1   **Contributions of associative and non-associative learning to the dynamics of defensive**

2   **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, 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.

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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

60   behavior change with the psychological distance of threat, consistent with the predatory

61   imminence continuum theory (Perusini and Fanselow, 2015).

62   However, the influence of non-associative elements on this ethological profile has

63   recently been discussed (Fanselow et al., 2019; Hersman et al, 2020; Trott et al. 2022). It has

64   been suggested that the inherent salience of the WN stimulus contributes more to WN-evoked

65   flight response than its predictive association with the US (Hersman et al, 2020). Others claim

66   that the immediate transition from freezing to flight behavior is a result of the rapid change and

67   relative increase in stimulus intensity from tone to WN that is caused by non-associative

68   sensitization, or by inherent stimulus properties, akin to an acoustic startle response (Fanselow

69   et al., 2019, Trott et al. 2022). In addition, sensitization and stimulus salience are known to

70   intensify freezing responses to auditory stimuli (Kamprath and Wotjak, 2004), and mice show

71   increased reactivity to a WN stimulus after experiencing stress (Hoffman et al., 2022). These

72   findings highlight the need to better elucidate the associative and non-associative elements of

73   Pavlovian fear conditioning that influence expression of defensive behavior.

74   To address this, we utilized four control groups for non-associative effects of

75   conditioning. To test the importance of the SCS-shock contingency, we utilized an unpaired

76   control procedure in which the US and the SCS were presented in a separated, pseudorandom,

77   and non-predictive fashion (Rescorla, 1967). To test the effects of sensitization by the shock, we

78   presented footshock alone during conditioning. To test the impact of stimulus intensity and

79   salience, we conducted paired and unpaired conditioning using a reversed SCS where the WN

80   preceded the tone. We compared these four control groups against a paired SCS-shock

81   conditioning group to determine the effects of associative learning on SCS-evoked fear

82   behavior. All groups went through two extinction sessions with SCS presentations alone to

83   elucidate the extent to which prior associative pairing affects de-escalating response strategies,

84   as well as to identify defensive behaviors that are expressed in the absence of a strict threat-

85   signaling association.

86   **Materials & Methods**

87   *Animal Subjects*

88   We used C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine, Stock #000664),

89   aged 3-6 months in this study. Equal numbers of males and females were used in all

90   experiments. All mice were individually housed on a 12 h light/dark cycle throughout the study

91   with *ad libitum* access to food and water. Behavioral experiments were performed during the

92   light cycle. All animal procedures were performed in accordance with institutional guidelines and

93   were approved by the Institutional Animal Care & Use Committee of Tulane University.

94   *Apparatus*

95   Behavioral testing was performed in two contexts. Context A consisted of a 30 cm

96   diameter transparent acrylic cylinder with a smooth acrylic floor, cleaned with 1% acetic acid

97   between each subject. Context B consisted of a modular fear conditioning chamber (ENV-

98   307W, Med Associates, Inc., Fairfax, Vermont) with metal grid flooring and walls of

99   polycarbonate, stainless steel, and polyurethane, cleaned with 70% ethanol solution between

100   sessions. Alternating current footshocks (ENV-414S, Med Associates, Inc.) were delivered to

101   the mice during conditioning in Context B. A programmable audio generator (ANL-926, Med

102   Associates, Inc.) generated auditory stimuli that were delivered at 75 dB in each context via an

103   overhead speaker (ENV-224AM, Med Associates, Inc.). A serial compound stimulus (SCS) was

104   used as previously described (Fadok et al., 2017, Borkar and Fadok, 2021, Borkar et al., 2020).

105   The SCS consisted of ten pips of tone (7.5 kHz, 0.5 ms at 1 Hz) followed by ten pips of white

106   noise (0.5 ms at 1 Hz), and the reversed SCS consisted of ten pips of white noise followed by

107   ten pips of tone. Behavioral protocols were generated using Med-PC software (Med Associates,

108   Inc.) to control auditory stimuli and shock with high temporal precision.

109   *Experimental Design: SCS Conditioning and Extinction Paradigm*

110   Mice were randomly allocated to one of five groups: Paired (PA), Paired Reverse (PA-

111   R), Unpaired (UN), Unpaired Reverse (UN-R), and Shock Only (SO). Behavioral testing took

112   place over 5 days. For all days of the paradigm, PA-R and UN-R mice experienced the reversed

113   SCS at identical presentation timings as their respective PA and UN counterparts. On Day 1

114   (Pre-Exposure), subjects were placed in Context A for a baseline period of 3 min, followed by 4

115   presentations of the SCS with a pseudorandom inter-stimulus interval (ISI) period of 90-100 s

116   and a period of 40 s following the final SCS presentation, totaling 590 s per session. Day 2 and

117   Day 3 (Conditioning) took place in Context B. On each conditioning day (CD1 and CD2), mice

118   were subjected to one of three conditions after a 3 min baseline period. For all groups, each

119   conditioning session lasted 820 s. PA mice (n=16 males, 16 females) and PA-R mice (n=5

120   males, 5 females) were presented with 5 pairings of the SCS co-terminating with a 1 s, 0.9 mA

121   footshock, with pseudorandom ISI periods of 90-150 s and a period of 60 s following the final

122   footshock of the session. UN mice (n=10 males, 10 females) and UN-R mice (n=5 males, 5

123   females) were presented with pseudorandom presentations of SCS and footshocks separate

124   from one another with ISI periods of 40-60 s, with a period of 90 s following the final stimulus of

125   the session. Stimuli were ordered such that the SCS could not reliably predict footshock. PA,

126   PA-R, UN, and UN-R mice all received the same number of SCS and footshock presentations,

127   only differing by SCS-footshock contingency. SO mice (n=10 males, 10 females) did not receive

128   presentations of the SCS during conditioning and were given 5 footshocks with pseudorandom

129   ISI periods of 120-160 s each session, with a period of 80 s following the final shock of the

130   session. For all groups, stimulus timing and ISI differed between CD1 and CD2 to avoid

131   predictable anticipation of stimuli before presentation. Days 4 and 5 (Extinction) took place in

132   Context B, and each session consisted of 16 presentations of the SCS with pseudorandom ISI

133   periods of 60-120 s, with a period of 50 s following the final SCS of the session. Each Extinction

134   session (Ext1 and Ext2) lasted 1910 s. Subjects were sacrificed after the conclusion of

135   behavioral testing.

136   *Behavioral Recording and Analysis*

137   All sessions were recorded to video using a camera (Pike, Allied Vision, Stadtroda,

138   Germany) mounted above the behavioral contexts with stimulus events encoded to the same

139   files using TTL pulses (Omniplex, Plexon, Dallas, Texas). Contour tracking (Cineplex, Plexon)

140   was used to automatically detect freezing based on frame-by-frame changes in pixels. Freezing

141   behavior was defined as a complete cessation of movement for at least 1 s, and results were

142   confirmed with an observer blinded to condition. By determining a calibration coefficient using

143   the known size of the behavioral context and the camera's pixel dimensions, speed (cm/s) was

144   extracted using the animal's center of gravity. An activity index was calculated for each animal

145   using a ratio of its speed during either the tone or WN stimulus (CS) period and its average

146   speed from the combined 10 s periods prior to each SCS presentation (pre-SCS) during the

147   session; the number of jumps performed during that stimulus period was then added to this ratio

148   ($Speed_{CS}/Speed_{avg\ pre-SCS}+Jumps$). Previously we calculated flight scores per trial using speed

149   from each trial's pre-SCS period (Fadok et al., 2017; Borkar et al., 2020), but here we utilized an

150   average from all pre-SCS periods in our calculations to avoid denominators that were close to or

151   equal to 0, a complication noted by other groups (Hersman et al., 2020). Reflecting this change,

152   we now refer to this measure of locomotor change as an "activity index" instead of a "flight

153   score" as before. Escape jumps and tail rattling behaviors were manually classified by an

154   observer blinded to condition. Jumps were defined as the period where the mouse had all four

155   paws above the chamber floor. Tail rattling was defined as rapid back-and-forth vibrations of the

156   tip and midsection of the tail. Darting behavior was detected and classified using machine

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157 learning software as described below and was defined as rapid bursts of movement across the

158 floor of the chamber. Distance traveled over pre-SCS, tone, and WN periods was calculated per

159 mouse by plotting its average speed per 0.5 s intervals and integrating the area under the curve.

160 When performing behavioral analyses that reported cumulative frequencies per group,

161 20 random subjects from the PA group were used to match the population sizes of the UN and

162 SO control groups. Due to the lower number of subjects in the PA-R and UN-R groups, they

163 were excluded from frequency-based comparisons.

164 *Analysis of Darting Using Machine Learning*

165 Darts were scored using the program Simple Behavior Analysis (SimBA, Nillson et al.,

166 2020) to generate a machine learning algorithm capable of automatically detecting the

167 occurrence of the behavior of interest. To generate this model, top-down footage (640x480 pixel

168 resolution, 30 frames per second) of 16 male and female C57BL/6J mice that underwent SCS

169 fear conditioning in Context B was collected and analyzed in DeepLabCut (DLC) (Mathis et al.,

170 2018) to assign 8 tracking points (Nose, L Ear, R Ear, Center of Mass, L Flank, R Flank, Tail

171 Base, Tail Tip) to subjects. The DLC markerless tracking model was generated using manually

172 assigned points from ~2,000 frames trained using the ResNet50 Neural Network for 125,000

173 iterations. 2,370 frames containing darting behavior were identified and added to the training set

174 for SimBA. The darting start point was defined as the first frame in which the mouse began

175 accelerating from a resting position, and the end point was defined as the last frame before the

176 mouse returned to a full stop. Once the model was generated, all videos from all subjects were

177 analyzed using a discrimination threshold of .37 and a minimum duration of 266 ms (8 frames).

