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# Behavioral and brain mechanisms mediating conditioned flight behavior in rats

Michael S. Totty<sup>1</sup>, Naomi Warren<sup>1</sup>, Isabella Huddleston<sup>1</sup>, Karthik R. Ramanathan<sup>1</sup>, Reed L. Ressler<sup>1</sup>, Cecily R. Oleksiak<sup>1</sup>, and Stephen Maren<sup>1,2</sup>

<sup>1</sup>*Department of Psychological and Brain Sciences and Institute for Neuroscience, Texas A&M University, College Station, TX, 77843-3474, USA*

<sup>2</sup>*Corresponding author*

Email: [maren@tamu.edu](mailto:maren@tamu.edu)  
Phone: 979-458-7960  
Address: Stephen Maren  
Department of Psychological and Brain Sciences  
Texas A&M University  
301 Old Main Dr.  
College Station, TX 77843-3474

*ORCID*

Michael S. Totty: <https://orcid.org/0000-0002-9292-8556>  
Karthik R. Ramanathan: <https://orcid.org/0000-0002-8843-3665>  
Reed L. Ressler: <https://orcid.org/0000-0003-0514-8269>  
Cecily R. Oleksiak: <https://orcid.org/0000-0001-5363-3467>  
Stephen Maren: <https://orcid.org/0000-0002-9342-7411>

38 **ABSTRACT**

39 Environmental contexts and associative learning can inform animals of potential threats, though it  
40 is currently unknown how contexts bias defensive transitions. Here we investigated context-  
41 dependent flight responses in the Pavlovian serial-compound stimulus (SCS) paradigm. We show  
42 here that SCS-evoked flight behavior in male and female rats is dependent on contextual fear.  
43 Flight was reduced in the conditioning context after context extinction and could be evoked in a  
44 different shock-associated context. Although flight was exclusive to white noise stimuli, it was  
45 nonetheless associative insofar as rats that received an equal number of unpaired USs did not show  
46 flight-like behavior. Finally, we found that inactivation of either the central nucleus of the  
47 amygdala (CeA) or bed nucleus of the stria terminalis (BNST) attenuated both contextual fear and  
48 flight responses. This work demonstrates that contextual fear summates with cued and innate fear  
49 to drive a high fear state and freeze-to-flight transitions.

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## 61 INTRODUCTION

62 The selection of appropriate defensive behavior is vital to survival in the face of threat. Associative  
63 learning allows animals and humans to adapt their behavior to avoid predicted danger, and  
64 environmental contexts are critical for discriminating between fear and safety. Traumatic events  
65 can lead to pathological fear and the dysregulation of contextual processing appears to be central  
66 to various psychopathologies, such as post-traumatic stress disorder (PTSD) (Liberzon and  
67 Abelson, 2016; Liberzon and Sripada, 2008; Maren et al., 2013). For example, context processing  
68 deficits in patients with PTSD can lead to the overgeneralization fear (Grillon and Morgan, 1999;  
69 Jovanovic et al., 2012; Kaczkurkin et al., 2017; Lis et al., 2020; Morey et al., 2020, 2015; Orr et  
70 al., 2000), deficits in the extinction of fear (Blechert et al., 2007; Jovanovic et al., 2012; Milad et  
71 al., 2009; Norrholm et al., 2011; Rougemont-Bücking et al., 2011; Steiger et al., 2015; Wicking et  
72 al., 2016), and the renewal of extinguished fear in safe contexts (Garfinkel et al., 2014). This  
73 suggests that a complete understanding of how contexts regulate conditioned defensive behavior  
74 is essential to identifying neural circuits relevant to fear and anxiety disorders.

75 Pavlovian fear conditioning has been used for decades to model aversive learning and  
76 memory in rodents. Previous work has revealed that contexts can both directly elicit conditioned  
77 fear responses and also act as occasion setters to gate the retrieval of aversive memories (Bouton,  
78 2002; Fraser and Holland, 2019; Maren et al., 2013). In both cases, fear responses in rodents  
79 typically manifest as defensive freezing behavior, which is used as the primary metric of  
80 conditioned fear in rodents. Predatory imminence theory posits that defensive behavior scales with  
81 threat proximity on a spatiotemporal scale such that freezing behavior is seen in post-encounter  
82 modes (once threat has been realized) whereas flight behavior is part of the circa-strike defensive  
83 mode (when threat is proximal) (Fanselow and Lester, 1988). This has been demonstrated in both

84 humans and rodents using naturalistic predator threats (Mobbs et al., 2007; Yilmaz and Meister,  
85 2013), but it remains unclear whether conditioned threats (such as auditory conditioned stimuli)  
86 can drive circa-strike behavior such as flight. Recently, Fadok and colleagues developed a  
87 modified auditory Pavlovian fear conditioning procedure that uses a serial-compound stimulus  
88 (SCS) to elicit both freezing and flight defensive modes in mice (Fadok et al., 2017). The SCS is  
89 comprised of a pure tone stimulus immediately followed by a white noise stimulus that elicits two  
90 conditioned responses (CRs): freezing and flight behavior, respectively. Moreover, they show that  
91 the switch between freezing and flight behavior is gated by microcircuitry within the central  
92 nucleus of the amygdala (CeA), a structure critical to the expression of Pavlovian CRs (Janak and  
93 Tye, 2015; Killcross et al., 1997). Interestingly, in the SCS paradigm flight behavior is normally  
94 limited to the conditioning chamber and freezing dominates when the SCS is presented in a  
95 different context. This procedure presents a unique opportunity to investigate mechanisms by  
96 which context and associative memory may scale between freezing and flight defensive modes.

97         In order to fully reveal the neural circuits that may underly pathological fear, we must  
98 understand the distinct neural circuits that underly various defensive modes and how they may be  
99 gated or modulated by context (Mobbs et al., 2020). Here we sought to determine the behavioral  
100 and neural mechanisms mediating the influence of context on the expression of defensive  
101 behaviors to an SCS. One possibility is that context serves as an occasion setter and promotes SCS-  
102 evoked flight behavior in the conditioning context, but not in other contexts. Another possibility  
103 is that direct context-US associations produce fear that summates with that to the SCS to elevate  
104 threat imminence thereby yielding flight. The occasion setting hypothesis predicts that flight would  
105 be specific to the conditioning context and would not be expressed elsewhere, whereas the  
106 summation hypothesis predicts that flight would be evoked in any shock-associated context,

