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

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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, the contributions of associative and non-associative mechanisms to dynamic defensive responses are currently unclear given the reductionist behavior elicited by most Pavlovian fear conditioning paradigms. To investigate defensive ethograms more thoroughly, we subjected male and female adult C57BL/6J mice to a Pavlovian threat conditioning paradigm that pairs 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 the paired SCS-footshock group with control groups that either received randomly presented SCS and footshock presentations (unpaired) or received only footshocks during conditioning (shock-only). After conditioning, only the paired group exhibited robust freezing during the tone period with switching to explosive flight responses comprised of jumping and darting behavior during the WN period. The unpaired group expressed no cue-induced freezing and significantly less freezing overall. Furthermore, the unpaired group reduced freezing but rarely showed jumping or darting during WN. Following conditioning, we observed how defensive behavior changed over two extinction sessions. During extinction, only the paired group decreased tone-induced freezing. During WN, the paired group rapidly transitioned from escape jumping to freezing and darting. Comparatively, the unpaired and shock-only groups displayed stress-induced tail rattling to SCS, eschewing freezing and jumping. These findings demonstrate that while non-associative factors promote some defensive responsiveness, associative pairings are required for robust cue-induced freezing and flight expression.

Introduction

Defensive responses have evolved to maximize survival (Anderson and Adolphs, 2014), and animals rapidly switch behaviors depending on threat imminence, context, and previous

experience with stimuli (Perusini and Fanselow, 2015). Understanding the neural 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 tractable methods with which to investigate how the nervous system controls complex behavior resulting from experience.

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). However, other defensive responses like escape jumping (Chu et al., 2022) and darting (Gruene et al., 2015) are less measured 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), 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 increasing 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 WN stimulus's inherent salience 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 from repeated tone-shock presentations is known to intensify subsequent freezing responses to conditioned 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 two important control groups for non-associative effects of conditioning. To test the importance of the SCS-shock contingency, we utilized a truly random control procedure in which the US and the SCS are presented in an unpaired and non-predictive fashion (Rescorla, 1967). To test for the effects of sensitization by the shock, we presented footshock alone during conditioning. We compared these groups against a paired SCS-shock conditioning group to quantify the effects of associative learning on SCS-evoked fear behavior. All three groups went through 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). 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 three groups: Paired (PA), Unpaired (UN), and Shock Only (SO). Behavioral testing took place over 5 days. 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-trial interval (ITI) 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), subjects 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) were presented with 5 pairings of the SCS co-terminating with a 1 s, 0.9 mA footshock, with pseudorandom ITI 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) were presented with pseudorandom presentations of SCS and footshocks separate from one another with ITI periods of 40-60 s, with a period of 90 s following the final stimulus of the session. Stimuli were ordered such that SCS could not reliably predict footshock. Both PA and UN groups 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 ITI 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 ITI 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 ITI 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 time in which all four paws were above the floor of the chamber. 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.

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 control groups.

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 (640x480 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 ($\alpha = 0.05$, 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 ($\alpha=0.05$). 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 the three 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. Fisher's exact test was used to assess if the relative proportions of each cohort differed in their performance of a specific behavior.

Results

SCS-evoked freezing and activity are affected by SCS-shock contingency

Equal numbers of male and female mice were randomly assigned to either a paired (**PA**), unpaired (**UN**), or shock-only (**SO**) group for fear and fear extinction conditioning (**Fig 1**). All data were statistically tested for sex differences and the significant results from these analyses are

listed in **Table 1**. Given that most comparisons of sex did not yield significant differences, data from male and female mice were pooled for statistical comparisons between groups.

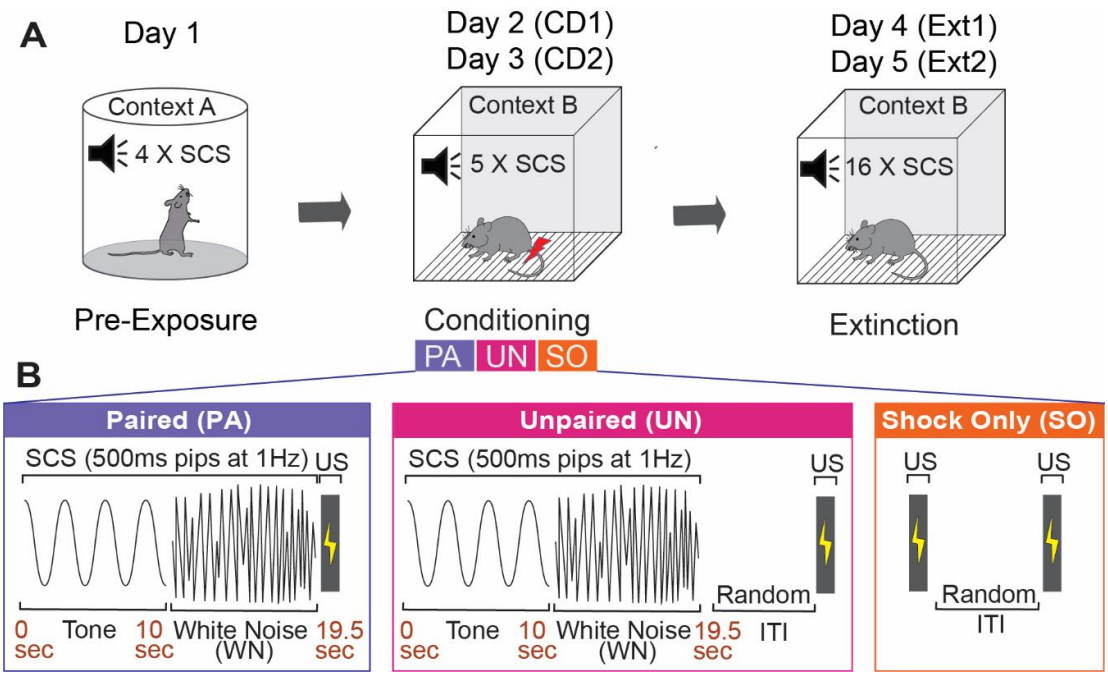


Figure 1. Experimental Design. **A,** Graphical representation of the three stages of the serial compound stimulus (SCS) conditioning paradigm and the context each stage took place in. Pre-Exposure to the SCS took place on Day 1 in Context A. Conditioning and Extinction took place in Context B. Animals only received footshock during Conditioning and were presented with shock-free SCS presentations during Extinction. **B,** Diagram illustrating the three SCS-shock association variants animals experienced during Conditioning. The Paired (PA) group experienced SCS co-terminating with shock, the Unpaired (UN) group experienced random presentations of the SCS and shock, and the Shock Only (SO) group experienced shock without SCS during Conditioning.

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
8	PA	Cohort proportion that tail rattled to WN in Ext2	Fisher's exact test	p=0.017	Males (6/10) tail rattled to WN more than females (0/10) in Ext2

Table 1. Statistical analysis of sex differences in behavior. To determine the extent to which sex influences behavior within each group, we conducted comparative analyses between males and females. The few significant sex differences we observed are detailed in the table. From left to right, the columns describe: Figure and/or panel that was analyzed for sex differences, the group that showed significant sex differences in behavior, the type of behavior analyzed, the statistical test performed, the p-value obtained, and the interpretation of the result. SCS, Serial compound stimulus; CD2, Conditioning Day 2; Ext2, Extinction Day 2; WN, white noise; PA, Paired; UN, Unpaired.