178 *Statistical Analysis*

179 Sample sizes for each group were justified via power analysis (α = 0.05, power = 80%).

180 Data were analyzed for statistical significance using Prism 9 (GraphPad Software Inc., San

181   Diego, California). For all tests, the definition of statistical significance was $p<0.05$. All data were

182   checked for normal distribution using the Shapiro-Wilk normality test ($\alpha=0.05$). For pairwise

183   comparisons between groups, unpaired t-test with Welch's correction was used to assess

184   behavioral differences since all relevant datasets had normal distributions. One-way analysis of

185   variance (ANOVA) was used to assess behavioral differences between all conditioning groups.

186   Two-way ANOVA was used to assess interactions of time point and conditioning variant

187   between groups, as well as interactions of stimulus and conditioning variant within groups.

188   When either ANOVA yielded significant interactions, Tukey's post-hoc multiple comparisons test

189   was used to detect significant behavioral differences between groups.

190   **Results**

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

192   *order*

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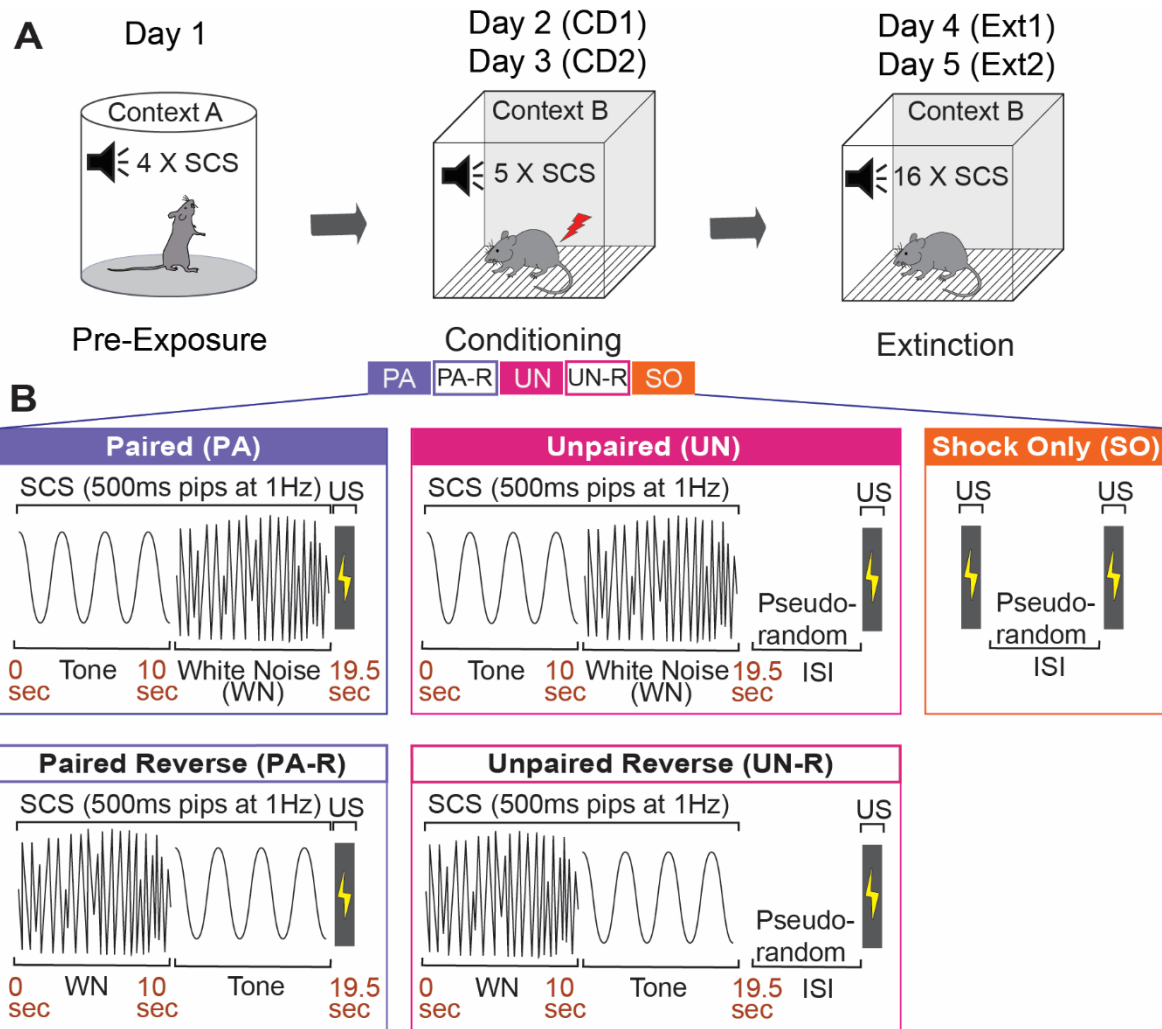
Figure 1. Experimental Design. **A,** Graphical representation of the three stages of the SCS conditioning paradigm. **B,** Five SCS-shock association variants were used during conditioning. SCS, Serial compound stimulus; CD1, Conditioning Day 1; CD2, Conditioning Day 2; Ext1, Extinction Day 1; Ext2,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

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

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204   male and female mice were pooled for statistical comparisons between groups. Additionally,

205   given the minimal sex differences observed within the PA, UN, and SO groups, we reduced the

206   number of subjects in the PA-R and UN-R groups and therefore did not statistically test for sex

207   differences.

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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

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210   **Table 1.** Statistical analysis of sex differences in defensive behavior.

211

212   Behavioral data from the PA, UN, PA-R, and UN-R groups during the second day of fear

213   conditioning (CD2) were compared to observe how conditioned defensive behavior differs

214   based on the associative value and stimulus order of the SCS. A two-way ANOVA was used to

215   analyze the effect of trial and group on freezing during the tone and WN. There was no

216   statistically significant interaction between trial and group for tone-induced freezing (**Fig. 2A**;

217    $F_{(12, 340)} = 0.65$, p=0.80); however, there was a significant main effect of trial ($F_{(4, 340)} = 6.3$,

218   p<0.0001) and group ($F_{(3, 340)} = 19.6$, p<0.0001). All groups showed little freezing to the WN (**Fig**

219   **2B**), and no significant interaction between trial and group ($F_{(12, 340)} = 0.76$, p=0.69) or main

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220   effect of trial ($F_{(4, 340)}$ = 2.07, p=0.08) were found. We did find a main effect of group ($F_{(3, 340)}$ =

221   3.00, p=0.03), which was attributed to greater freezing from the PA-R group during Trial 1.

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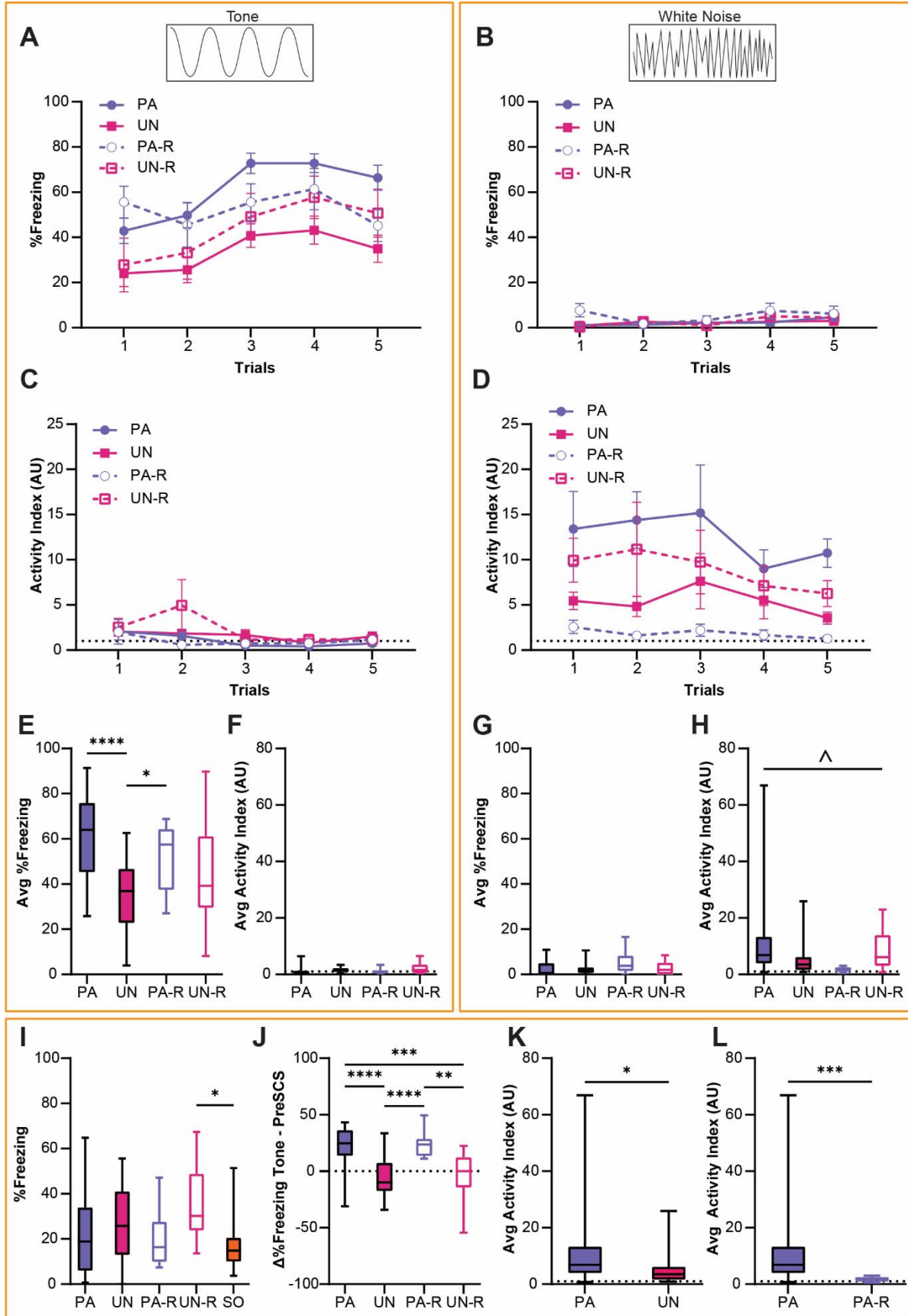
Figure 2. Stimulus-evoked freezing and activity during CD2 are affected by SCS-shock contingency. **A,** Trial-by-trial freezing during the tone period. **B,** Trial-by-trial freezing during the WN period. **C,** Trial-by-trial activity index during the tone period. **D,** Trial-by-trial activity index 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 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.