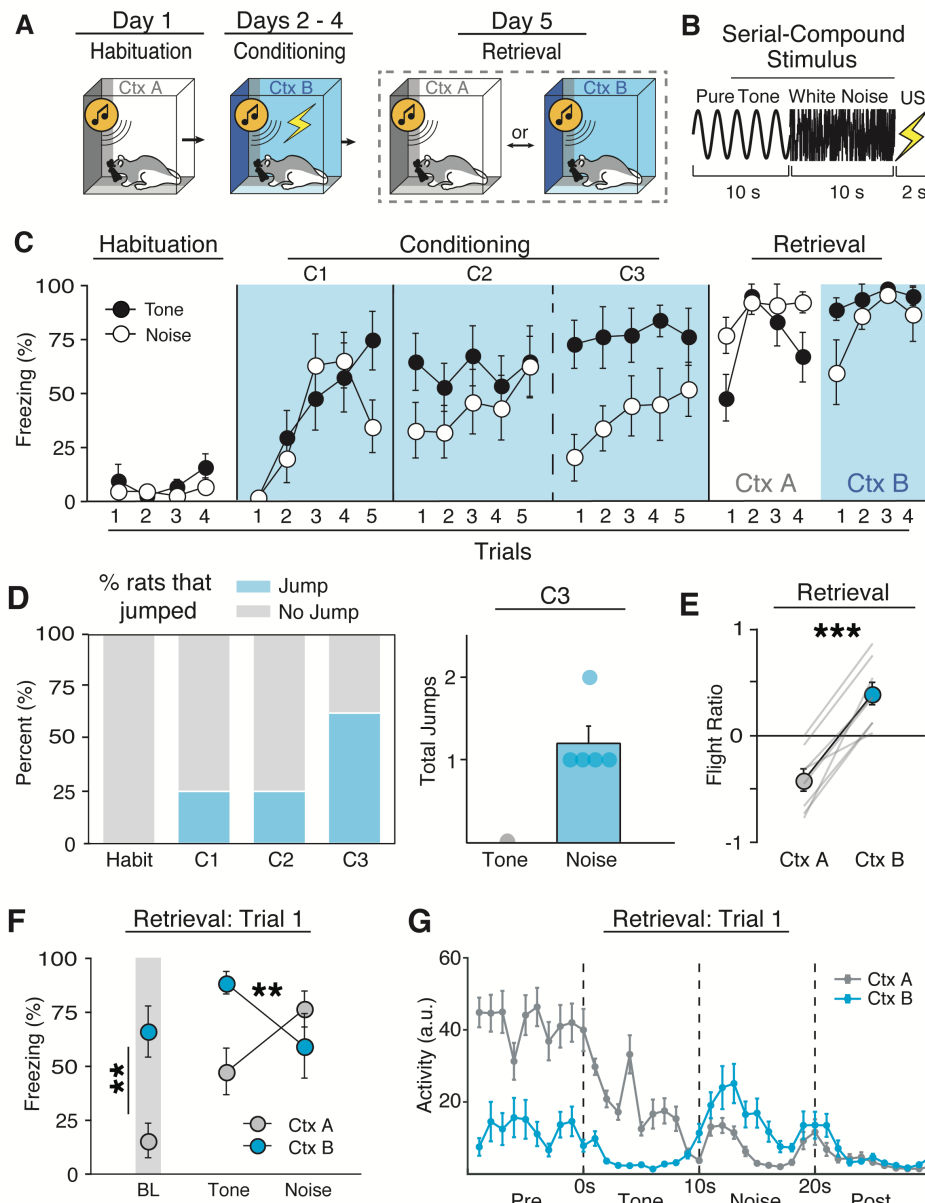
107 regardless of whether it had hosted SCS-shock trials. In a series of experiments to test these  
108 competing hypotheses, we found that rats displayed flight behavior when the SCS was presented  
109 in a US-associated context different than the conditioning context. Moreover, extinguishing fear  
110 to the conditioning context suppressed flight behavior in that context. We further provide evidence  
111 that SCS-evoked flight is a conditioned response by showing that flight-like behavior cannot be  
112 explained by sensitization or fear-potentiated startle. Finally, we show that pharmacologically  
113 inactivating either the CeA or BNST, brain regions that are critical to the expression of contextual  
114 fear, reduces flight-like behavior. We thus argue that SCS-evoked flight behavior is a high fear  
115 state driven by the summation of cued, contextual, and innate fear.

116

## 117 **RESULTS**

### 118 **A conditioned serial-compound stimulus evokes flight behaviors in rats.**

119 Previous work shows that SCSs can evoke flight behavior in mice, but it is unknown if this  
120 behavior occurs in rats. Therefore, we first sought to determine if rats show flight-like behavior to  
121 an SCS using the behavioral protocol first described by Fadok and colleagues (Fadok et al., 2017).  
122 In this procedure (**Figure 1A**), rats were first habituated to four SCS presentations (tone→white  
123 noise; each stimulus consisted of 10 sec trains of 500 ms pips with an inter-pip interval of 500 ms)  
124 in context A (Day 1), then conditioned with five SCS-US presentations for the next three days in  
125 context B (Days 2-4), and finally tested with four SCS-alone presentations in both context A and  
126 B (Days 5 and 6; counterbalanced). For this experiment, we quantified 1) freezing as a percentage  
127 of time, 2) average motor activity, as well as 3) the number of jumps (all four paws leaving the  
128 floor) and darts (rapid movement from one position to another) during both tone and noise  
129 components of the SCS. Freezing and activity were quantified automatically



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131 **Figure 1.** Rats display context-dependent flight-like behavior to a conditioned SCS. **A**, Schematic of the behavioral  
 132 design used in Experiment 1. **B**, Schematic representation of the serial-compound stimulus (SCS). **C**, Average freezing  
 133 data for tone and noise stimuli during each SCS presentation during Habituation, Conditioning, and Retrieval. Rats  
 134 showed lower freezing to the noise on the second and third day of conditioning. **D**, Percentages of rats that showed at  
 135 least one jump during an SCS for each respective day of behavioral testing. Of the rats that showed at least one jump,  
 136 jumps were exclusive to noise stimuli (C3). **E**, Average flight ratio in which positive numbers represent increased  
 137 movement to the noise relative to tone, whereas negative numbers represent decrease activity relative to tone. Rats  
 138 displayed flight like behavior when tested in the conditioning, but not habituation context. **F**, Average freezing data  
 139 during Retrieval shows that rats tested in the conditioning context showed high freezing during baseline and the first  
 140 tone presentation but decrease to the noise, whereas rats tested in the habituation context showed low freezing to  
 141 baseline and the tone which increase to the noise. **G**, Averaged motor activity data from 10s before SCS onset to 10s  
 142 after SCS offset in both the Habituation (Ctx A) and Conditioning (Ctx B) contexts. All data are represented as mean  
 143  $\pm$  SEM; \*, \*\*, and \*\*\* denotes  $p < .05$ ,  $p < .01$ , and  $p < .001$ , respectively.

144 online by digitizing voltages emitted by force transducers under each chamber; jumps and darts  
145 were scored offline from video recordings of the sessions by observer's blind to the experimental  
146 conditions.

147 As shown in **Figure 1C**, prior to conditioning, SCS presentations produced low levels of  
148 freezing and there was no difference in stimulus type on either freezing [ $F(1, 6) = 1.31, p = .295$ ]  
149 or activity [ $F(1, 6) = 2.57, p = .160$ ]. All rats showed increased freezing behavior throughout the  
150 first day of conditioning [main effect of trial,  $F(4, 24) = 8.94, p < .0001$ ]. Although there was no  
151 main effect of stimulus type [ $F(1, 6) = 1.91, p = 2.16$ ], there was a trial x stimulus type interaction  
152 [ $F(4, 24) = 3.33, p = .026$ ] with noise producing a decrease in freezing relative to the tone stimulus  
153 on the last trial of the first conditioning session (**Figure 1C**). This suggested that noise onset was  
154 associated with a reduction in freezing. Indeed, on the second day of conditioning rats showed less  
155 freezing to the noise CS [ $F(1, 6) = 10.12, p = .019$ ], which was mirrored by an increase in activity  
156 [ $F(1, 6) = 15.27, p = .008$ ]. Although the rats displayed a clear switch in defensive behavior upon  
157 noise onset, the number of jumps to the noise were low with only ~25% of rats displaying at least  
158 one jump (**Figure 1D**). During this third and final conditioning session, all rats showed an even  
159 greater decrease in freezing and increase in activity upon noise onset [Freezing: main effect of  
160 stimulus type,  $F(1, 6) = 52.23, p = .0004$ ; Activity: main effect of stimulus type,  $F(1, 24) = 41.15,$   
161  $p = .001$ ], and ~60% of the rats performed at least one jump. Importantly, jumps were nearly  
162 exclusive to noise presentations, with only three total jumps observed during tone presentations  
163 across all three days of conditioning. Despite a clear increase in activity, rats emitted only a small  
164 number of total jumps with only one rat jumping multiple times during the third conditioning  
165 session (**Figure 1D**).

166           Recent reports have noted that aversive CSs can elicit darting behavior, particularly in  
167 female rats (Gruene et al., 2015). However, we seldomly observed darting behavior to the SCS in  
168 male or female rats (3 or less total darts across all animals each day). Moreover, there were no sex  
169 differences in freezing [main effect:  $F(1, 6) = .769, p = .414$ ] or activity [main effect:  $F(1, 6)$   
170  $= .598, p = .469$ ] evoked by the SCS across conditioning. In summary, a conditioned SCS elicits a  
171 clear switch from freezing to activity with infrequent jumps in both male and female rats. It appears  
172 that an increase in motor activity (and decrease in freezing) is the dominant mode of SCS-evoked  
173 flight-like behavior in rats, compared to frequent jumping previously observed in mice (Fadok et  
174 al., 2017; Hersman et al., 2020). We will therefore use white noise-evoked decreases in freezing  
175 from here on as the primary metric for flight behavior in rats.