Behavioral data from the PA and UN groups during the second day of fear conditioning (CD2) were compared to observe how conditioned defensive behavior differs based on the associative value 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 ($F_{(4, 250)} = 0.50$, $p=0.74$); however, there was a significant main effect of trial ($F_{(4, 250)} = 7.9$, $p<0.0001$) and group (**Fig 2A**, $F_{(1, 250)} = 58.9$, $p<0.0001$). Neither the PA nor UN group showed much freezing to the WN (**Fig 2B**), and no significant interaction

between trial and group ($F_{(4, 250)} = 0.35$, $p=0.86$), main effect of trial ($F_{(4, 250)} = 1.3$, $p=0.26$), or main effect of group ($F_{(1, 250)} = 0.0005$, $p=0.98$) were found.

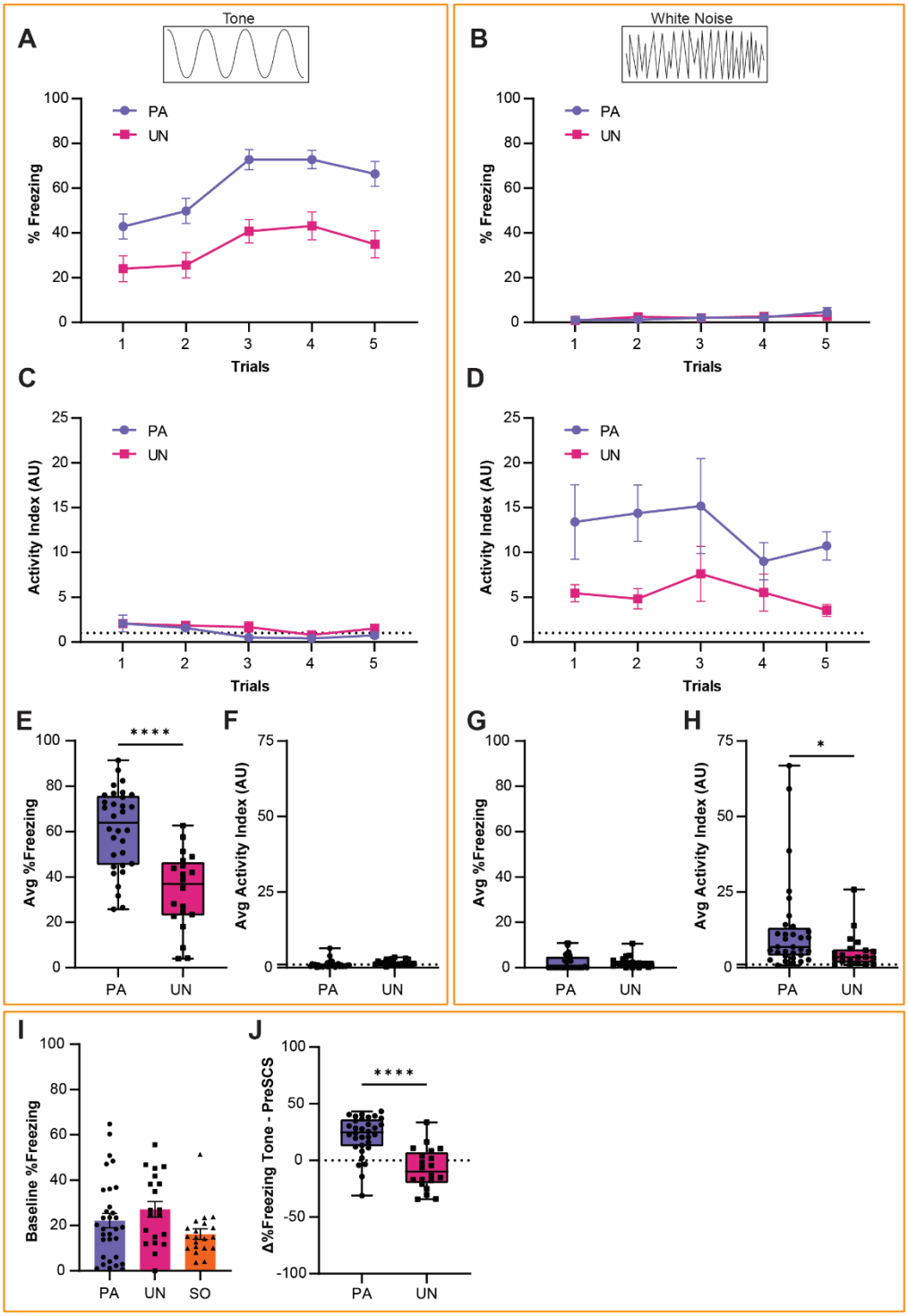


Figure 2. SCS-evoked freezing and activity during CD2 are affected by SCS-shock contingency. **A,** Trial-by-trial freezing to the tone period. **B,** Trial-by-trial freezing to the WN period. **C,** Trial-by-trial activity index to the tone period. **D,** Trial-by-trial activity index to the WN period. **E,** Comparison of average percentages of freezing to the tone period from all trials of CD2. **F,** Comparison of average activity index scores to the tone period from all trials of CD2. **G,** Comparison of average percentages of freezing to the WN period from all trials of CD2. **H,** Comparison of average activity index scores to the WN period from all trials of CD2. **I,** Comparison of baseline contextual freezing levels between all groups during CD2. **J,** Comparison of the difference in average freezing between pre-SCS and tone periods for the PA and UN groups from all trials of CD2. SCS, Serial compound stimulus; pre-SCS, 10 s prior to SCS; CD2, Conditioning Day 2; WN, white noise; PA, Paired; UN, Unpaired. Figures **2A-2D** were analyzed using two-way ANOVA. Figures **2E-2H** were analyzed using unpaired t-test with Welch's correction. Figures **2I** and **2J** were analyzed using one-way ANOVA. Data from Figures **2A-2D** and **2I** are presented as Mean ± SEM. Data from Figures **2E-2H** and **2J** are presented as box-and-whisker plots from min to max. *p<0.05; ****p<0.0001.

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 the PA and UN groups were very low during the tone period (**Fig 2C**), and there was no significant interaction between trial and group ($F_{(4, 250)} = 0.46$, $p=0.76$). The activity index during the tone decreased over trials, concomitant with the observed increase in freezing behavior (main effect of trial, $F_{(4, 250)} = 2.8$, $p=0.03$), but there was no significant effect of group ($F_{(1, 250)} = 2.8$, $p=0.09$). While the WN-evoked activity indices in the PA and UN groups showed no significant trial by group interaction ($F_{(4, 250)} = 0.23$, $p=0.92$), or main effect of trial ($F_{(4, 250)} = 0.58$, $p=0.68$), a significant main effect of group (**Fig 2D**, $F_{(1, 250)} = 11.6$, $p=0.0008$) was observed.

Welch's unpaired t-test was used to compare average freezing and activity indices between the PA and UN groups. The PA group showed significantly higher freezing to the tone than the UN group (**Fig 2E**, $t_{(42.04)} = 5.5$, $p<0.0001$), but there was no significant difference between groups in their activity indices during tone (**Fig 2F,** $t_{(49.12)} = 1.78$, $p=0.08$). On the contrary, while there were no differences in WN-evoked freezing between PA and UN groups (**Fig 2G**, $t_{(49.04)} = 0.02$, $p=0.98$), PA mice displayed a significantly higher activity index to WN (**Fig 2H**, $t_{(43.08)} = 2.4$, $p=0.02$).