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

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255   groups (**Fig 2G**, $F_{(3, 68)}$ = 2.62, p=0.058), there was a significant interaction when analyzing

256   activity indices during WN (**Fig 2H**, $F_{(3, 68)}$ = 3.04, p=0.035). However, post-hoc comparisons did

257   not yield significant pairwise differences.

258   An ordinary one-way ANOVA was used to test for between-group differences in

259   contextual freezing during the initial three minutes of the session preceding the first SCS

260   presentation, and Tukey's multiple comparisons test was used for post-hoc comparisons. There

261   was a main effect of group for baseline contextual freezing (**Fig 2I**, $F_{(3, 68)}$ = 3.18, p=0.017), but

262   significant differences were found only between the UN-R and SO groups (UN-R vs SO,

263   p=0.013). Welch's unpaired t-test was used to compare the differences in freezing during the

264   pre-SCS and tone periods to determine the extent to which the tone increased freezing. The PA

265   and PA-R groups showed significantly greater increases in freezing from pre-SCS to tone

266   compared to the UN and UN-R groups (**Fig 2J**, $F_{(3, 68)}$ = 15.92, p<0.0001; PA vs UN, p<0.0001;

267   PA vs UN-R, p=0.0009 ; PA-R vs UN, p<0.0001; PA-R vs UN-R, p=0.0055).

268   Given the interaction found in **Fig 2H,** we performed Welch's unpaired t-test to conduct

269   pairwise comparisons between average activity indices during WN. WN-evoked activity was

270   higher in the PA group compared to the UN group (**Fig 2K**, PA vs UN, $t_{(43.08)}$ = 2.36, p=0.023)

271   and the PA-R group (**Fig 2L**, PA vs PA-R, $t_{(31.62)}$ = 3.89, p=0.0005). Additionally, the PA-R group

272   displayed lower WN-evoked activity than both of the UN groups (PA-R vs UN, $t_{(20.65)}$ = 2.65,

273   p=0.015; PA-R mean ± SEM = 1.86 ± 0.28; UN mean ± SEM = 5.39 ± 1.31) and the UN-R group

274   (PA-R vs UN-R, $t_{(9.215)}$ = 2.75, p=0.022; UN-R mean ± SEM = 8.84 ± 2.52). There was no

275   difference between the UN and UN-R groups with regards to WN-evoked activity (UN vs UN-R,

276    $t_{(14)}$ = 1.214, p=0.24). While the PA and UN groups significantly differed in average activity

277   indices during WN, the PA and UN-R groups did not (PA vs UN-R, $t_{(30.42)}$ = 0.9962, p=0.33; PA

278   mean ± SEM = 12.54 ± 2.73).

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279   Overall, these data show that the respective changes in defensive behavior during tone

280   and WN were significantly affected by the explicit pairing of SCS and shock during fear

281   conditioning, and that the order of tone and WN presentation influenced the intensity of WN-

282   evoked responses.

283   *Associative pairing of the SCS and shock elicits escape jumping and darting responses to WN*

284   *after conditioning*

285   Although the UN or UN-R groups did not receive an associative pairing between SCS

286   and shock like the PA and PA-R groups, all groups still displayed increased activity indices to

287   WN (**Fig 2D**). To determine if this behavioral response was due to defensive flight or a more

288   basic locomotor response, we investigated the occurrence of escape jumping and darting

289   behaviors during WN presentation on CD2. A substantial percentage of PA mice jumped during

290   WN on every trial, and these jumps were distributed across the entire WN period (**Fig 3A, B**). In

291   contrast, an exceedingly small percentage of the UN group jumped during WN (**Fig 3C**), and

292   when jumps occurred, they occurred at the onset of the WN (**Fig 3D**). Both groups responded to

293   shock with a similar number of jumps (**Fig 3B, D**). Trial-by-trial, PA mice displayed more

294   jumping behavior across the WN period (**Fig 3E**) compared to UN mice (**Fig 3F**). Like the UN

295   group, the reverse SCS groups also displayed lower jumping percentages to WN, with jumps in

296   the PA-R group occurring rarely near the transition from WN to tone, (**Fig 3G**) and jumps in the

297   UN-R group occurring near WN onset (**Fig 3H**). The PA group had the largest percentage of

298   mice that jumped to WN during CD2 compared to all other groups (**Fig 3I**). Lastly, all groups

299   exhibited jumps to shock (**Fig 3J**), with greater percentages of the unpaired and reverse cohorts

300   responding with jumps.
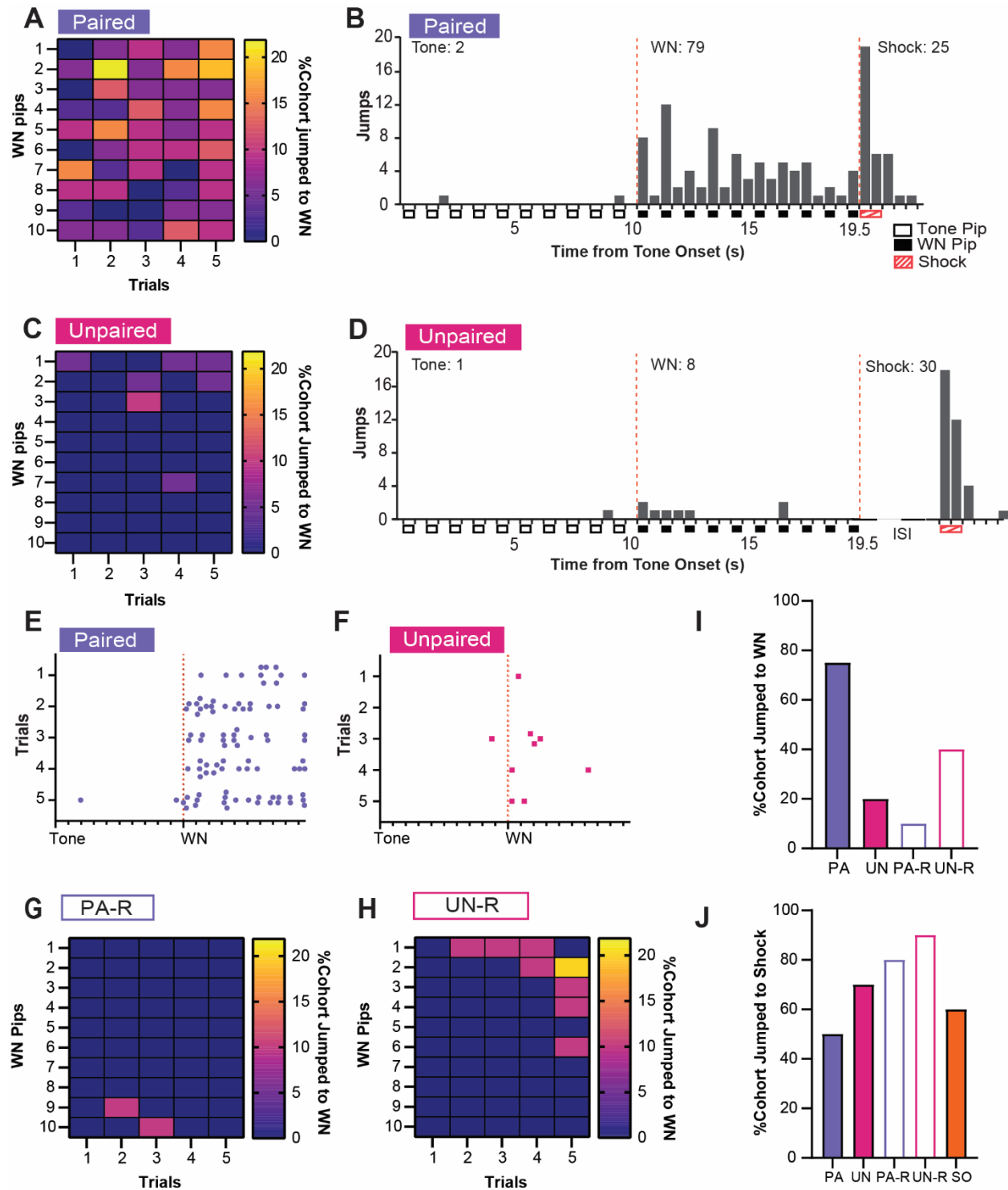
Figure 3. Associative pairings of the SCS and shock lead to robust escape jumping during WN. **A,** The percentage of the PA group that exhibited jumping during WN on CD2. Data are distributed across 1 s bins, each coinciding with one of the ten pips of WN that occurred during each SCS presentation. **B,** The cumulative distribution of jumps from 20 randomly selected
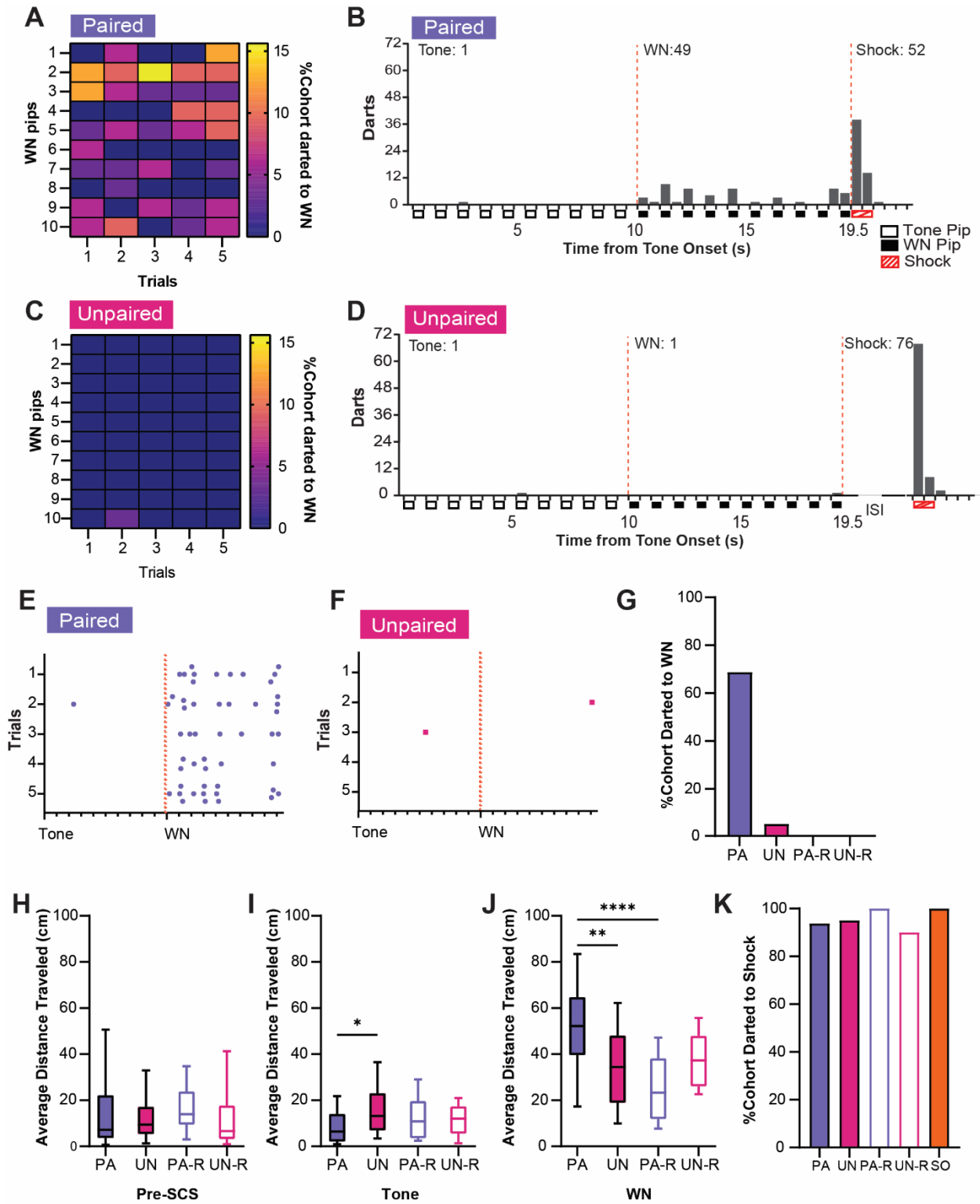
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307 subjects of the PA group across the duration of the SCS from all 5 trials of CD2. Empty boxes
308 represent each 0.5 s pip of tone, filled boxes represent each 0.5 s pip of WN, and striped boxes
309 represent the 1 s shock stimulus. The vertical dotted lines depict the onset and termination of
310 the WN period. Total jumps per stimulus are listed above histogram bars. **C,** The percentage of
311 the UN group that exhibited jumping during WN on CD2. Data are distributed across 1 s bins,
312 each coinciding with one of the ten pips of WN that occurred during each SCS presentation. **D,**
313 The cumulative distribution of jumps from the UN group across the duration of the SCS from all
314 5 trials of CD2. Empty boxes represent each 0.5 s pip of tone, filled boxes represent each 0.5 s
315 pip of WN, and striped boxes represent the 1 s shock stimulus. The vertical dotted lines depict
316 the onset and termination of the WN period. ISI represents the period between SCS and shock.
317 Total jumps per stimulus are listed above histogram bars. **E,** The distribution of jumps across
318 the duration of the SCS from 20 randomly selected subjects of the PA group for each trial of
319 CD2. Each dot represents a single jump event, and each tick on the x-axis represents the onset
320 of each pip of tone or WN. The vertical dotted line depicts the onset of the WN period. **F,** The
321 distribution of jumps across the duration of the SCS from the UN group for each trial of CD2.
322 Each dot represents a single jump event, and each tick on the x-axis represents the onset of
323 each pip of tone or WN. The vertical dotted line depicts the onset of the WN period. **G,** The
324 percentage of the PA-R group that exhibited jumping during WN on CD2. Data are distributed
325 across 1 s bins, each coinciding with one of the ten pips of WN that occurred during each SCS
326 presentation. **H,** The percentage of the UN-R group that exhibited jumping during WN on CD2.
327 Data are distributed across 1 s bins, each coinciding with one of the ten pips of WN that
328 occurred during each SCS presentation. **I,** Total percentage of cohort that jumped during WN
329 over the whole CD2 session. **J,** Total percentage of cohort that jumped to shock over the whole
330 CD2 session.