176

#### 177 **SCS-evoked flight behavior in rats is context-dependent.**

178 Next, we sought to determine if flight behavior in rats is context-dependent as previously shown  
179 in mice (Fadok et al., 2017). In previous work, flight behavior was observed in the conditioning  
180 context, but not when the SCS was presented in the habituation context. To test this, conditioned  
181 rats were placed into either the habituation or conditioning context (Ctx A and B, respectively)  
182 and presented the SCS four times without the US. Although there was no difference in overall  
183 freezing between contexts [ $F(1, 6) = .95, p = .367$ ] or stimulus type [ $F(1, 6) = .14, p = .720$ ], rats  
184 displayed a clear decrease in freezing upon noise onset in the conditioning context (**Figure 1C &**  
185 **F**), whereas they instead froze more to the noise presentation in the habituation context [context X  
186 stimulus type interaction,  $F(1, 6) = 17.82, p = .006$ ]. This was again mirrored by activity levels  
187 [ $F(1, 6) = 14.12, p = .009$ ]. In other words, rats displayed an increase in activity upon noise onset  
188 in the conditioning context, but not the habituation context (**Figure 1G**). There was no main effect



189 of sex for either freezing [ $F(1, 6) = .434, p = .535$ ] or activity levels [ $F(1, 6) = .770, p = .414$ ].  
190 Interestingly, flight only occurred during the first trial of retrieval testing and rats froze at high  
191 levels for the remainder of the test trials [main effect of trial,  $F(3, 18) = 8.25, p = .001$ ]. This is  
192 reminiscent of the rapid extinction of flight behavior previously reported in mice (Fadok et al.,  
193 2017).

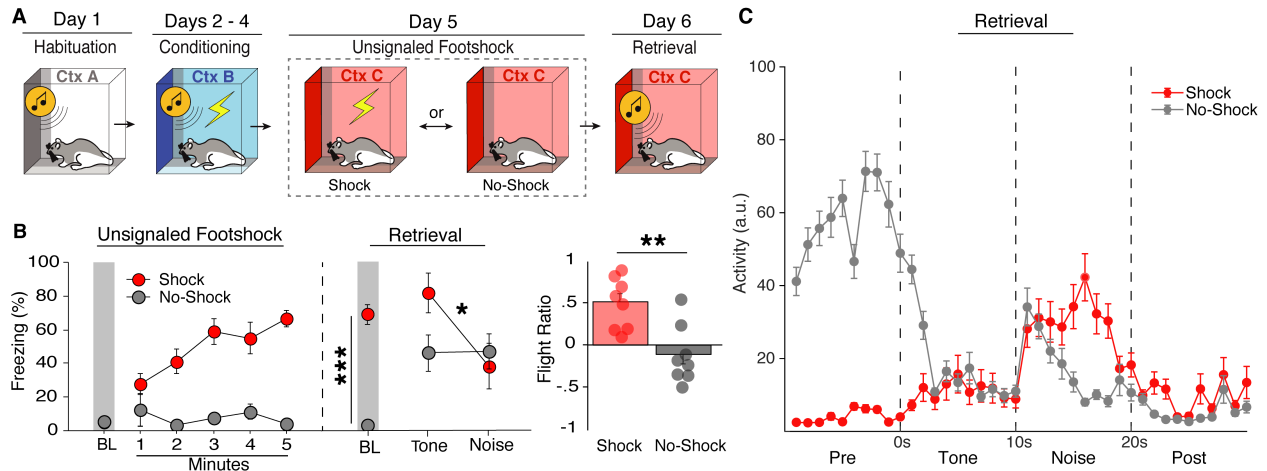
194 **Figure 1G** shows that when tested in the conditioning context rats exhibited low levels of  
195 activity to the tone but increased their activity upon white noise onset. However, in the habituation  
196 context, rats exhibited low levels of activity throughout the duration of the SCS. To further  
197 quantify this, we computed a “flight ratio”, which was the ratio of the difference of noise and tone  
198 load-cell activity to the sum of noise and tone load-cell activity for the first retrieval test trial  
199 (further described in the methods). This metric spans a scale from -1 to 1 whereby increased  
200 activity during noise relative to tone is represented as positive values and decreased relative  
201 activity is represented as negative values. As shown in **Figure 1E**, flight ratios were greater to the  
202 noise compared to the tone in the conditioning context relative to the habituation context [ $F(1, 6)$   
203 = 86.26,  $p < .0001$ ]. There was once again no main effect of sex [ $F(1, 6) = .041, p = .911$ ]. This  
204 shows that the SCS-driven flight behavior observed in rats is limited to the conditioning context.  
205 Collectively, these results demonstrate that a conditioned SCS drives flight-like behavior in rats  
206 manifest as a reduction in freezing punctuated by infrequent jumping behavior, and this pattern of  
207 responding to the SCS was context-dependent, as has previously been reported in mice.

208

### 209 **Flight-like behavior depends on context-US associations.**

210 We next investigated what properties of the test context gates flight behavior. One possibility is  
211 that context serves as an occasion setter, informing the animal about the SCS-US association in

212 the conditioning context. Alternatively, flight may be driven by a high fear state resulting from  
 213 the summation of SCS-US and context-US associations. To discriminate among these possibilities,  
 214 we explored whether conditioned flight would be expressed in an excitatory context that had never  
 215 hosted SCS-US trials (i.e., a context in which animals experienced unsignaled shocks).  
 216



217 **Figure 2.** Flight-like behavior depends on context-US associations. **A**, Behavioral design for Experiment 2. **B**,  
 218 Average freezing data shows rats that received unsignaled footshocks (Shock) froze at high levels, whereas rats that  
 219 were merely exposed to the context (No-Shock) froze at low levels. For Retrieval testing, Shock animals showed  
 220 higher baseline freezing and a decrease in freezing upon white noise onset whereas No-Shock animals showed low  
 221 baseline levels and remained freezing at moderate levels throughout the SCS. **C**, Average flight ratio shows that rats  
 222 that Shock animals showed a bigger flight response than No-Shock animals. **D**, Averaged activity data during the first  
 223 trial of Retrieval for Shock and No-Shock animals. All data are represented as mean  $\pm$  SEM; \*, \*\*, and \*\*\* denotes  
 224  $p < .05$ ,  $p < .01$ , and  $p < .001$ , respectively.  
 225  
 226

227 To this end, rats first underwent habituation and conditioning as previously described  
 228 (**Figure 2A**). There was once again very little freezing to the SCS prior to conditioning and no  
 229 difference in stimulus type [ $F(1, 12) = 1.607$ ,  $p = .229$ ]. Rats displayed increased freezing across  
 230 conditioning sessions [main effect of day:  $F(2, 24) = 9.809$ ,  $p = .0008$ ] as well as noise-elicited  
 231 decreases in freezing [day X stimulus type interaction:  $F(1, 12) = 14.109$ ,  $p < .0001$ ]. Next, to test  
 232 if flight depends on a context-US association, rats were separated into two groups that would either  
 233 receive five unsignaled USs in a novel context (context C) or would merely be exposed to the same

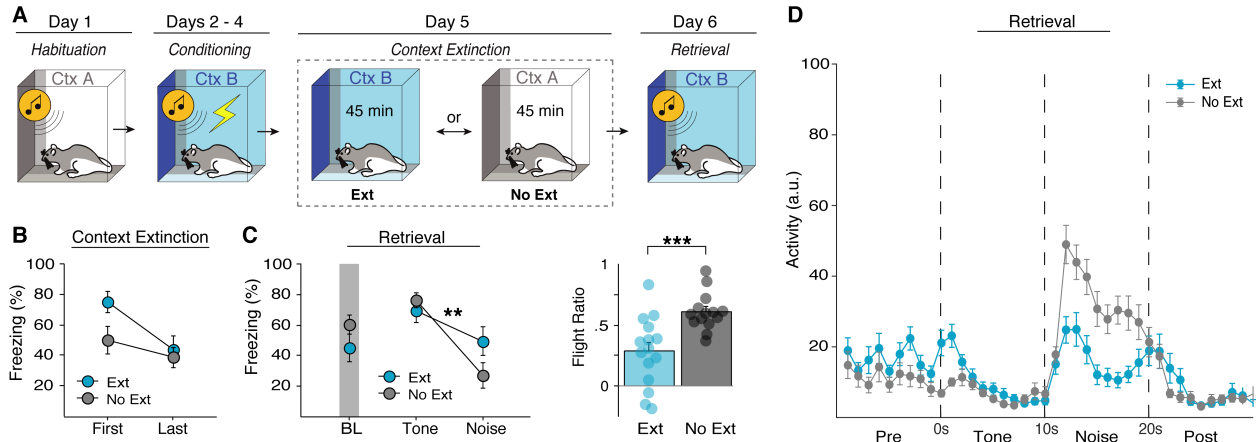
234 context for an equal amount of time (**Figure 2A, Day 5**). As shown in **Figure 2B**, rats that received  
235 unshocked USs (Shock group) showed increased freezing across the session, whereas rats that  
236 were not shocked (No-Shock group) froze at low levels [minutes X group interaction:  $F(5, 60) =$   
237  $11.364, p < .0001$ ].