An ordinary one-way ANOVA was used to test for differences in contextual freezing during the first three minutes of the session preceding SCS presentation (baseline) across the PA, UN, and SO groups. All three groups displayed similar amounts of baseline contextual freezing (**Fig 2I**, $F_{(2, 69)} = 3.5$, $p=0.09$). Welch's unpaired t-test was used to compare the differences in average freezing during pre-SCS and tone periods between the PA and UN groups. PA mice showed greater increases in freezing from pre-SCS to tone than UN mice (**Fig 2J**, $t_{(39.22)} = 5.755$, $p<0.0001$).

Overall, these data show that the respective changes in defensive behavior during tone and WN were significantly elevated by the explicit pairing of SCS and shock during fear conditioning.

SCS-shock contingency elicits jumping and darting responses to white noise after conditioning

Although the UN group did not receive an associative pairing between SCS and shock like the PA group, both groups still displayed increased activity indices to WN (**Fig 2D**). To determine if this behavioral response was due to defensive flight or a simpler locomotor response, we investigated the occurrence of escape jumping and darting behaviors within the PA and UN groups 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 similarly to shock (**Fig 3B, D**). Trial-by-trial, PA mice displayed more jumping behavior across the WN period (**Fig 3E**) compared to UN mice (**Fig 3F**). Lastly, increasing numbers of the PA group jumped as the session progressed, and many more PA mice jumped during the WN presentations compared to the UN group (**Fig 3G**).

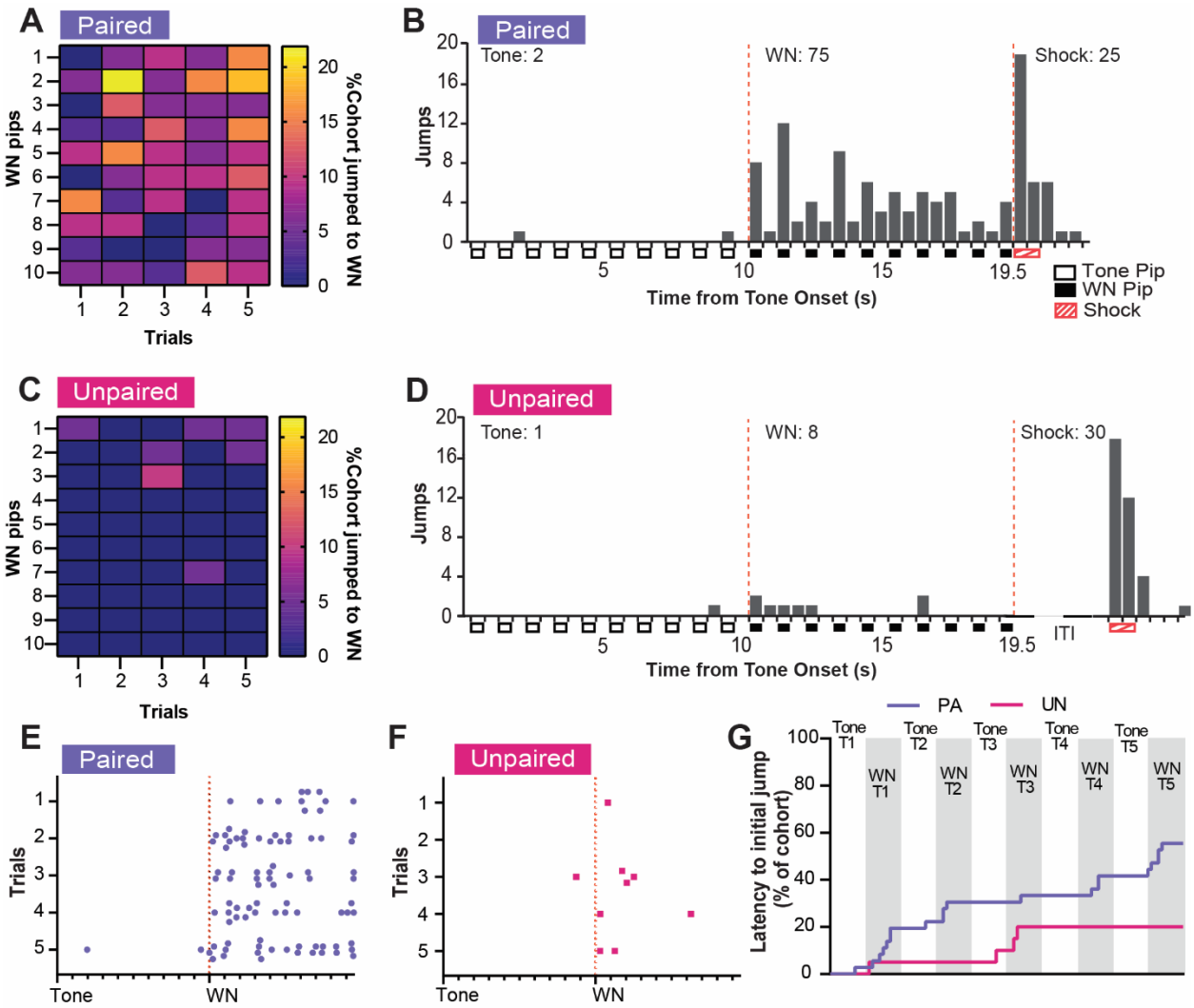


Figure 3. SCS-shock contingency elicits jumping responses to white noise during CD2. **A,** Heatmap showing the percentage of the PA group that exhibited jumping to WN per trial on CD2. Jumping is distributed across 1 s bins, each coinciding with one of the ten pips of WN that occurred during each SCS presentation. **B,** Histogram detailing the cumulative distribution of jumps from 20 randomly selected subjects of the PA group across the duration of 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,** Heatmap showing the percentage of the UN group that exhibited jumping responses to WN per trial on CD2. Jumping is distributed across 1 s bins, each coinciding with one of the ten pips of WN that occurred during each SCS presentation. **D,** Histogram detailing the cumulative distribution of jumps from the UN group across the duration of 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. ITI represents the period between SCS and shock. Total jumps per stimulus

are listed above histogram bars. **E,** Dot plot showing the distribution of jumps across the duration of 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 lines depict the onset of the WN period. **F,** Dot plot showing the distribution of jumps across the duration of 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 lines depict the onset of the WN period. **G,** Survival graph showing when subjects of the PA and UN groups, represented as percentages of their respective cohorts, first exhibited jumping behavior to either portion of SCS. SCS, Serial compound stimulus; ITI, Inter-trial interval; CD2, Conditioning Day 2; WN, white noise; PA, Paired; UN, Unpaired.

Fisher's exact test was used to compare the proportions of mice from the PA and UN groups that jumped during WN. A significantly larger proportion of PA mice jumped to WN compared to UN mice (PA 24/32 vs UN 4/20, p=0.0002). The proportion of PA and UN mice that exhibited escape jumping to tone was not statistically different (PA 4/32 vs UN 1/20, p=0.64), and similar numbers of PA and UN mice jumped in response to the shock (PA 16/32 vs UN 14/20, p=0.25).

The same 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**). Finally, more of the PA group displayed darting during the first WN presentation, and most of the PA group showed darting (**Fig 4G**), compared to the UN group.