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332   Similar analyses were performed for darting behavior. A high percentage of PA mice

333 showed darting during WN (**Fig 4A**), darts were specific for the WN, and they were spread

334 across the stimulus period (**Fig 4B**). Mice in the UN group almost never darted during the tone

335 or WN (**Fig 4C, D**). Further, PA mice displayed darts across the WN period on every trial (**Fig

336 4E**), whereas UN mice did not (**Fig 4F**). The PA group also had the largest percentage of mice

337 that darted during WN compared to all other groups (**Fig 4G**). Interestingly, we did not detect

338 darting from the reverse groups during the SCS in CD2. The non-PA groups only rarely

339 expressed escape jumping or darting, yet they did have elevated activity indices (**Fig 2D**).

340 Therefore, we measured the average distance traveled over the WN period, and we used a one-

341 way ANOVA to determine if these higher activity levels were due to a simpler locomotor

342 response. The groups did not differ in distance traveled during preSCS (**Fig 4H**, $F_{(3, 68)} = 0.28$,

343 p=0.84), but we did observe differences during tone (**Fig 4I**, $F_{(3, 68)} = 3.34$, p=0.024) and WN

344 (**Fig 4J**, $F_{(3, 68)} = 9.83$, p<0.0001). During the tone, the UN group had significantly greater

345   distance traveled than the PA group (PA vs UN, p=0.014), which is reflective of the elevated

346   freezing during the tone in the PA group (**Fig 2E**). During the WN, the PA group had greater

347   distance traveled than the UN (PA vs UN, p=0.0018) and the PA-R groups (PA vs PA-R,

348   p<0.0001), but not the UN-R group (PA vs UN-R, p=0.083). Finally, all groups darted to shock in

349   similar percentages (**Fig 4K**).

350

351

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

354 1 s bins, each coinciding with one of the ten pips of WN that occurred during each SCS
355 presentation. **B,** The cumulative distribution of darts from 20 randomly selected subjects of the
356 PA group across the duration of the SCS. Empty boxes represent each 0.5 s pip of tone, filled
357 boxes represent each 0.5 s pip of WN, and striped boxes represent the 1 s shock stimulus. The
358 vertical dotted lines depict the onset and termination of the WN period. Total darts per stimulus
359 are listed above the histogram. **C,** The percentage of the UN group that exhibited darting
360 responses during WN. Data are distributed across 1 s bins, each coinciding with one of the ten
361 pips of WN that occurred during each SCS presentation. **D,** The cumulative distribution of darts
362 from the UN group across the duration of SCS. Empty boxes represent each 0.5 s pip of tone,
363 filled boxes represent each 0.5 s pip of WN, and striped boxes represent the 1 s shock stimulus.
364 The vertical dotted lines depict the onset and termination of the WN period. ISI represents the
365 period between SCS and shock. Total darts per stimulus are listed above the histogram. **E,** The
366 distribution of darts across the duration of SCS from 20 randomly selected subjects of the PA
367 group. Each dot represents a single dart event, and each tick on the x-axis represents the onset
368 of each pip of tone or WN. The vertical dotted lines depict the onset of the WN period. **F,** The
369 distribution of darts across the duration of SCS from the UN group. Each dot represents a single
370 dart event, and each tick on the x-axis represents the onset of each pip of tone or WN. The
371 vertical dotted lines depict the onset of the WN period. **G,** The total percentage of each group
372 that jumped during WN over the whole session. **H**, Average distance traveled during the
373 preSCS period. **I**, Average distance traveled during the tone period. **J**, Average distance
374 traveled during the WN period. **K**, The total percentage of each group that jumped to shock over
375 the whole session. Data from Figures **4H-4J** are presented as box-and-whisker plots from min
376 to max. *p<0.05; **p<0.01; ****p<0.0001.

377

378   In summary, associative pairings of SCS and shock produced significant cue-induced

379 freezing to the tone, as well as robust jumping and darting behaviors that occurred over the

380 entirety of WN presentations. Altering the contingency between WN and shock in the unpaired

381 or reversed SCS conditions profoundly reduced these defensive behaviors, but the increased

382 activity in all groups during WN suggests that inherent properties of the WN interact with non-

383 associative processes to induce locomotor responses. This is reflected in the increased

384 distance traveled during WN in all groups compared to the tone period. Representative

385 behavioral responses of the PA, UN, PA-R, and UN-R groups to the SCS during conditioning