238 For retrieval testing, all rats were placed back into context C and after a 3-minute baseline  
239 period were presented one SCS-alone trial. Shock animals showed much higher levels of fear to  
240 the context via freezing during the baseline period compared to No-Shock animals [ $F(1, 12) =$   
241  $107.324, p < .0001$ ]. Upon SCS presentation, Shock animals displayed a dramatic switch from  
242 freezing to activity upon noise onset (**Figure 2C**), whereas No-Shock animals decreased freezing  
243 momentarily, but quickly reverted back to freezing [group X stimulus type interaction:  $F(1, 14) =$   
244  $5.928, p = .0289$ ]. This was mirrored by the flight ratio [ $F(1, 12) = 12.926, p = .0037$ ] (**Figure 2B**).  
245 In other words, conditioned animals presented the SCS in a shock-associated context displayed  
246 flight-like behavior, whereas animals tested in a neutral context did not. No sex differences were  
247 seen during retrieval [ $F(1, 12) = .498, p = .494$ ] nor at any other point in this experiment. Thus, by  
248 showing that flight behavior can be evoked in a shock-associated context different from the  
249 original conditioning context, these results demonstrate SCS-driven flight-like behavior depends  
250 on context-US associations rather than occasion setting by the conditioning context. This suggests  
251 that flight to an SCS is driven by a high fear state gated by summation of SCS-US and context-US  
252 association.

253

#### 254 **Extinguishing contextual fear reduces flight-like behavior.**

255 If contextual fear drives flight to an SCS, then extinguishing that fear should reduce flight  
256 behavior. To test this, we habituated and conditioned rats as previously described (**Figure 3A**),



257

258 **Figure 3.** Extinguishing contextual fear reduces flight-like behavior. **A**, behavioral design for Experiment 3. **B**,  
 259 Average freezing data showing that rats that underwent context extinction (Ext) froze at high levels at the beginning  
 260 of extinction which reduced by the end of extinction. Rats that did not undergo extinction (No-Ext) did not show a  
 261 reduction in freezing from the first to last block of context exposure. **C**, Ext animals did not show a significant  
 262 reduction in baseline freezing, but did show a reduced flight response as shown by the reduced flight ratio. **D**, Averaged  
 263 activity data showing that Ext animals showed reduced activity during the white noise stimulus compared to No-Ext  
 264 animals. All data are represented as mean  $\pm$  SEM; \*, \*\*, and \*\*\* denotes  $p < .05$ ,  $p < .01$ , and  $p < .001$ , respectively.  
 265

266 and then extinguished the conditioning context prior to retrieval testing. As in the previous  
 267 experiments all rats showed similarly low levels of freezing to both stimuli prior to conditioning  
 268 [ $F(1, 25) = 1.674, p = .2075$ ], increased freezing across conditioning days [main effect:  $F(2, 50) =$   
 269  $44.685, p < .0001$ ], and displayed noise-elicited decreases in freezing during conditioning [main  
 270 effect:  $F(1, 25) = 69.535, p < .0001$ ]. Of note, female rats in this experiment showed slightly higher  
 271 levels of freezing during habituation [main effect:  $F(1, 25) = 5.208, p = .0313$ ], but no sex  
 272 differences were observed across conditioning days [ $F(1, 25) = .768, p = .3891$ ]. After  
 273 conditioning, rats were either placed back into the conditioning context to extinguish contextual  
 274 fear for 45 min (Ext) or they were exposed to the habituation context for an equal amount of time  
 275 (No-Ext) (**Figure 3A, Day 5**). Although there was not a significant group X time interaction during  
 276 extinction [ $F(1, 27) = 2.994, p = .095$ ], planned comparisons revealed that Ext animals showed a  
 277 significant reduction in freezing [ $F(1, 14) = 16.930, p = .0011$ ], whereas No-Ext animals showed  
 278 stable and lower levels of freezing during the session [ $F(1, 13) = 1.611, p = .2266$ ].

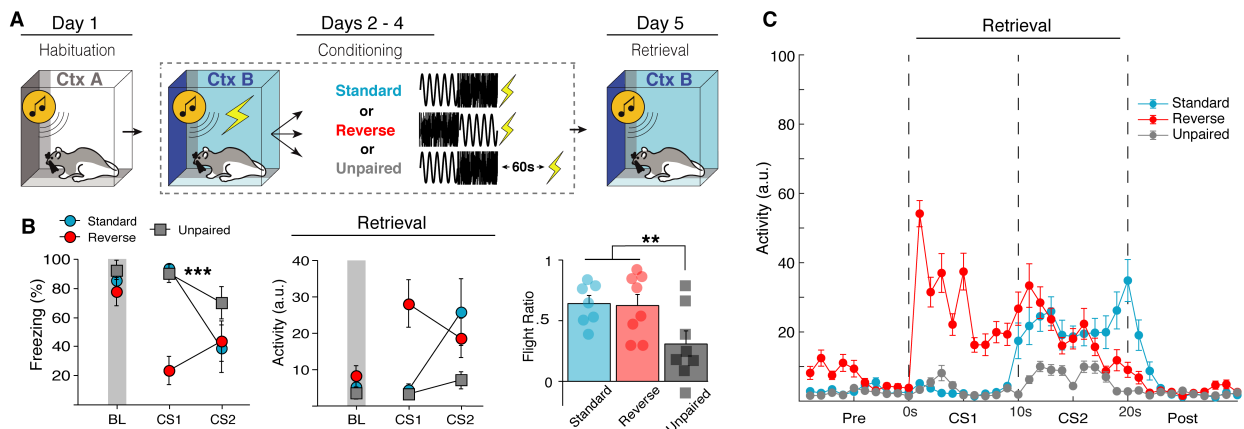
279 For retrieval testing, all rats were placed back into the conditioning context and presented  
280 four SCS-alone trials. Although baseline freezing was similar between groups [ $F(1, 27) = 1.857$ ,  
281  $p = .1843$ ], NoExt rats showed a greater reduction in freezing to noise onset relative to Ext animals  
282 [stimulus type X group interaction,  $F(1, 25) = 15.880$ ,  $p = .0005$ ], which was again mirrored by  
283 changes in activity [ $F(1, 25) = 6.995$ ,  $p = .0139$ ], and flight ratio [ $F(1, 25) = 14.212$ ,  $p = .0009$ ]  
284 (**Figure 3C**). In other words, context extinction reduced flight-like behavior (**Figure 3D**). There  
285 was no main effect of sex for any of these metrics during retrieval testing [ $F(1, 25) = 1.388$ ,  $p$   
286  $= .2498$ ]. These results provide converging evidence that SCS-driven flight-like behavior is driven  
287 by summation of fear to the SCS and conditioning context.