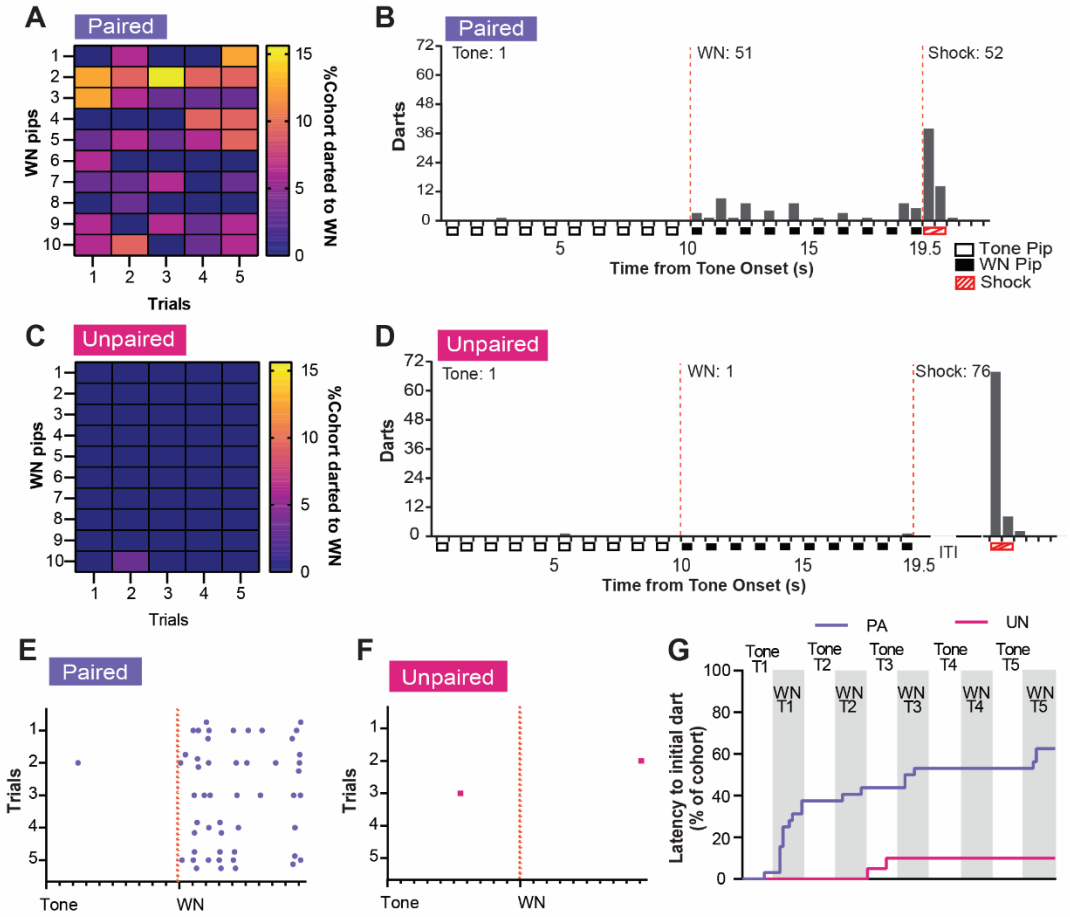
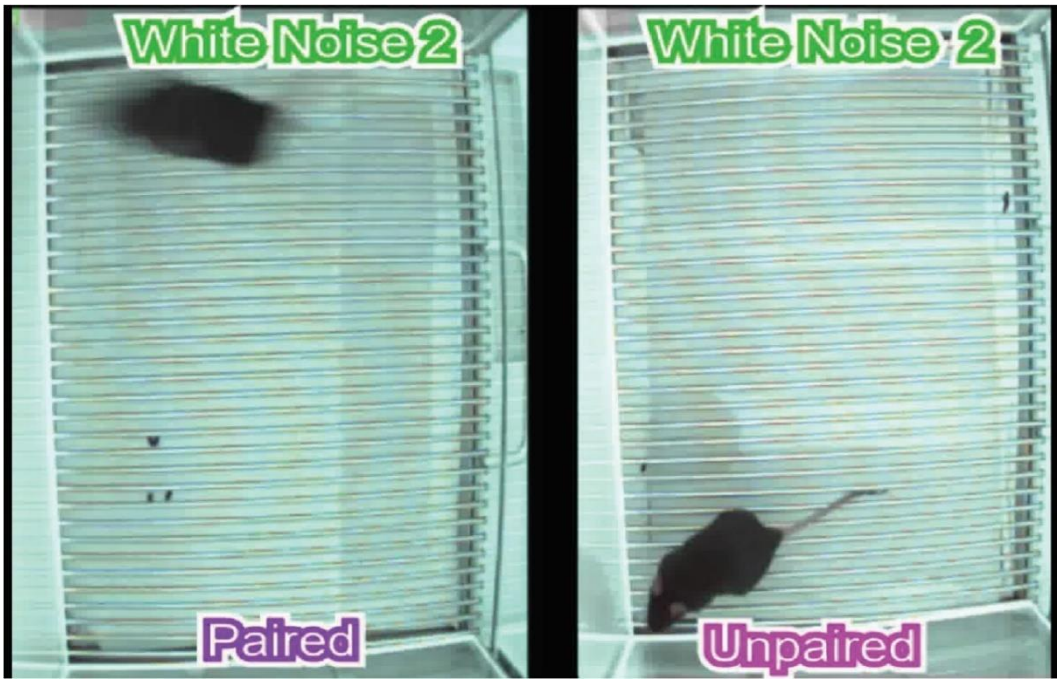


Figure 4. SCS-shock contingency elicits darting responses to white noise during CD2. **A,** Heatmap showing the percentage of the PA group that exhibited darting responses to WN per trial on CD2. Darting is distributed across 1 s bins, each coinciding with one of the ten pips of WN that occurred during each SCS presentation. **B,** Histogram detailing the cumulative distribution of darts from 20 randomly selected subjects of the PA group across the duration of 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 darts per stimulus are listed above histogram bars. **C,** Heatmap showing the percentage of the UN group that exhibited darting responses to WN per trial on CD2. Darting is distributed across 1 s bins, each coinciding with one of the ten pips of WN that occurred during each SCS presentation. **D,** Histogram detailing the cumulative distribution of darts from the UN group across the duration of 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. ITI represents the period between SCS and shock. Total darts per stimulus are listed above histogram bars. **E,** Dot plot showing the distribution of darts across the duration of SCS from 20 randomly selected subjects of the PA group for each trial of CD2. 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,** Dot plot showing the distribution of darts across the duration of SCS from the UN group for each trial of CD2. 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,** Survival graph showing when subjects of the PA and UN groups, represented as percentages of their respective cohorts, first exhibited darting behavior to either portion of SCS. SCS, Serial compound stimulus; ITI, inter-trial interval; CD2, Conditioning Day 2; WN, white noise; PA, Paired; UN, Unpaired.

Fisher's exact test was used to compare the proportions of mice from the PA and UN groups that darted. A significantly larger proportion of PA mice darted to WN compared to UN mice (PA 22/32 vs UN 1/20, $p<0.0001$). PA and UN mice did not differ in darting to tone (PA 2/32 vs UN 1/20, $p>0.99$) or shock (PA 30/32 vs UN 19/20, $p>0.99$).