386 are provided in **Video 1** and **Video 2.**

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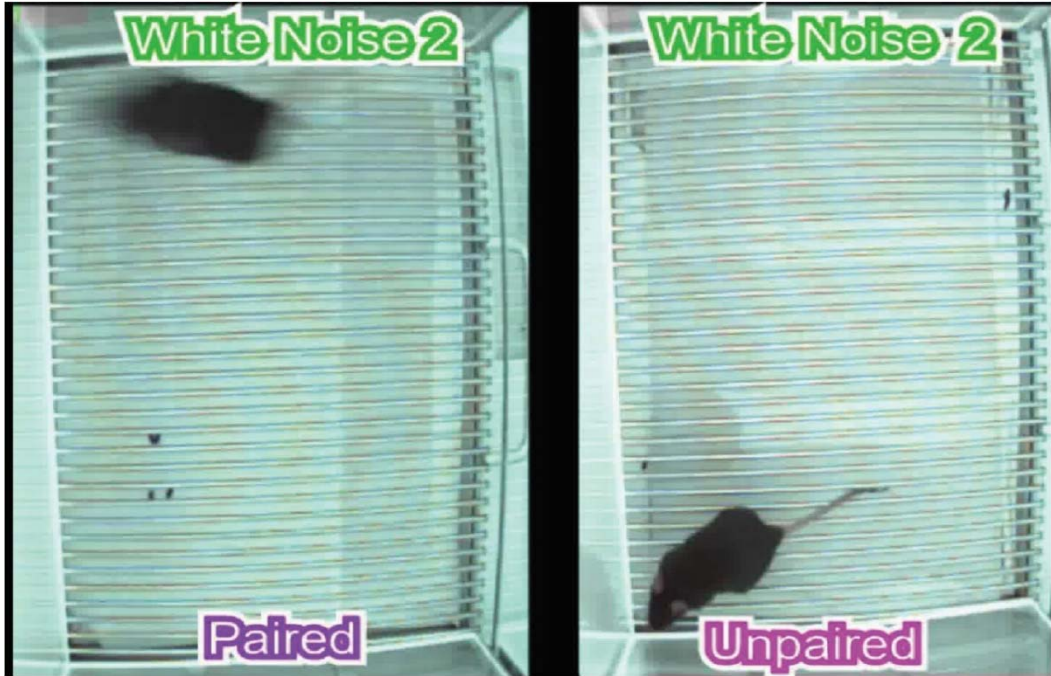
388 **Video 1.** Representative comparison of PA and UN groups' response to SCS during
389 conditioning. The video features audio of the SCS, which consists of 10 pips of tone followed by
390 10 pips of WN.

391

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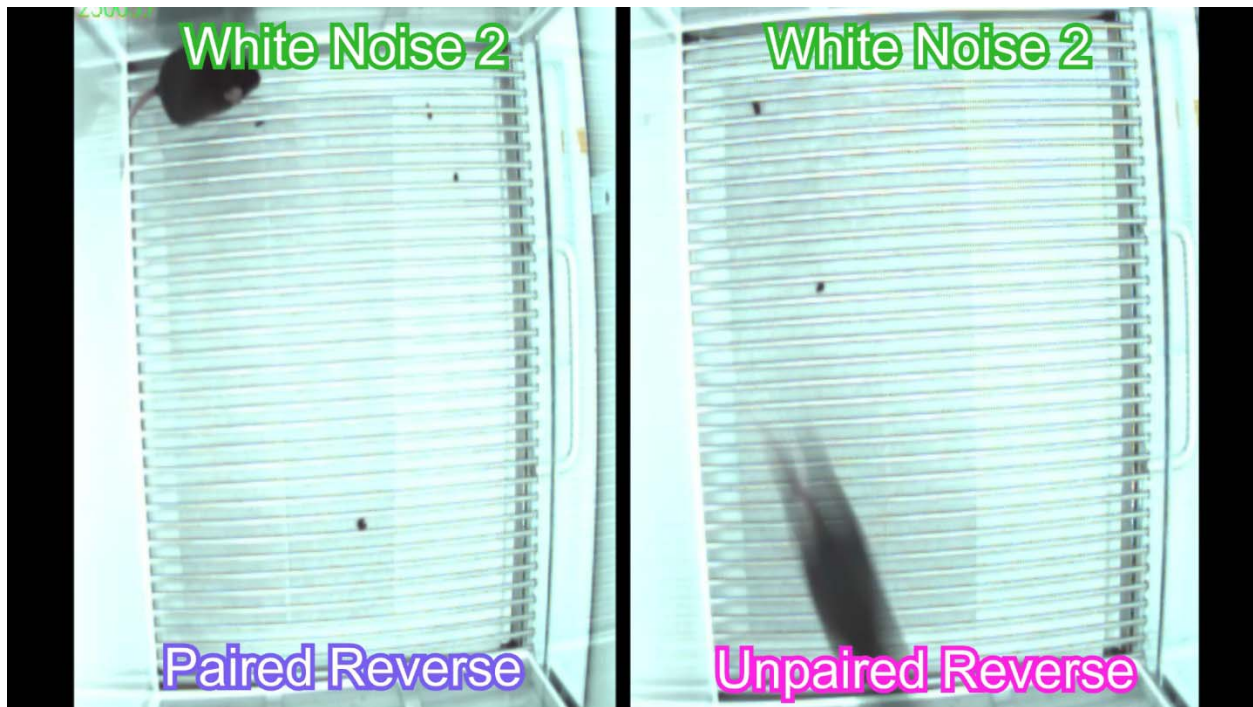
392 **Video 2.** Representative comparison of PA-R and UN-R groups' response to reverse SCS

393 during conditioning. The video features audio of the reverse SCS, which consists of 10 pips of

394 WN followed by 10 pips of tone.

395

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

397 We next analyzed how the defensive ethogram of each group changed over the course

398 of two extinction sessions. A two-way ANOVA was used to analyze the effect of trial and group

399 on freezing during the tone, and Tukey's multiple comparisons test was used for post-hoc

400 comparisons. When analyzing tone-evoked freezing across extinction within the PA, UN, and

401 SO groups (**Fig 5A**), a significant interaction between trial and group ($F_{(62, 2208)}$ = 2.3, p<0.0001)

402 was found. For every trial except the last, the PA group exhibited a higher level of freezing

403 during tone compared to the control groups (p<0.05 for Trials 1-15, for both sessions). Tone-

404 evoked freezing presented similarly between the PA-R and UN-R groups (Fig 5B), yielding a

405 significant interaction between trial and group ($F_{(31, 576)}$ = 1.63, p=0.018). The PA-R group

406 maintained consistently high freezing during the tone across both extinction sessions, freezing

407 more than the UN-R group for nearly all trials (p<0.05 for Trials 1, 3-16 on Ext1, all trials on

408 Ext2).

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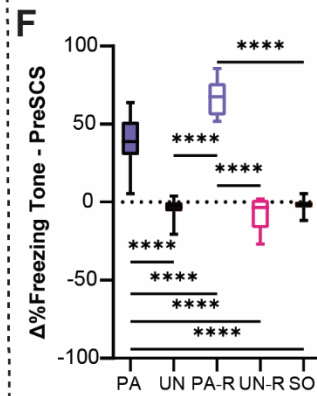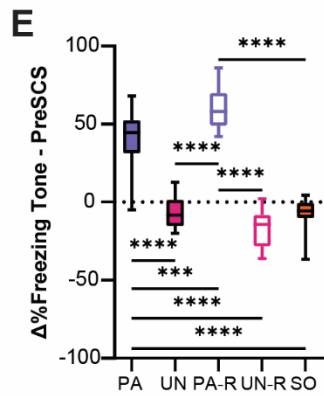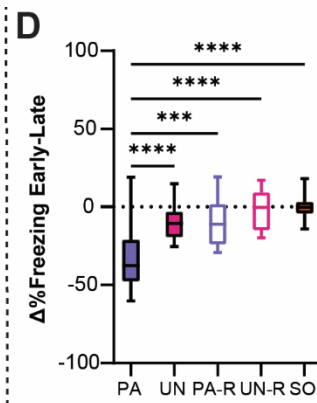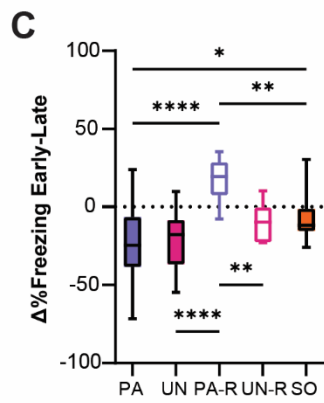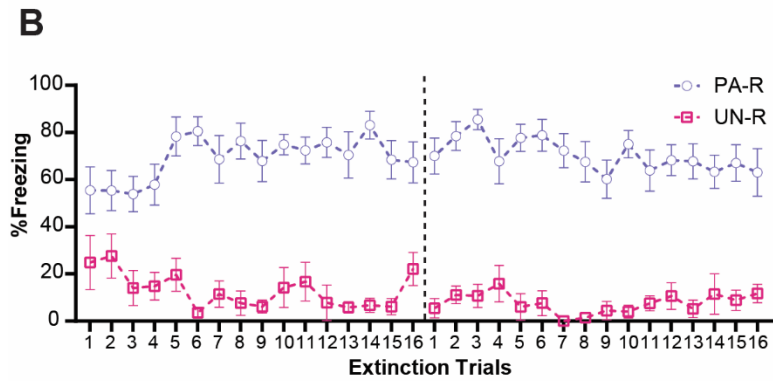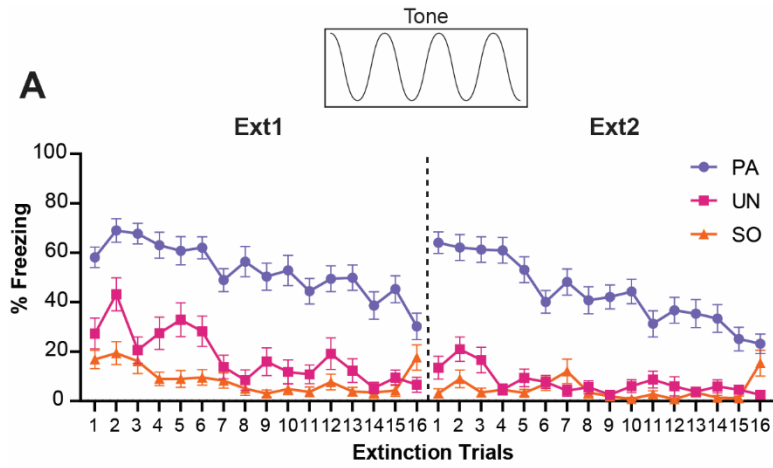
Figure 5. Tone-evoked freezing in the PA group is reduced by extinction learning. **A,** Percent freezing during the tone period for the PA, UN, and SO groups. **B,** Percent freezing during the tone period for the PA-R and UN-R. **C**, The difference in average freezing during the tone period between 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.