288

### 289 **Flight-like responses in rats are specific to white noise and not due to sensitization.**

290 One outstanding question is whether flight behavior in rats is driven by threat imminence or  
291 stimulus salience. Predatory imminence theory posits that defensive responding scales with threat  
292 proximity, and thus, white noise may elicit flight in the SCS paradigm because it is temporally  
293 proximal to the shock US. However, recent work shows that SCS-elicited flight behavior in mice  
294 is driven by stimulus salience, specifically intensity and high frequency components of white noise  
295 (Hersman et al., 2020). Indeed, this work shows in mice that flight behavior is specific to white  
296 noise regardless of whether it precedes or follows the pure tone component of the SCS. Moreover,  
297 Hersman and colleagues show that flight behavior does not require SCS-US pairings, insofar as an  
298 unpaired SCS-US procedure also produced flight to the SCS. This suggests that sensitization or  
299 pseudoconditioning might contribute to flight to the SCS. We therefore sought to determine  
300 whether the temporal order of the stimuli in the SCS influences the emergence of flight behavior,  
301 and if flight-like behavior in rats occurs after unpaired SCS-US trials. To test this (**Figure 4A**),

302 after habituation animals underwent either a standard SCS-US procedure described thus far  
 303 (Standard), a standard SCS with a 60-s delay before the US (Unpaired), or a reversed order SCS  
 304 (noise-tone) immediately followed by a US (Reversed). All groups were then tested by presenting  
 305 the same SCS that they were conditioned with (either standard or reversed) without US  
 306 presentation in the conditioning context. To accurately reflect freeze-to-flight transitions in this  
 307 experiment the flight ratio was calculated for each group as activity during white noise relative to  
 308 the 10-sec period prior to white noise onset for the first retrieval test trial. Thus, for Standard and  
 309 Unpaired groups the flight ratio is the same as previously described, the ratio of the difference of  
 310 noise and tone load-cell activity to the sum of noise and tone load-cell activity; however, for the  
 311 Reversed group this becomes the ratio of the difference of noise and pre-SCS load-cell activity to  
 312 the sum of noise and pre-SCS load-cell activity.  
 313



314 **Figure 4.** Flight-like responses in rats are specific to white noise and not due to sensitization. **A**, Behavioral design  
 315 for Experiment 4. **B**, Average freezing and activity data showing that flight-like behavior is specific to the white noise  
 316 stimulus regardless of the temporal of order of the SCS. In a Reversed order SCS, rats show a decrease in freezing  
 317 and corresponding increase in activity to the first stimuli (noise) rather than the second (tone). The data additionally  
 318 show that unpairing the SCS and US with a 60-s gap (Unpaired) prevents flight-like behavior compared to Standard  
 319 SCS-US controls. This all further shown by averaged activity time across time (**C**). All data are represented as mean  
 320  $\pm$  SEM; \*, \*\*, and \*\*\* denotes  $p < .05$ ,  $p < .01$ , and  $p < .001$ , respectively.  
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324 Prior to conditioning, there was no main effect of either group [ $F(2, 17) = .858, p = .4416$ ],  
 325 sex [ $F(1, 17) = 2.232, p = .1535$ ], or stimulus type [ $F(1, 17) = .536, p = .4742$ ]; though female rats

326 in the Reversed group did show increased freezing to tone presentations across habituation which  
327 yielded a trial X sex interaction [ $F(3, 51) = 3.777, p = .0160$ ]. All animals showed increased  
328 freezing across conditioning sessions and all groups showed decreased freezing to the noise  
329 relative to the tone, including animals in which the SCS order was reversed [day X stimulus type  
330 X group interaction:  $F(4, 34) = 15.466, p < .0001$ ]. This suggests that flight responses are indeed  
331 specific to white noise, rather than determined by threat proximity. Female rats in this experiment  
332 generally showed higher freezing levels across conditioning [main effect of sex:  $F(1, 17) = 5.509,$   
333  $p = .0313$ ], although no interactions with sex were seen. During retrieval testing, Unpaired animals  
334 showed a reduced flight response to white noise [main effect:  $F(2, 17) = 7.646, p = .0043$ ]  
335 compared to both Standard [ $p = .0103$ ] and Reversed groups [ $p = .0106$ ] (**Figure 4B**). No main  
336 effect of sex was seen [ $F(1, 17) = 1.397, p = .2535$ ]. In summary, we show here that flight behavior  
337 is also exclusive to white noise in rats and suggests that footshock sensitization cannot account for  
338 flight behavior. This reaffirms previous findings reported in mice that innately aversive auditory  
339 stimuli drive flight response in the SCS paradigm, not threat imminence.

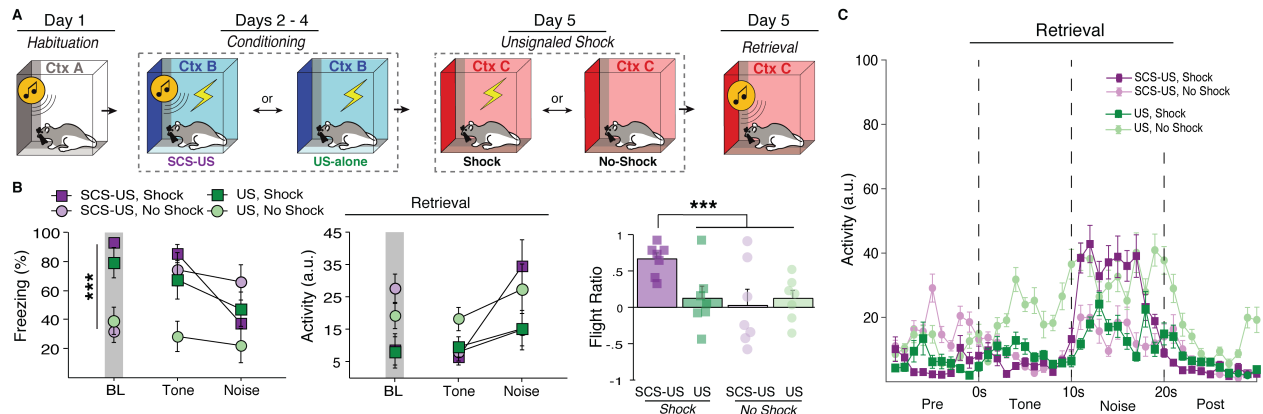
340

#### 341 **An unconditioned SCS fails to evoke flight behavior in a threatening context.**

342 As shown in the previous experiment, flight behavior is specific to white noise and appears to be  
343 driven by stimulus salience. This raises an alternative possibility that SCS-evoked flight may be  
344 nonassociative. White noise is commonly used as an acoustic startle stimulus and it is well known  
345 that a startle response can be potentiated when presented in a shock-associated context, a process  
346 known as fear-potentiated startle (Davis and Walker, 2014; Luyten et al., 2011; McNish et al.,  
347 1997). Although we showed in the last experiment that unpaired SCS/US presentations fail to  
348 produce robust flight behavior, an unpaired CS can come to act as a conditioned inhibitor (i.e.,

349 safety signal) which may have reduced flight behavior (Rescorla, 1969). Thus, we next  
 350 investigated whether an excitatory context might drive a potentiated startle to the noise that could  
 351 account for SCS driven reductions in freezing and concomitant flight behavior.

352



353 **Figure 5.** An unconditioned SCS fails to evoke flight behavior in a threatening context. **A**, Behavioral design for  
 354 Experiment 5. **B**, Averaged freezing and activity data showing that rats that received US-alone trials throughout  
 355 conditioning (US) and tested in a US-associated context (Shock) had a reduced flight response in comparison to rats  
 356 that received SCS-US pairings. This is shown by increased freezing and decreased activity to the white noise stimulus,  
 357 and a reduced flight ratio. Rats that were tested in a neutral context (No-Shock) also showed reduced flight responses  
 358 compared to SCS-US/Shock animals. This is further shown by the average activity trace of each group (**D**). All data  
 359 are represented as mean  $\pm$  SEM; \*, \*\*, and \*\*\* denotes  $p < .05$ ,  $p < .01$ , and  $p < .001$ , respectively.