In summary, associative pairings of SCS and shock produced significant cue-induced freezing to the tone, and robust jumping and darting behaviors that occurred over the entirety of WN presentation, while altering this contingency in the random unpaired condition fundamentally eliminated these defensive behaviors. Representative behavioral responses of the PA and UN groups to the SCS during conditioning are provided in **Video 1.**



Video 1. Representative comparison of PA and UN groups' response to SCS during conditioning. Video 1 shows defensive responses to the last SCS during CD2 for the PA group and the UN group. The video features audio of the SCS, which consists of 10 pips of tone followed by 10 pips of WN. SCS, Serial compound stimulus; CD2, Conditioning Day 2; WN, white noise; PA, Paired; UN, Unpaired.

Tone-evoked freezing is associative and 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 (**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 towards tone compared to the control groups ($p<0.05$ for Trials 1-15, for both sessions).

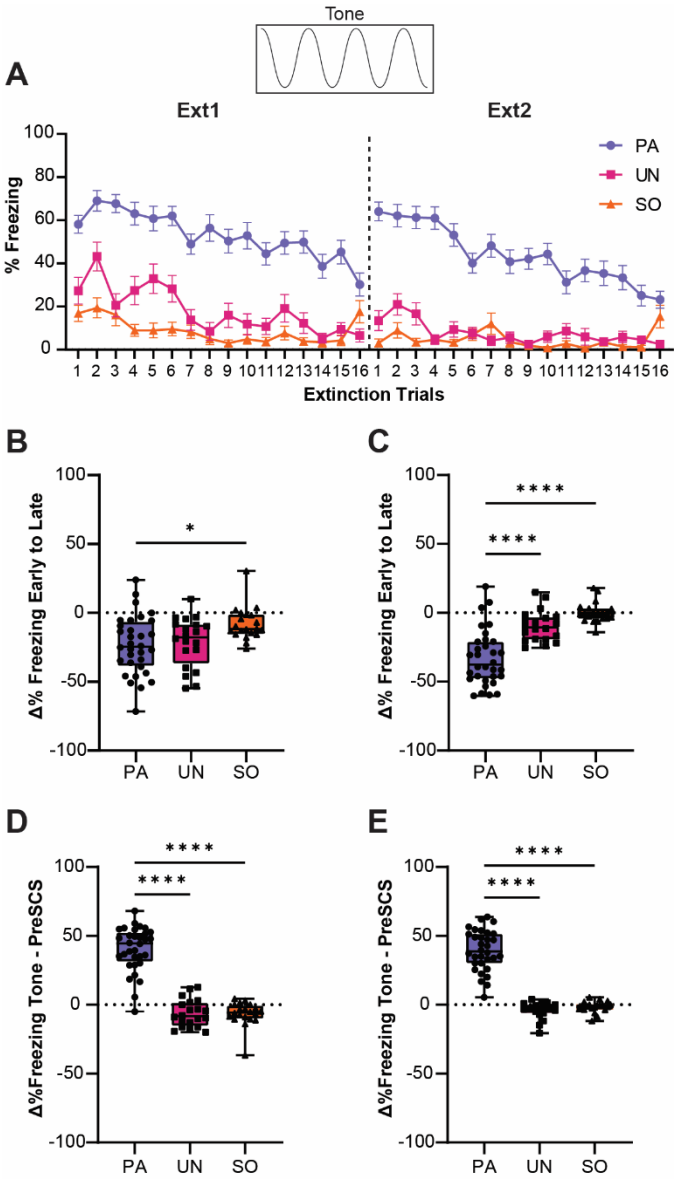


Figure 5. Tone-evoked freezing is associative and is reduced by extinction learning. **A,** Trial-by-trial percentage of freezing to the tone period for the PA, UN, and SO groups during Ext1 and Ext2. **B,** Comparison of the difference in average freezing to the tone period from early and late 4-trial bins of Ext1. **C,** Comparison of the difference in average freezing to the tone period of SCS from early and late 4-trial bins of Ext2. **D,** Comparison of the difference in average freezing between pre-SCS and tone periods during Ext1. **E,** Comparison of the difference in average freezing between pre-SCS and tone periods during Ext2. SCS, Serial compound stimulus; Ext1, Extinction Day 1; Ext2, Extinction Day 2; PA, Paired; UN, Unpaired; SO, Shock Only. Figure **5A** was analyzed using two-way ANOVA. Figures **5B**, **5C**, **5D**, and **5E** were analyzed using one-way ANOVA. Data from Figure **5A** are presented as Mean ± SEM. Data from Figures **5B**, **5C**, **5D**, and **5E** are presented as box-and-whisker plots from min to max. *p<0.05, ****p<0.0001.

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 5B**, $F_{(2, 69)} = 3.5$, $p=0.01$), with the PA and SO groups differing significantly in their change in tone-evoked freezing (PA vs SO, $p=0.01$). No other differences in freezing decreases were found (PA vs UN, $p=0.90$; UN vs SO, $p=0.07$). A significant difference between groups was also detected for the second extinction session (**Fig 5C**, $F_{(2, 69)} = 8.4$, $p<0.0001$). The PA group displayed significantly larger decreases in tone-evoked freezing compared to the UN (PA vs UN, $p<0.0001$) and SO (PA vs SO, $p<0.0001$) groups (**Fig 5C**). There was no significant difference detected between the UN and SO groups (UN vs SO, $p=0.10$).

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 5D, E**). For both the first (**Fig 5D**, $F_{(2, 69)}$

= 4.36) and second (**Fig 5E**, $F_{(2, 69)}$ = 18.21) sessions of extinction, only the PA group increased freezing levels during the tone ($p<0.0001$ for all PA-related comparisons), whereas the UN and SO groups had equivalent freezing during the pre-SCS and tone periods (Ext1, UN vs SO, $p=0.99$; Ext2, UN vs SO, $p=0.76$).

Taken together these data suggest that the PA group associated the tone with threat, while freezing in the UN and SO groups was more indicative of contextual fear.

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. All three groups had elevated activity indices, yet only the PA group showed decreases in WN-evoked activity over extinction (**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 control 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 a value of 1 across extinction trials, indicating levels of movement that were higher during the WN than the pre-SCS period.