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 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

25

437   was used to analyze the effect of group, and Tukey's multiple comparisons test was used for

438   post-hoc comparisons. For both the first (**Fig 5E**, $F_{(4, 87)}$ = 105.5, p<0.0001) and second (**Fig 5F**,

439    $F_{(4, 87)}$ = 137.5, p<0.0001) sessions of extinction, only the PA and PA-R groups increased

440   freezing levels during the tone (p<0.0001 for all PA and PA-R comparisons to other groups for

441   both sessions). The PA-R group showed a greater change in freezing from pre-SCS to tone

442   than the PA group (Ext1, PA vs PA-R, p=0.0003; Ext2, PA vs PA-R, p<0.0001), whereas the

443   UN, UN-R, and SO groups had equivalent freezing during the pre-SCS and tone periods

444   (p>0.25 for all pairwise comparisons that excluded the PA and PA-R groups for both sessions).

445   Taken together, these data suggest that the PA and PA-R groups associated the tone with

446   threat, while freezing in the UN, UN-R, and SO groups was more indicative of contextual fear.

447   Interestingly, while pairing either order of SCS with shock resulted in greater freezing during

448   tone, we observe a phenotype more resistant to extinction within the PA-R group. These data

449   indicate that the extinction of cue-induced freezing in the conditioned flight paradigm depends

450   on its proximity to conditioned threat.

451

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

453   Activity indices were calculated to analyze the effect of extinction learning on behavioral

454   responses during the WN. The PA, UN, and SO groups had elevated activity indices in the early

455   trials of extinction training, yet only the PA group showed a decrease in WN-evoked activity (**Fig**

456   **6A**). A two-way ANOVA was used to analyze the effect of trial and group, and Tukey's multiple

457   comparisons test was used for post-hoc comparisons. There was a significant interaction

458   between trial and group ($F_{(62, 2208)}$ = 1.9, p<0.0001). Starting with the fifth trial of the first

459   extinction session, the PA group expressed significantly less activity to WN compared to the UN

460   and SO groups (p<0.05 compared to UN and SO for Trials 5, 6, and 8-13), and PA activity index

461   scores remained below 1 for the entirety of the second extinction session (p<0.05 compared to

26

462   UN and SO for Trials 1, 2, and 4-16). For the UN and SO groups, WN-evoked activity indices

463   remained above 1 across extinction trials, indicating levels of movement that were higher during

464   the WN than the pre-SCS period. There was no interaction between trial and group (**Fig 6B**,

465    $F_{(31, 576)}$ = 0.7, p=0.89) for the PA-R and UN-R groups. There was an effect of group ($F_{(1, 576)}$ =

466   64.7, p<0.0001), with activity being higher in the UN-R compared to the PA-R group. There was

467   no significant effect of trial ($F_{(31, 576)}$ = 0.66, p=0.92).
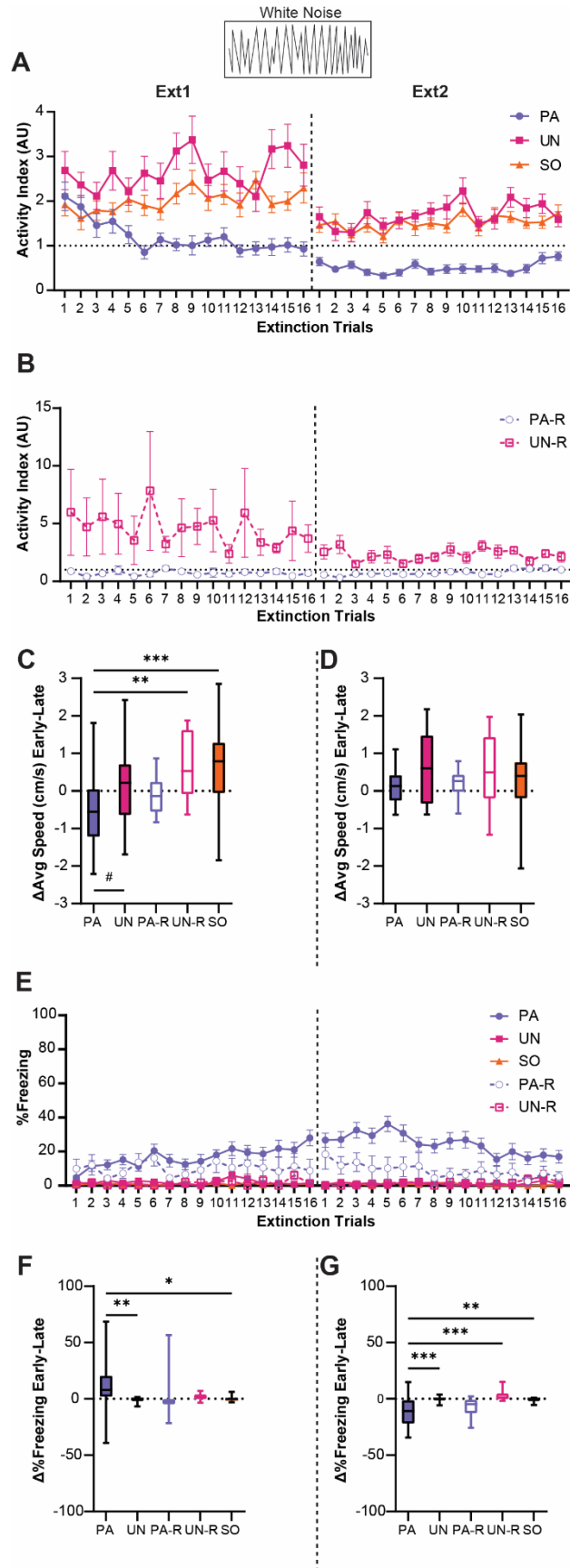
Figure 6. Stimulus-induced flight is associative and is partially replaced by freezing during extinction. **A,** Trial-by-trial activity during the WN period for the PA, UN, and SO groups during Ext1 and Ext2. **B,** Trial-by-trial activity during the WN period for the PA-R and UN-R groups during Ext1 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.

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, 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-

496   10). When comparing changes in WN-evoked freezing between the first and last four trials for

497   the first extinction session (**Fig 6F**, $F_{(4, 87)}$ = 4.2, p=0.0036), the PA group displayed significant

498   increases compared to the UN and SO groups (PA vs UN, p=0.0086; PA vs SO, p=0.017), but

499   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

500   the second session (Fig 6G, $F_{(4, 87)}$ = 8.1, p<0.0001), the PA group exhibited a significant

501   decrease in WN-evoked freezing compared to the UN, UN-R, and SO groups (PA vs UN,

502   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,

503   p=0.85).Collectively, these findings show changes in the magnitudes and modes of behavior to

504   WN within the PA group across extinction, indicating that WN-evoked flight in the PA group can

505   be extinguished and is associative.

506   *Stimulus evoked escape jumping and darting during extinction*

507   To determine if the activity measured during extinction was related to defensive flight or

508   a more basic locomotor response, we examined the expression of jumping and darting

509   behaviors between groups. Within the first four trials of the first extinction session, PA mice

510   displayed the most jumping behavior during WN, with the SO group displaying only two jumps

511   occurring near the onset of WN, and the UN group displaying no jumps (**Fig 7A**). When

512   examining darting behavior within the first four trials of extinction, minimal darting was observed

513   during the tone period, the PA and SO groups displayed darting behavior spread across the WN

514   period, while the UN group darted only a few times (**Fig 7B**). Jumps and darts were not present