361  
 362 All animals were first habituated with four SCS-alone trials. No differences were seen between  
 363 groups [ $F(1, 24) = .604$ ,  $p = .445$ ], stimulus type [ $F(1, 24) = 3.205$ ,  $p = .0860$ ], or sex [ $F(1, 24)$   
 364  $= .927$ ,  $p = .4445$ ]; although females showed slightly higher freezing across habituation trials [trial  
 365 X sex interaction:  $F(3, 72) = 2.870$ ,  $p = .0423$ ]. To test if SCS driven flight can be explained by  
 366 fear-potentiated startle (**Figure 5A**), animals either underwent standard SCS-US conditioning or  
 367 received an equal number of unsignaled USs across three consecutive days. The day after  
 368 conditioning, animals were placed into a novel context C where they either received five US-alone  
 369 presentations or context exposure, similar to Experiment 2. Finally, all animals were presented  
 370 SCS-alone trials in context C on the last day of experimentation. This creates a 2x2 design where  
 371 animals were conditioned with either SCS-US or US-alone trials and were subsequently tested in  
 372 either a threatening (Shock) or neutral context (No-Shock). If SCS driven flight is merely a



373 potentiated startle response, we would expect both animals conditioned with SCS-US and US-  
374 alone trials to exhibit flight-like behavior in the shock-associated context.

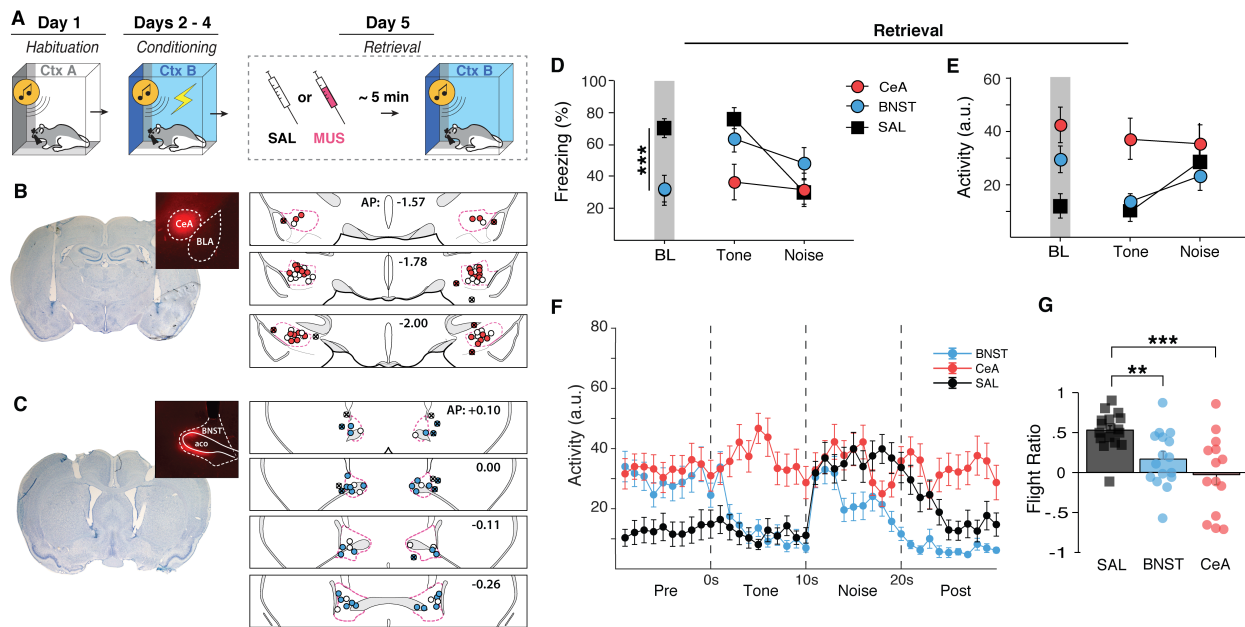
375       During SCS-US conditioning, all animals showed increased freezing across sessions [ $F(2,$   
376  $24) = 70.058, p < .0001$ ] and lower levels of freezing to noise than tone [ $F(1, 12) = 29.132, p$   
377  $= .0002$ ], though the difference in freezing between stimuli were similar across days [day X  
378 stimuli:  $F(2, 24) = 2.784, p = .0818$ ]. Animals that received US-alone trials also showed increased  
379 freezing across sessions [ $F(2, 24) = 22.404, p < .0001$ ]. No sex differences were observed during  
380 conditioning. One day after conditioning, animals either received five unsignaled footshocks in a  
381 novel context C or were exposed to the context for an equal amount of time. Animals that received  
382 footshocks showed an increase in freezing behavior relative to No-Shock animals [ $F(5, 120) =$   
383  $10.762, p < .0001$ ]. Although there was no main effect of sex [ $F(1, 24) = .445, p = .5111$ ], there  
384 was a trial X group X sex interaction [ $F(35, 120) = 2.507, p = .0338$ ], again driven by slightly  
385 higher freezing in female No-Shock animals. On the day of retrieval testing (**Figure 5B**), Shock  
386 animals showed much higher levels of baseline freezing than No-Shock animals [ $F(1, 23) =$   
387  $34.997, p < .0001$ ] and there was no difference in SCS-US and US-alone groups [ $F(1, 23) = .145,$   
388  $p = .7070$ ] or sex [ $F(1, 23) = .064, p = .8025$ ]. At the first SCS presentation, all groups froze at a  
389 high level except for the US-alone/No-Shock group [conditioning X unsignaled shock interaction:  
390  $F(1, 23) = 4.062, p = .0557$ ]. Upon white noise onset, the SCS-US/Shock group showed a dramatic  
391 increase in activity greater than all other groups [flight score main effect:  $F(3, 24) = 3.632, p$   
392  $= .0272$ ], including the US-alone/Shock group [ $p = .0193$ ] (**Figure 5B**). Indeed, the US-  
393 alone/Shock group showed similar flight-like behavior as animals that were never shocked in that  
394 context. We therefore conclude that SCS driven flight behavior does require a direct SCS-US  
395 pairing in this paradigm and thus cannot be attributed to sensitization or fear potentiated-startle.

396 **Muscimol inactivation of the central or extended amygdala attenuates flight behavior**

397 If SCS-evoked flight depends on context-US associations, then inactivating brain regions that are  
398 critical for this process should block flight behavior. The bed nucleus of the stria terminalis  
399 (BNST) has been shown to be critical for the expression of contextual but not cued fear (Davis et  
400 al., 2010; Goode and Maren, 2017; Sullivan et al., 2004), whereas the CeA is critical for both  
401 contextual and cued fear (Janak and Tye, 2015; Maren and Quirk, 2004). Based on this, we  
402 reasoned that inactivation of the either CeA or BNST would block freezing to the conditioning  
403 context and SCS-driven flight responses.

404 All rats were implanted with cannula targeting either the BNST or CeA one week prior to  
405 SCS habituation and conditioning (**Figure 6A-C**). During habituation, there were main effects of  
406 stimulus type [ $F(1, 40) = 5.145, p = .029$ ] and trial [ $F(3, 120) = 3.284, p = .023$ ] with animals  
407 showing increased freezing to tones at the end of habituation. All animals showed increased  
408 freezing to the SCS across conditioning days [ $F(2, 80) = 117.994, p < .0001$ ] with decreased  
409 freezing to the white noise stimulus [main effect of stimulus type:  $F(1, 40) = 356.902, p < .0001$ ].  
410 No sex differences were seen across habituation or conditioning. Immediately prior to retrieval  
411 testing rats received local infusions of either the GABA<sub>A</sub> agonist muscimol (MUS) or saline  
412 (SAL). Inactivation of either the CeA [ $p = .0013$ ] or BNST [ $p = .0012$ ] reduced baseline freezing  
413 relative to SAL controls [ $F(1, 40) = .901, p = .3482$ ], an indication of diminished contextual fear.  
414 CeA animals showed lower freezing to the tone [main effect:  $F(2, 40) = 5.457, p = .008$ ] compared  
415 to both SAL [ $p = .0028$ ] and BNST animals [ $p = .038$ ] (**Figure 6D**). Upon noise onset, there was  
416 not a stimulus type X group interaction [ $F(1, 40) = .901, p = .3482$ ], but planned comparisons  
417 reveal that SAL animals [ $p < .0001$ ], but not CeA [ $p = .6165$ ] or BNST [ $p = .1321$ ], showed a  
418 reduction in freezing behavior (**Figure 6D**). This is reflected in the flight ratios [main effect:  $F(2,$

