditioning and extinction? How might they differ between the PA and UN groups? What differences might emerge when an animal is shifting their defensive behavior from freezing to darting, for example? Answering these questions would require intensive additional experiments, therefore more discussion of possible neural mechanisms that might be recruited during this task would be appreciated, given the scope of the subject area.*

We agree that understanding the neural circuits recruited during these behaviors and across conditioning and extinction is of vital importance. We are actively working on these questions, and we have published on the role of central amygdala circuits (Fadok et al. 2017) as well as on top-down control of flight by the medial prefrontal cortex (Borkar et al. 2024). Because the current manuscript is focused on learning mechanisms influencing defensive behavior, we would prefer to focus our discussion on that, rather than speculating on possible neural mechanisms. However, we have added a statement in the Discussion (LINES 706-707) emphasizing that future studies should investigate the neuronal mechanisms contributing to threat associations and different defensive behaviors.

> *(2) Were any vocalizations observed during conditioning or extinction phases? If not, could you speculate how type and occurrence of vocalizations might correlate with the different defensive responses observed?*

Audible vocalizations were only observed during footshock presentations (squeaks). Unfortunately, we do not have the proper specialized recording equipment to monitor the full spectrum of mouse vocalizations, especially those in the ultrasonic range. Thus, we cannot speculate on the nuances of vocalizations in mice with respect to this behavioral paradigm. To the best of our knowledge, mice have not been reported to emit specific ultrasonic calls during conditioned threat like those of rats. That said, it would be of interest to determine if mice emit different vocalizations during different defensive behaviors.

> *(3) The transition from freezing to flight during the SCS is thought to be due to the close proximity of threat imminence between the WN CS and shock US. What if you switched the order of the SCS stimuli to WN followed by tone stimuli? If the salience of the WN stimulus is truly driving the jumping behavior, then it would be observed even if the WN stimulus preceded the pure tone stimulus and that would bring additional evidence that it is the associative value of the stimuli rather than its salience that's driving the defensive behaviors. What do you predict you would observe in rodents that were given a WN-tone SCS paired and unpaired in the same design of this study?*

As suggested by the reviewer, we collected data from reverse-SCS paired and unpaired groups and reported our findings within the manuscript. Our detailed findings are also discussed above. Overall, we find that a combination of stimulus salience and temporal proximity, and a summation of non-associative and associative mechanisms, are necessary to elicit explosive flight behavior (escape jumping and darting).

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