515   within the second extinction session for any group.
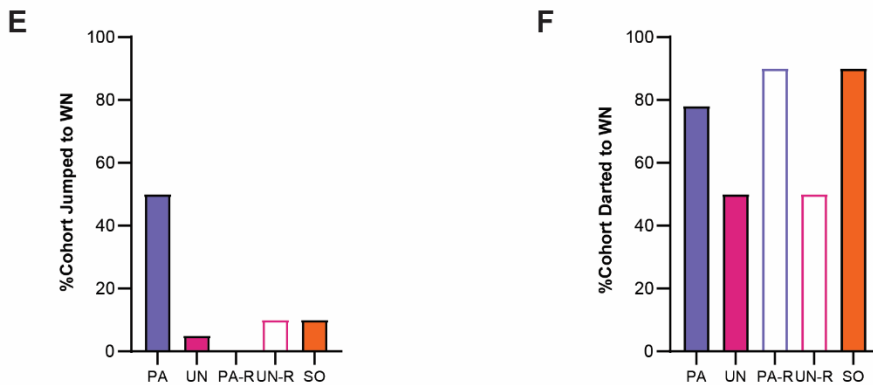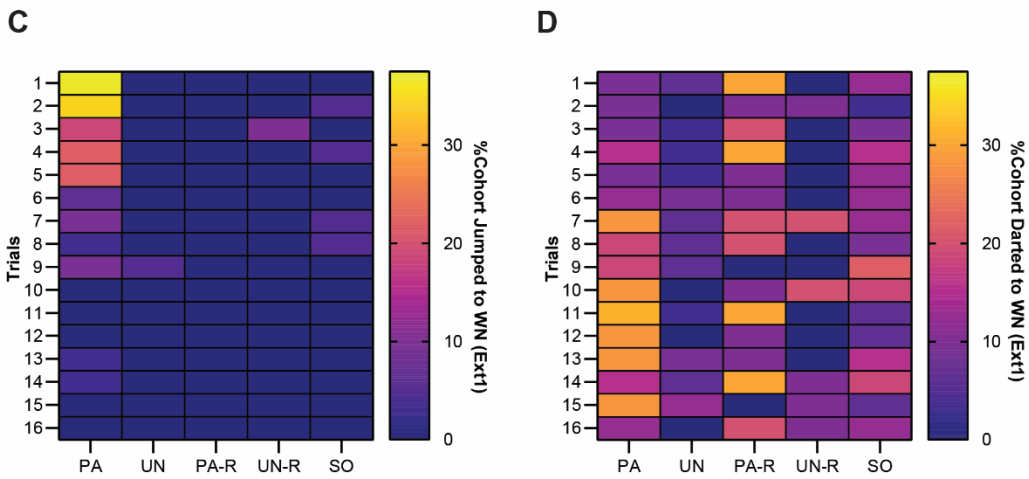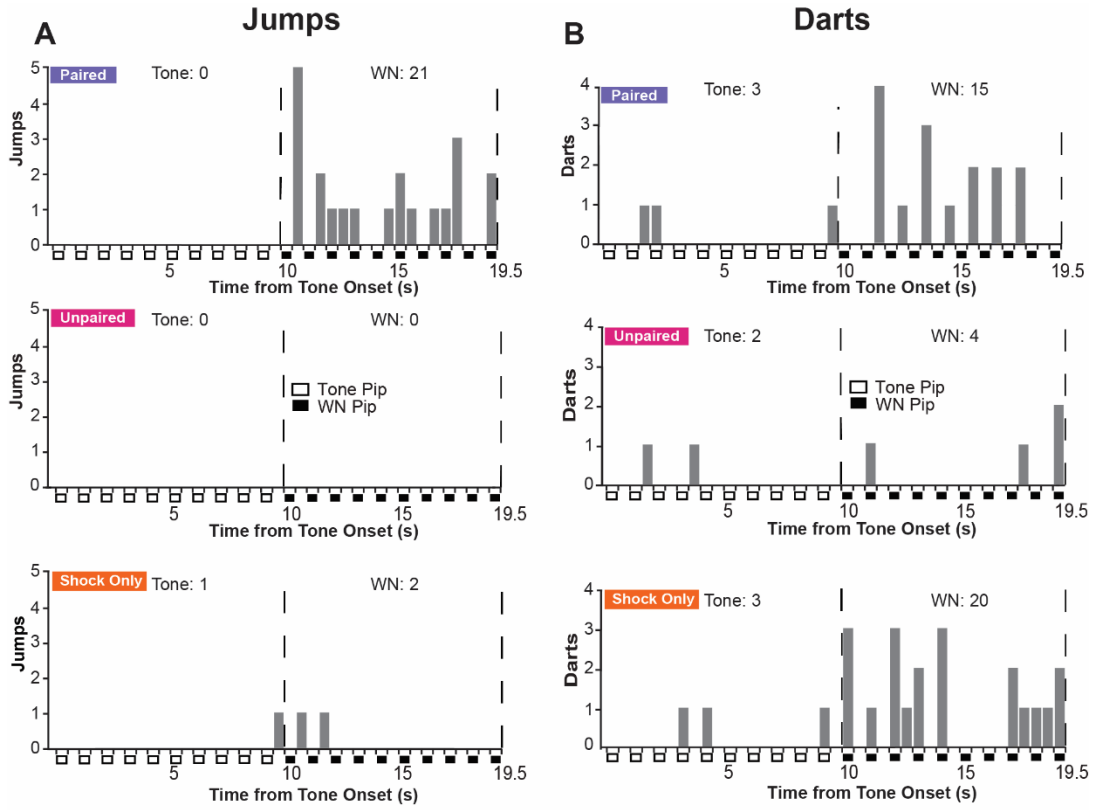
Figure 7. Stimulus-evoked escape jumping and darting during extinction. **A,** The cumulative distribution of jumps from the first 4 trials of Ext1 for 20 randomly selected subjects from the PA group (*top*), the UN group (*middle*), and the SO group (*bottom*). Empty boxes represent each 0.5 s pip of 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.

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.**
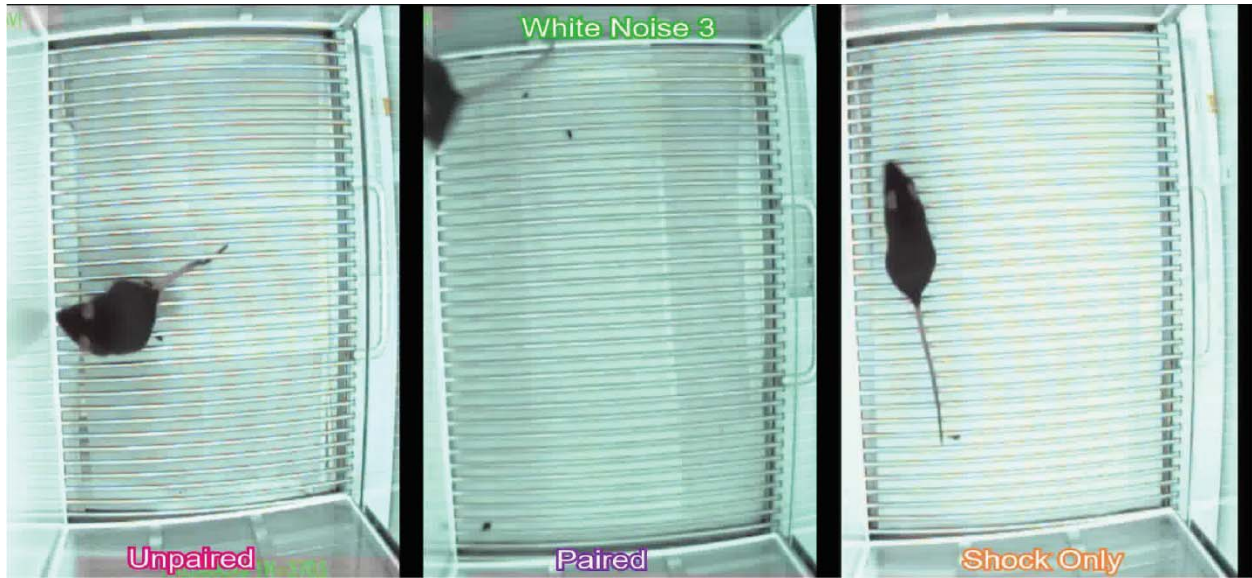
Video 3. Representative comparison of PA, UN, and SO groups' responses to the SCS during an early and late extinction trial of the first extinction session. The video features audio of the SCS, which consists of 10 pips of tone followed by 10 pips of WN.


Video 4. Representative comparison of PA-R and UN-R groups' responses to the reverse SCS during an early and late extinction trial of the first extinction session. The video features audio of the reverse SCS, which consists of 10 pips of WN followed by 10 pips of tone.

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552

Tail rattling is a non-associative behavioral response during extinction

554   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

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**).

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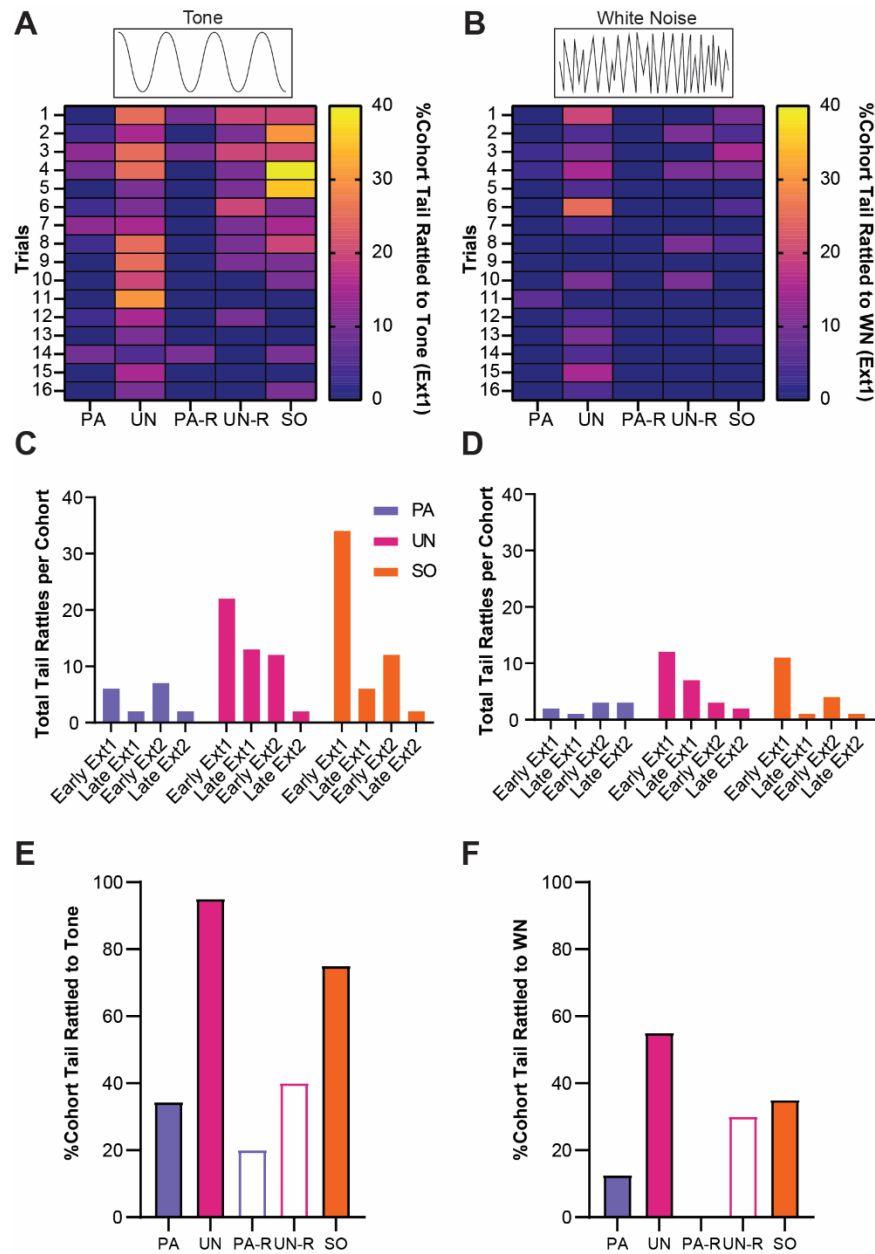
Figure 8. Tail rattling is a non-associative behavioral response during extinction. **A,** The percentage of each group that exhibited tail rattling to the tone. **B,** The percentage of each group that exhibited tail rattling to the WN. **C,** Cumulative tail rattling during tone across early and late 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.