419 40) = 8.773,  $p = .0007$ ] that show both CeA [ $p < .0001$ ] and BNST inactivation [ $p = .0067$ ] blunted  
 420 flight response (**Figure 6G**). This can further be seen in averaged activity plots (**Figure 6E-F**). No  
 421 sex differences were seen during retrieval. In summary, inactivating either the CeA or BNST was  
 422 sufficient to block both contextual fear and SCS-evoked flight responses. This provides further  
 423 evidence that SCS flight is a high fear state gated by a summation of contextual and cued fear.  
 424



425  
426

427 **Figure 6.** Pharmacological inactivation of either the BNST or CeA disrupts flight-like behavior. **A**, Behavioral  
 428 design for Experiment 6. Histological summary of CeA (**B**) and BNST (**C**) cannula placements with representative  
 429 thionin-stained sections and drug spread with fluorescent muscimol. Labeled anterior-posterior coordinates are  
 430 relative to bregma. **D**, averaged freezing data showing that CeA and BNST animals both showed reduced baseline  
 431 freezing. BNST animals increased freezing to tone presentation and remained at higher freezing levels during white  
 432 noise. CeA animals remained at low levels of freezing during the SCS. **E** and **F**, averaged activity levels during the  
 433 first SCS presentation showing that BNST animals showed a reduced flight response and CeA animals did not  
 434 increase activity from tone to noise at all despite a lack of freezing behavior. This is reflected in the flight ratio (**G**).  
 435 All data are represented as mean  $\pm$  SEM; \*, \*\*, and \*\*\* denotes  $p < .05$ ,  $p < .01$ , and  $p < .001$ , respectively.  
 436

## 437 DISCUSSION

438 Here we investigated context-dependent flight behavior evoked by a serial-compound stimulus  
 439 conditioning procedure in male and female rats. First, we show that context-dependent flight-like

440 behavior can be evoked in rats using the SCS procedure, although jumping was less frequent than  
441 previously reported in mice (Fadok et al., 2018). We further found that flight occurs in any shock-  
442 associated context and that extinguishing contextual fear to the conditioning chamber suppresses  
443 flight-like behavior, demonstrating that SCS-evoked flight behavior reflects the summation of  
444 cued and contextual fear. Although flight is specific to white noise, we found that fear-potentiated  
445 startle and sensitization could not account for SCS-evoked flight. That is, neither unpaired nor  
446 neutral SCS presentations were sufficient to drive flight behavior in a shock-associated context,  
447 even when animals had received an equal number of prior footshocks. Finally, we show that  
448 pharmacological inactivation of brain regions that are critical for the expression of contextual fear,  
449 either the CeA or BNST, is sufficient to block the expression of flight behavior. Together, these  
450 data demonstrate that conditioned flight behavior in the SCS paradigm is driven by a high fear  
451 state via a combination of contextual and cued fear.

452       Until now only mice had been used to study flight responses in the SCS paradigm and it  
453 was unknown if rats would also elicit flight behavior to an SCS (Dong et al., 2019; Fadok et al.,  
454 2017; Hersman et al., 2020). We found that the SCS procedure indeed evoked context-dependent  
455 flight responses similar to reports in mice, although jumping is less frequent in rats. One important  
456 limitation of our study is that we used indirect measures of motion, opposed to other work that  
457 used direct measurements of speed via camera which makes direct comparisons of locomotion  
458 difficult (Dong et al., 2019; Fadok et al., 2017). Despite this, visual comparison of the increase in  
459 activity seen here to those in previous reports appear similar (Dong et al., 2019; Fadok et al., 2017;  
460 Hersman et al., 2020). Additionally, increases in activity and jumping seen here were both specific  
461 to the white noise stimulus. It is currently unclear why rats display infrequent jumps although we  
462 speculate that this may be a species-specific difference.

463 Previous work suggests that female rats display more active defensive behaviors, such as  
464 defensive darting, in response to an aversive CS (Gruene et al., 2015). Based on this we expected  
465 that female rats may be more likely to show flight-like behavior compared to males in the SCS  
466 paradigm. However, we did not observe significant sex differences in SCS-evoked jumping or  
467 activity levels. Darting behavior was seldom observed during SCS presentations in both female  
468 and male rats. This could be due to scoring differences as original reports used an automated  
469 detection method (Gruene et al., 2015). Conversely, other work shows that female mice actually  
470 exhibit increased freezing in the SCS paradigm, though the average speed of male and female mice  
471 during the SCS did not differ (Borkar et al., 2020). We do report here that female rats frequently  
472 show increased freezing during SCS habituation and conditioning, although the effects were small  
473 and not always present across experiments. Assessing divergent defensive strategies in male and  
474 female rats may require machine learning-based behavioral scoring methods (Mathis et al., 2018;  
475 Pereira et al., 2020).

476 As others have shown (Hersman et al., 2020), we found that rats display flight behavior  
477 specifically to white noise, even when the order of the SCS is reversed. We further show that flight  
478 is not due to sensitization and that flight cannot be explained by fear potentiated startle. This  
479 supports previous work demonstrating that stimulus salience determines flight behavior in mice  
480 (Hersman et al., 2020). This previous work specifically shows that it is the high-frequency  
481 component and intensity (>80 dBs) of white noise that evoke flight. Indeed, loud, high-frequency  
482 stimuli appear to innately produce flight behavior in mice. However, it was also shown that  
483 sensitization by previous US presentations actually reduces the frequency of flight behavior due  
484 to increased competition with freezing behavior (Mongeau et al., 2003). So, how do we reconcile  
485 that a direct SCS-US association is necessary to drive flight behavior to a white noise stimulus in

486 rats? We believe that the most parsimonious explanation is that flight in the SCS paradigm is  
487 driven by a high fear state in which a threshold is reached such that a freeze-to-flight transition  
488 occurs. Specifically, SCS flight is driven by the summation of cued, contextual, and innate fear;  
489 although all three are not always necessary to elicit flight behavior. For example, flight in mice  
490 can be evoked innately to loud, high-frequency stimuli (i.e., without conditioned fear) (Mongeau  
491 et al., 2003). Additionally, flight to an SCS can be evoked without a salient high-frequency  
492 component by increasing the intensity above 90 dBs (Hersman et al., 2020). In the SCS paradigm,  
493 auditory stimuli are presented at 75-80 dB which appears to be just below the threshold to innately  
494 evoke flight responses to white noise (Fadok et al., 2017; Hersman et al., 2020). Coupled with our  
495 findings that SCS flight requires both SCS-US and context-US associations, we propose that cued  
496 and contextual fear act in sum with stimulus salience to cause a freeze-to-flight transition in the  
497 SCS paradigm.

498 In line with our behavioral results, we found that reversible inactivation of either the CeA  
499 or BNST is sufficient to disrupt not only contextual fear, but also context-dependent flight  
500 responses in the SCS paradigm. The finding that CeA inactivation disrupted both contextual and  
501 cued fear is supported by decades of work demonstrating that the CeA is critical to the expression  
502 of conditioned responses (Janak and Tye, 2015; Killcross et al., 1997; Ressler et al., 2020).  
503 Moreover, our finding that inactivating the BNST disrupted defensive freezing to the conditioning  
504 context, but not the SCS, is in line with previous work showing that the BNST mediates fear to  
505 unpredictable threats (Davis et al., 2010; Goode et al., 2019, 2015; Goode and Maren, 2017;  
506 Resstel et al., 2008; Sullivan et al., 2004; Walker et al., 2009; Zimmerman and Maren, 2011). In  
507 the original report by Fadok and colleagues, they found that SCS-elicited flight is gated by neurons  
508 in the CeA expressing corticotrophin-releasing hormone (CRH+) that inhibit somatostatin-

509 expressing neurons (SOM+) (Fadok et al., 2017). Stimulation of SOM+ neurons can elicit freezing  
510 behavior in naïve animals (Li et al., 2013; Penzo et al., 2015, 2014; Yu et al., 2016), however,  
511 flight responses evoked by stimulation of CRH+ requires prior conditioning (Fadok et al., 2017).  
512 This raises the possibility that SCS-evoked flight behavior may be evoked indirectly via the  
513 inhibition of CeA-driven freezing behavior, and thus, may not directly require the CeA. We extend  
514 this literature by showing that the CeA is indeed required for SCS-evoked flight behavior by  
515 showing that reversible inactivation CeA attenuates flight responses. Collectively, this findings  
516 detail for this first time how conditioned auditory and contextual fear may sum with stimulus  
517 salience to drive circa-strike behavior.