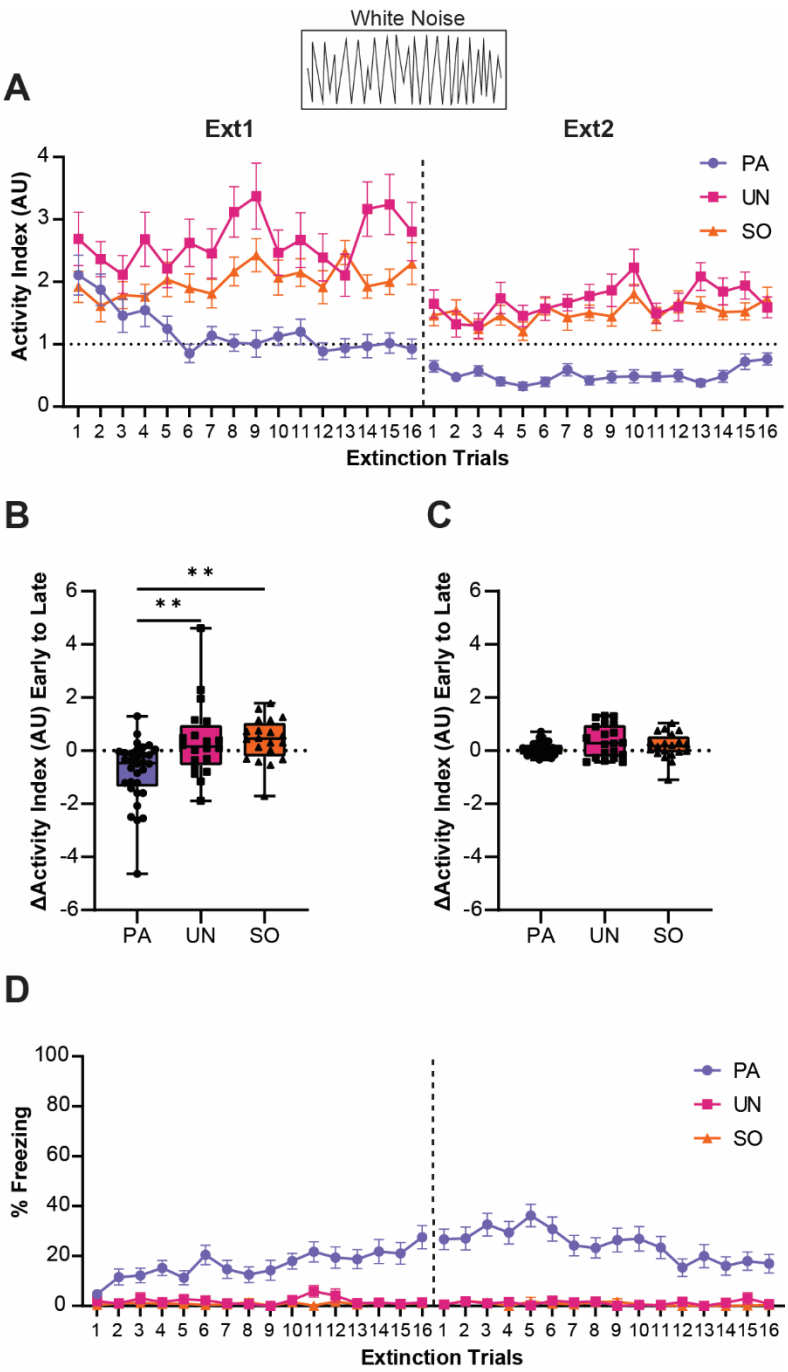


Figure 6. Stimulus-induced flight is associative and is partially replaced by freezing during extinction. **A,** Trial-by-trial activity index to the WN period for the PA, UN, and SO groups during Ext1 and Ext2. **B,** Comparison of the difference in average activity index scores to the WN period from early and late 4-trial bins of Ext1. **C,** Comparison of the difference in average activity index scores to the WN period from early and late 4-trial bins of Ext2. **D,** Trial-by-trial percentage of

freezing to the WN period of SCS for the PA, UN, and SO groups during Ext1 and Ext2. SCS, Serial compound stimulus; Ext1, Extinction Day 1; Ext2, Extinction Day 2; WN, white noise; PA, Paired; UN, Unpaired; SO, Shock Only. Figures **6A** and **6D** were analyzed using two-way ANOVA. Figures **6B** and **6C** were analyzed using one-way ANOVA. Data from Figures **6A** and **6D** are presented as Mean ± SEM. Data from Figures **6B** and **6C** are presented as box-and-whisker plots from min to max. **p<0.01.

An ordinary one-way ANOVA was used to analyze the effect of group on changes in WN-elicited activity during the first extinction session, and Tukey's multiple comparisons test was used for post-hoc comparisons. When comparing relative decreases in WN-evoked activity between early and late trials on the first day of extinction, the PA group showed a significant decrease compared to the UN and SO groups (**Fig 6B**, $F_{(2, 69)} = 0.7976$, p=0.0003; PA vs UN, p=0.003; PA vs SO, p=0.002; UN vs SO, p=0.99). There were no significant differences between groups during the second extinction session (**Fig 6C**, $F_{(2, 69)} = 10.9$, p=0.07).

Interestingly, as WN-evoked activity decreased during extinction, the PA group developed and maintained a freezing response to WN, while the UN and SO groups displayed almost no freezing to WN (**Fig 6D**). A significant interaction between trial and group was detected ($F_{(62, 2208)} = 1.9$, p<0.0001), and the PA group displayed greater freezing than the control groups for most of the first extinction session (p<0.05 for Trials 2, 4, and 6-16) and for all trials of the second session (p<0.05 for all trials).

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 observed during conditioning 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 simpler locomotion, 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 across the entire WN presentation period, 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**).

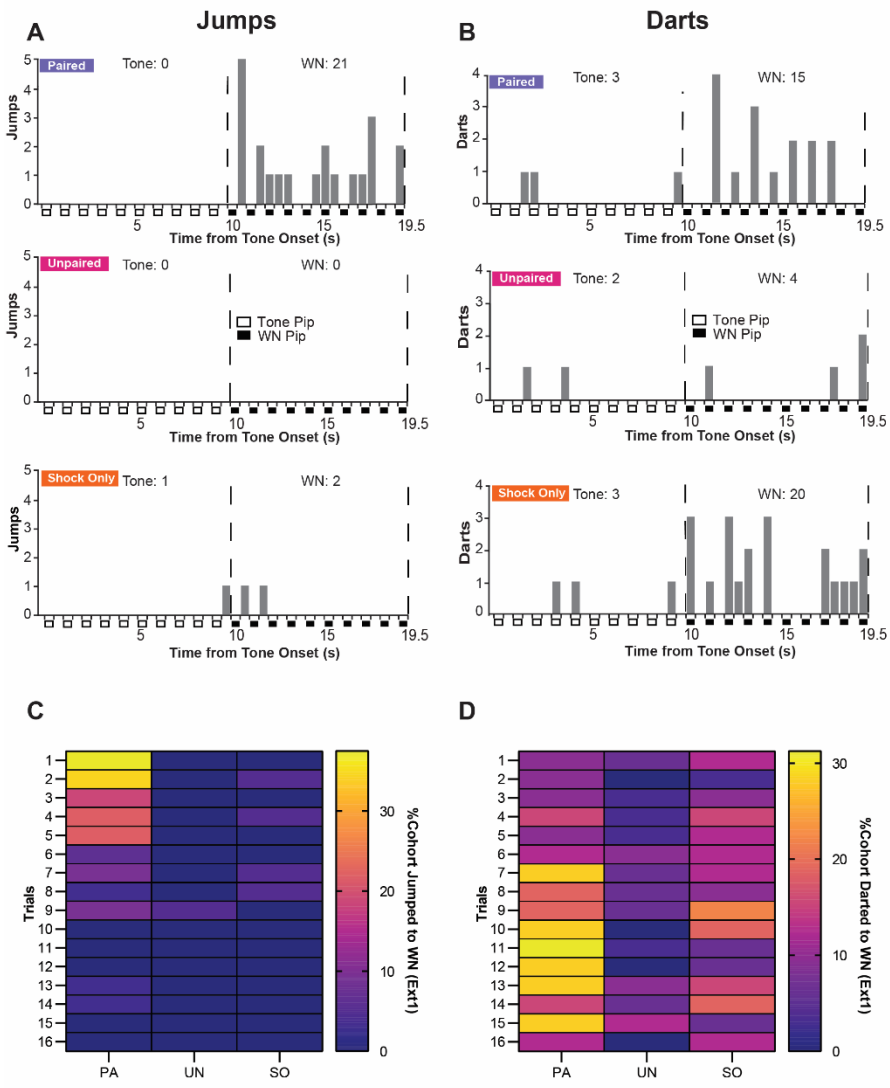


Figure 7. Stimulus-evoked escape jumping and darting during extinction. **A,** Histograms detailing the cumulative distribution of jumps across the duration of SCS from the first 4 trials of Ext1 for