577   These data suggest that tail rattling during the SCS is mostly a non-associative

578   defensive response that is suppressed when the SCS predictably signals threat.

579   **Discussion**

580   This study investigated the contributions of associative and non-associative processes to

581   the expression of cue-induced defensive behaviors. The results signify that associative pairings

582   and a proximal stimulus-threat association during fear conditioning produce maximal expression

583   of cue-induced freezing and flight responses. Non-associative elements such as cue salience,

584   change in stimulus intensity, and shock-induced sensitization elicit stress-associated behaviors

585   like tail rattling and activity bursts, as hypothesized before (Trott et al., 2022), but the addition of

586   the WN-threat associative pairing contributes significantly to eliciting high-intensity defensive

587   responses like escape jumping. Therefore, these associative and non-associative factors

588   combine to produce distinct behavioral transitions between freezing and flight.

589   During conditioning, we observed distinct ethograms for the PA and UN groups in

590   response to the SCS. Freezing to tone and activity to WN were both significantly higher in the

591   PA group compared to the UN group (**Fig 2E, K**), highlighting the impact of SCS-shock

592   contingency on the magnitude of defensive responses. Notably, comparable results were

593   previously reported in rats conditioned using similar parameters (Totty et al., 2021). Additionally,

594   we found that the PA and PA-R groups showed significant increases in freezing from pre-SCS

595   to tone, while in the UN and UN-R groups, freezing to tone was no greater than contextual

596   freezing, suggesting that the paired groups placed associative value on the tone (**Fig 2J**).

597   Lastly, the PA group displayed much more intense flight behavior during WN than the PA-R

598   group (**Fig 2L**), indicating that the proximity of WN to the footshock threat affects defensive

599   scaling.

600   Previous studies have factored jumping behaviors into normalized measures to gauge

601   conditioned flight behavior (Fadok et al., 2017; Hersman et al., 2020; Borkar et al., 2020), but

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602   given the increased activity indices in the PA, UN, and UN-R groups, we examined if the escape

603   jumping we observed was associative or non-associative (**Fig 3**). We found that the PA group

604   exhibited consistent jumping responses to the WN stimulus during conditioning that were not

605   reproduced in the UN, PA-R, or UN-R groups. Others have found that a salient stimulus is

606   sufficient to induce conditioned jumping responses after multiple sessions (Furuyama et al.,

607   2023). Given that the PA-R group did not display consistent jumping behavior to WN or tone,

608   this suggests that a robust jumping response to WN in the SCS paradigm is not due to the non-

609   associative salience of the WN as others have suggested (Hersman et al., 2020) but is instead

610   due to its association with imminent threat.

611   Darting has been reported to be increased in rodents that undergo stress and fear

612   conditioning, and it is more prevalent in female rats (Gruene et al., 2015, Brzozowska et al.,

613   2017). Previous studies have measured darting to WN as a darts per minute measure (Morena

614   et al., 2021; Hoffman et al., 2022; Trott et al., 2022) or as part of a composite escape score

615   (Hersman et al., 2020), but our data suggests that examining darting requires a more detailed

616   analysis. We found that the PA group exhibited darting across the entire WN period during

617   conditioning, while the response was virtually nonexistent in the UN, PA-R, and UN-R groups

618   (**Fig 4**). This is consistent with findings where conditioned darting has been shown to occur

619   more often several seconds after a CS, rather than at its onset (Mitchell et al., 2023). This

620   indicates that darting during conditioning is not caused by the salience of the tone-WN

621   transition, but from an associative learned response. Additionally, the absence of darting in the

622   PA-R group indicates that the temporal proximity of the WN to the shock also influences darting.

623   Our data show that the control groups engage primarily in simple locomotor behaviors induced

624   by stimulus salience (**Fig 4J**). Overall, darting behaviors during conditioning contribute to the

625   higher activity score observed during WN in the PA group, and our data suggests that these

626   contributions are the result of associative learning and threat imminence, rather than non-

627   associative stimulus salience.

628   During extinction, the PA and PA-R groups showed the highest level of freezing to tone

629   compared to the non-associative control groups (**Fig 5A, B**). This indicates that the paired

630   groups had the strongest association between the tone stimulus and threat. This is further

631   reinforced by the larger difference between tone-evoked freezing and pre-SCS freezing in the

632   PA and PA-R groups (**Fig 5E, F**). Freezing during tone in the non-associative control groups, on

633   the other hand, is no greater than freezing in the interstimulus intervals. Interestingly, tone-

634   evoked freezing in the PA group underwent within-session freezing, while that of the PA-R

635   group was resistant to extinction (**Fig 5A-D**). The sustained tone-evoked freezing over multiple

636   extinction sessions in the PA-R group is likely a product of the temporal proximity of the cue to

637   the footshock, which is set at a greater intensity (0.9 mA) in the conditioned flight paradigm than

638   is traditionally used in Pavlovian threat conditioning (typically 0.2-0.6 mA). Because freezing

639   during the tone is not elevated over contextual freezing levels in the unpaired and SO groups,

640   the reductions in freezing during the tone during extinction in these groups can be attributed to

641   reduced freezing overall.

642   In response to WN during extinction, the PA group transitioned from explosive circa-

643   strike flight responses containing escape jumps to a combination of anticipatory post-encounter

644   freezing and darting (**Fig 6A, Fig 7**). This likely reflects a larger perceived psychological

645   distance from threat that influences defensive strategy (Perusini and Fanselow, 2015). The

646   control groups showed only slight changes in WN-evoked activity over extinction, similar to

647   responses of stressed mice to an unfamiliar WN (**Fig 4**; Hoffman et al., 2022). The lack of

648   decrease in WN-evoked activity over extinction in the PA-R group compared to the PA group,

649   and the lack of the control groups transitioning to freezing behavior, suggests that WN-evoked

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650   flight in the PA group is associative and is dependent on the perceived threat value of the WN

651   stimulus.

652   An examination of jumping and darting behaviors over the first extinction session reveals

653   a distinct ethogram in the PA group (**Fig 7**). The presence of jumping during early extinction

654   trials in the PA group indicates that the WN initially signaled imminent threat, resulting in an

655   explosive circa-strike escape response. However, the change from jumping to darting may

656   reflect a change in perceived threat imminence in the same vein as the observed change from

657   flight to freezing. This phenomenon was only observed in the PA group, indicating that this

658   behavioral change is associative and is consistent with the predatory imminence continuum.

659   This jumping behavior to WN is associative and can be extinguished, making it a suitable

660   measure for future studies interested in how the nervous system controls experience-dependent

661   high-intensity fear reactions.

662   The elevated activity indices from the UN and SO groups are similar to other studies that

663   report heightened activity to WN after multiple footshocks, or a sudden change in stimulation

664   (Hoffman et al., 2022; Trott et al.,2022). However, given the lack of darting and jumping from the

665   UN group during extinction (**Fig 7A, B**), their increased activity is due to other locomotor

666   behaviors unrelated to flight. The SO group maintained a consistent level of darting throughout

667   the first extinction session, which contributed to their overall increased activity index (**Fig 7B, D**).

668   Given that darting can be elicited in stressed mice (Brzozowska et al., 2017), it is probable that

669   shock sensitization can prime an animal to dart more readily to an unfamiliar, but highly salient,

670   stimulus upon stimulus transition. Indeed, darting behavior has been shown to change based on

671   multiple parameters, decreasing both with increased shock intensity (Mitchel et al., 2023) and

672   with prolonged extinction training (Demars et al., 2022), suggesting that darting is both an

673   associative conditioned response to stimuli associated with threat and a response resulting from

674   non-associative sensitization.

39

675   Within the UN, UN-R, and SO control groups, we observed a higher degree of tail rattling

676   responses during extinction compared to the PA and PA-R groups, and tail rattling was more

677   prominent during the tone period (**Fig 8**). Tail rattling has been observed in mice when

678   determining hierarchical relationships (Haber and Simmel 1976, Terranova et al., 1998,

679   Dorofeikova et al., 2023), anticipating fighting (Miczek et al., 2001), and encountering looming

680   threat (Salay et al., 2018). Thus, tail rattling may be a behavior elicited in stressed mice in the

681   face of uncertain threat. Previously, we found that tail rattling occurs most often during early

682   trials of fear conditioning, with a prominent decline later in conditioning (Borkar et al., 2020).

683   This is consistent with our results from the UN, UN-R, and SO groups, who displayed greater

684   tail rattling to unpaired (UN, UN-R groups) or novel (SO group) SCS presentations within the

685   conditioning context, with reductions in tail rattling over the course of extinction exposure. Taken

686   together, this suggests that tail rattling is not a behavior exhibited during post-encounter or

687   circa-strike levels of threat, but rather within stressful scenarios where danger is uncertain but

688   anticipated. Future studies that are interested in measuring defensive responses to threat

689   signals such as context, odor, or innately aversive sensory stimuli should consider measuring

690   tail rattling as a marker of non-associative anticipatory fear.

691   Current studies are investigating behaviors beyond freezing within classical Pavlovian

692   conditioning paradigms (Tryon et al., 2021; Laine et al., 2022), but responses like jumping and

693   darting are not always reliably elicited during CS presentation (Colon et al., 2018; Akmese et al.,

694   2023; Biddle and Knox, 2023). Using the SCS flight conditioning paradigm, we elicit a robust

695   continuum of consistent associative defensive responses during CS presentation that are

696   seldom observed within classical Pavlovian conditioning. While behaviors like darting and tail

697   ratting can occur due to non-associative stimulus-inherent properties, transitions between

698   freezing and jumping are robustly present when associative factors signal threat imminence.

699   Future studies can utilize this paradigm to investigate neuronal mechanisms that contribute to

700　threat association and direct dynamic responses to threat, with important implications for

701　developing new treatments for those that suffer from fear and anxiety disorders.

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809 **Conflict of interest statement**: The authors declare no competing financial interests.