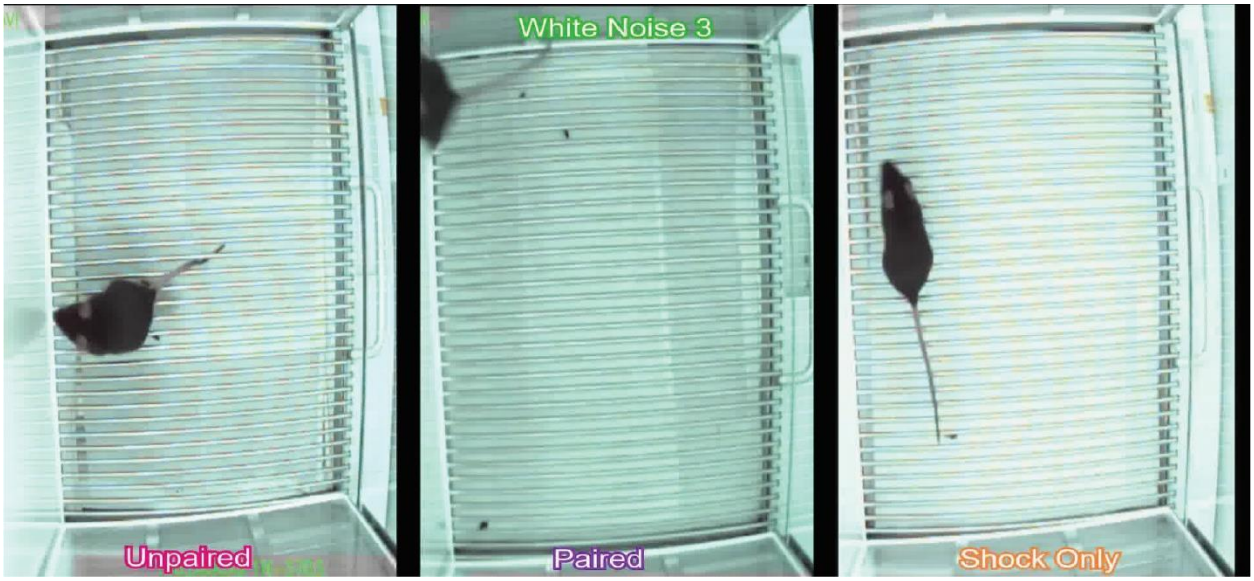
20 randomly selected subjects from the PA group, the entire UN group, and the entire SO group. Empty boxes represent each 0.5 s pip of tone, filled boxes represent each 0.5 s pip of WN, and the vertical dotted lines represent the onset and termination of the WN period. Total jumps per stimulus are listed above histogram bars. **B,** Histograms detailing the cumulative distribution of darts across the duration of SCS from the first 4 trials of Ext1 for 20 randomly selected subjects from the PA group, the entire UN group, and the entire SO group. 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 histogram bars. **C,** Heatmap showing the percentage of each group that exhibited jumping responses to the WN period of SCS per trial on Ext1. **D,** Heatmap showing the percentage of each group that exhibited darting responses to the WN period of SCS per trial on Ext1. SCS, Serial compound stimulus; Ext1, Extinction Day 1; WN, white noise; PA, Paired; UN, Unpaired; SO, Shock Only.

We used Fisher's exact test to compare the proportions of mice that jumped and darted during WN across the first extinction session. The PA group showed the largest proportion of mice jumping during WN, and this percentage decreased over trials (**Fig 7C**). Statistically, the proportion of mice that jumped to WN was significantly higher in the PA group compared to the UN (PA 16/32 vs UN 1/20, $p=0.0007$) and SO (PA 16/32 vs SO 2/20, $p=0.006$) groups. UN and SO groups did not differ in jumping behaviors to WN (UN 1/20 vs SO 2/20, $p>0.9999$).

Over the course of the first extinction session, the percentage of the PA and SO groups displaying darting behavior to WN increased (**Fig 7D**), with statistically similar proportions of the PA and SO groups darting during WN (PA 25/32 vs SO 18/20, $p=0.45$) and significantly more of the SO group darting to WN than the UN group (UN 10/20 vs SO 18/20, $p=0.01$; PA 25/32 vs UN 10/20, $p=0.07$).

These data suggest that jumping to WN is an associative defensive response that switches to darting as psychological distance of threat increases. 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 2.**



Video 2. Representative comparison of PA, UN, and SO groups' response to SCS during an early and late extinction trial. Video 2 shows how defensive responses to SCS change from early extinction to late extinction during the first extinction session. The video features audio of the SCS, which consists of 10 pips of tone followed by 10 pips of WN. SCS, Serial compound stimulus; Ext1, Extinction Day 1; WN, white noise; PA, Paired; UN, Unpaired; SO, Shock Only.

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 and during extinction (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 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. 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 groups displayed similar levels of tail rattling by the end of the second session.

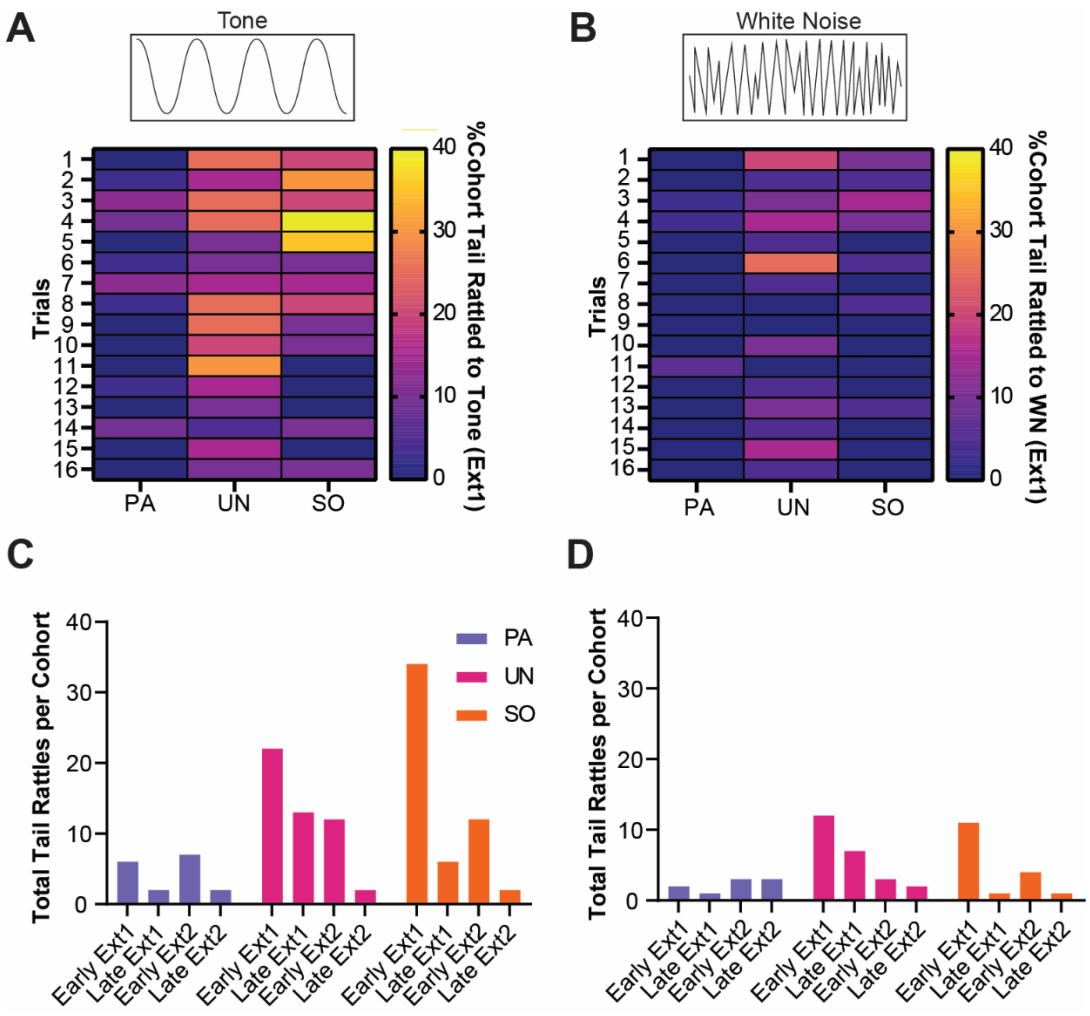


Figure 8. Tail rattling is a non-associative behavioral response during extinction. **A,** Heatmap showing the percentage of each group that exhibited tail rattling behaviors to the tone period of SCS per trial on Ext1. **B,** Heatmap showing the percentage of each group that exhibited tail rattling behaviors to the WN period of SCS per trial on Ext1. **C,** Histogram detailing the cumulative amounts of tail rattles during the tone period of SCS across early and late 4-trial bins of Ext1 and

Ext2. **D,** Histogram detailing the cumulative amounts of tail rattles during the WN period of SCS across early and late 4-trial bins of Ext1 and Ext2. SCS, Serial compound stimulus; Ext1, Extinction Day 1; Ext2, Extinction Day 2; WN, white noise; PA, Paired; UN, Unpaired; SO, Shock Only.

We used Fisher's exact test to compare the proportions from all groups that tail rattled during each portion of the SCS during the first extinction session. The PA group displayed lower proportions of tail rattling during tone compared to both UN (PA 11/32 vs UN 19/20, $p<0.0001$) and SO groups (PA 11/32 vs SO 15/20, $p=0.0095$). The UN and SO groups displayed similar tail rattling levels to tone (UN 19/20 vs SO 15/20, $p=0.18$). Additionally, the UN group tail rattled more to WN than the PA group (PA 4/32 vs UN 11/20, $p=0.002$), but neither group differed from the SO group (PA 4/32 vs SO 7/20, $p=0.08$; UN 11/20 vs SO 7/20, $p=0.34$).

These data highlight that tail rattling to the SCS is a non-associative defensive response that is generally suppressed when the SCS predictably signals incoming threat.

Discussion

This study investigated the contributions of associative and non-associative processes to the expression of learned, cue-induced defensive behaviors. The results signify that associative pairings during fear conditioning are vital for the expression and extinction of cue-induced defensive response scaling. Although non-associative elements like cue salience and shock sensitization elicit some stress-associated behaviors, as hypothesized before (Trott et al., 2022), they do not contribute significantly to high intensity cue-evoked defensive responses. Additionally, extinction of cue-evoked freezing and flight also depends on associative pairings. Therefore, the associative pairing formed during conditioning, not stimulus salience or shock sensitization, is the main driver of 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, highlighting the impact of SCS-shock contingency on the magnitude of defensive responses. Notably, similar results were previously reported in rats conditioned using similar parameters (Totty et al., 2021). Additionally, we found that the PA group showed an increase in freezing from pre-SCS to tone, while the UN group's freezing to tone was no greater than contextual freezing, indicating that the PA group placed associative value on the tone.

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 both the PA and UN groups, we examined if jumping within our SCS paradigm was associative or non-associative. We found that the PA group exhibited consistent jumping responses to the WN stimulus during conditioning that were not reproduced in the UN group. This indicates that a robust jumping response to WN in the SCS paradigm is not due to the salience of the WN, but its association with imminent threat.

Darting has been reported to be increased in rodents that undergo stress and fear conditioning (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 across the whole WN period requires a more nuanced analysis. We found that the PA group exhibited darting across the whole WN period during conditioning, while the response was almost nonexistent in the UN group. This matches 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 is not caused by the salience of the tone-WN transition, but from an associative learned response. We hypothesize that since the WN stimulus does not signal incoming threat, the UN group is not inclined to respond defensively during its presentation, instead engaging in standard locomotion and breaks in freezing induced by stimulus

salience. Overall, we identify jumping and darting behaviors that contribute to the high flight seen during WN in the PA group, and we provide data that suggests that these contributions are the result of associative learning rather than stimulus salience.

During extinction, the PA group showed the highest freezing to tone as well as the greatest within-session extinction of freezing compared to the control groups. This indicates that the PA group had the strongest association between the tone stimulus and threat, and this association was capable of being extinguished. This is further reinforced by the PA group's larger difference between tone-evoked freezing and pre-SCS freezing, indicating that freezing in the control groups during tone is no different than freezing to the context. Just as in conditioning, the PA group demonstrates a strong associative response to the tone stimulus that manifests in strong yet extinguishable freezing.

As the PA group learned that the WN no longer signaled imminent threat, they adjusted their behavior from explosive circa-strike flight to a combination of anticipatory post-encounter freezing and darting. This would indicate that their defensive strategy reflects a larger psychological distance from perceived threat, as the predatory imminence continuum describes (Perusini and Fanselow, 2015). The UN and SO groups showed only slight changes in WN-evoked activity over extinction, maintaining an elevated activity index and matching responses of stressed mice to an unfamiliar WN (Hoffman et al., 2022). Given that the magnitude and mode of WN-evoked behavior was altered over extinction in the PA group, we conclude that WN-evoked flight observed during conditioning and extinction is associative and is dependent on the perceived threat-signaling 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. The presence of jumping during early extinction trials in the PA group indicates that the WN initially signaled imminent threat, warranting 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 could also follow the predatory imminence continuum. Through our findings, we show that mice can be conditioned to display explosive escape jump behaviors when given an imminence-related predictor of threat, something not achievable with traditional single-cue Pavlovian conditioning paradigms. 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 relative lack of darting and jumping from the UN group during extinction, their increased activity is due to small locomotor movements, not flight behavior. The SO group maintained a consistent level of darting throughout the session, which contributed to their overall increased activity index. Given that darting can be elicited in stressed mice (Brzozowska et al., 2017), it is probable that shock sensitization can prime a stressed animal to dart more readily to WN upon stimulus transition. However, the change from jumping to darting midway through the first extinction session in the PA group suggests that the darting observed in these mice is not the result of sensitization. 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 with intermediate threat value and a defensive response resulting from sensitization.

Within the UN and SO control groups, we observed a higher degree of tail rattling responses to the SCS during extinction compared to the PA group, and tail rattling was more prominent during the tone period. 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 that anticipate an uncertain threat. Previously we found that tail rattling to SCS occurs most often early on during fear conditioning, when the threat association is weaker, and this behavior declines as the association fully develops (Borkar et al., 2020). This is in line with our results from the UN and SO groups, who displayed greater tail rattling to unrelated or novel SCS presentations within the stressful context. 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 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 this SCS paradigm, we elicit a robust continuum of consistent associative defensive responses during CS presentation that are seldom observed within classical Pavlovian conditioning. 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.

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