518         Considering this, what neural structure may then be responsible for the proposed threshold-  
519 like mechanism gating flight behavior? The periaqueductal grey (PAG) is a midbrain structure that  
520 is critical to defensive responding downstream of both the CeA and BNST (Tovote et al., 2016,  
521 2015) and appears to have a specialized role in mediating escape behavior (Lefler et al., 2020).  
522 Functional differences in the PAGs dorsal-ventral axis exists such that the ventral PAG mediates  
523 freezing behavior and the dorsal PAG mediates flight behavior (Assareh et al., 2016; Carrive,  
524 1993; Franklin, 2019; Vianna et al., 2001). For example, stimulation of the dorsal PAG can result  
525 in both flight and freezing behavior, whereas stimulation of the ventral PAG results exclusively in  
526 freezing (Assareh et al., 2016; Carrive, 1993; Chou et al., 2018; Kim et al., 2013; Vianna et al.,  
527 2001). Importantly, recent work in mice has indeed shown that the dorsal PAG performs a synaptic  
528 threshold mechanism for computing escape behavior in a looming-disc paradigm (Evans et al.,  
529 2018). Based on this, we speculate that concurrent CeA and BNST input to the PAG could drive  
530 the threshold-like mechanism underlying context-dependent flight behavior (Nagy and Paré,  
531 2008). Alternatively, BNST projections to the CeA may represent necessary inputs to drive CeA

532 CRH+ neurons to gate flight behavior (Fadok et al., 2017; Gungor et al., 2015; Gungor and Paré,  
533 2016; Yamauchi et al., 2018). Future work should investigate these pathways and their potential  
534 role in mediating flight behavior in the SCS paradigm.

535 To summarize, we have shown that rats display flight-behavior in the SCS paradigm  
536 similarly to mice, although rats show less frequent escape-like behaviors such as jumping and  
537 darting. Flight-like behavior evoked by the SCS is specific to white noise, gated by contextual fear,  
538 and cannot be accounted for by sensitization or fear potentiated startle. We conclude that SCS  
539 conditioning results in a high fear state driven by the summation of cued, contextual, and innate  
540 fear that drives a freeze-to-flight transition. Future work should investigate the neural mechanisms  
541 underlying the transition from post-encounter to circa-strike defensive behaviors and how this is  
542 driven by a combination of conditioned and innately aversive stimuli (Fanselow and Lester, 1988;  
543 Mobbs et al., 2020). This may reveal important clinical implications for psychiatric disorders that  
544 are characterized by high fear states and the dysregulation of contextual processing, such as panic  
545 disorder and PTSD (Goddard, 2017; Maren et al., 2013).

546

## 547 **MATERIALS AND METHODS**

548 **Subjects.** Experiments used adult Long-Evans rats ( $n = 163$ ) acquired from Envigo (Indianapolis,  
549 IN; 200-240 g upon arrival). Males and females were used in equal numbers throughout all  
550 experiments. All animals were housed in a climate-controlled vivarium and kept on a fixed  
551 light/dark cycle (lights on starting at 7:00 AM and off at 9:00 PM; experiments took place during  
552 the light phase of the cycle). Rats were individually housed in clear plastic cages (with bedding  
553 consisting of wood shavings; changed weekly) on a rotating cage rack. Group assignments for  
554 behavioral testing was randomized for cage position on the racks. Animals had access to standard



555 rodent chow and water *ad libitum*. Animals were handled by the experimenter(s) (~30 sec/day) for  
556 five consecutive days prior to the start of any surgeries or behavior. All procedures were in  
557 accordance with the US National Institutes of Health (NIH) Guide for the Care and Use of  
558 Laboratory Animals and were approved by the Texas A&M University Institutional Animal Care  
559 and Use Committee.

560

561 **Apparatuses.** All behavioral testing occurred within one of two rooms in the laboratory. Each  
562 behavioral room housed eight identical rodent conditioning chambers (30 cm × 24 cm × 21 cm;  
563 MED Associates, Inc.). Each chamber was housed in a larger, external sound-attenuating cabinet.  
564 Rear walls, ceilings, and the front doors of the testing chambers were made of Plexiglas, while  
565 their sidewalls were made of aluminum. Grid floors of the chambers were comprised of nineteen  
566 stainless steel bars (4 mm in diameter) and spaced 1.5 cm apart (center to center). The grid floors  
567 were attached to an electric shock source and a solid-state grid scrambler for delivery of the US  
568 (MED Associates, Inc.). A speaker attached to each chamber was used to deliver the auditory CS.  
569 As needed for each context, the chambers were equipped with 15 W house lights, and small fans  
570 were embedded in the cabinets (providing background noise of ~70 dB). An aluminum pan was  
571 inserted beneath the grid floor to collect animal waste. A small camera was attached to the top of  
572 the cabinet for video monitoring of behavior.

573         Measurements of freezing and motor activity were performed using an automated system  
574 (Maren, 1998). Specifically, each behavioral testing chamber rested on a load-cell platform that  
575 was sensitive to cage displacement due to each animal's movements. During behavioral testing,  
576 load-cell activity values (ranging from -10 to +10 V) were collected and digitized at 5 Hz using  
577 Threshold Activity Software (MED Associates, Inc.). Offline conversions of the load-cell activity

578 values were performed to generate absolute values ranging from 0 to 100; lower values indicate  
579 minimal cage displacement, which coincided with freezing behaviors in the chambers.  
580 Accordingly, freezing bouts were defined as absolute values of  $\leq 10$  for 1 s or more. The  
581 percentage of freezing behavior during the pre-SCS baseline and SCS trials was computed for each  
582 behavioral session. Motor activity was analyzed by directly reporting the absolute values generated  
583 by the Threshold Activity Software (i.e., larger values indicated more movement in the cage).  
584 Jumping and darting behavior were manually scored off-line from video recordings by an  
585 experimenter blind to experimental conditionings.

586         Unique contexts (A, B, and C) were used for various phases of behavior testing. Chamber  
587 assignments were unique to each context and group assignments were counterbalanced across test  
588 chambers when possible. For each experiment contexts A and B were assigned to different  
589 behavioral testing rooms. For context A, the test chamber and pans beneath the grid floors were  
590 wiped down with an ammonium hydroxide solution (1%). The cage lights were turned off,  
591 chamber fans were turned on, and the cabinet doors were left open. Black Plexiglas panels were  
592 also placed over the grid floors. The behavioral room was lit with white light (red lights were  
593 turned off). Animals were transported to and from the chambers using white plastic transport  
594 boxes. For context B, an acetic acid solution (3%) was used to wipe down and scent the chambers,  
595 the cage lights were turned on, the chamber fans were turned off, and the cabinet doors were closed.  
596 The behavioral room was lit with dim red light (white room lights were turned off). Rats were  
597 transported to and from context B using black plastic transport boxes that included a layer of clean  
598 bedding. For context C, an ethanol solution (70%) was used to wipe down and scent the chambers,  
599 the cage lights were turned on, the chamber fans were turned on, and the cabinet doors were open.  
600 The behavioral room was lit with white lights (red lights remained off) and rats were transported

601 to and from context C in white plastic transport boxes with clean bedding. Testing in context C  
602 was always performed in the same behavioral room as context A.

603

604 **Experimental Design.** Overviews of each behavioral experiment are provided in the figures. The  
605 auditory serial-compound stimulus (SCS) used for all experiments was comprised of ten 500 ms  
606 pure tone pips (80 dB, 7 kHz) presented at a frequency of 1 Hz (500 ms inter-pip intervals, 10 s  
607 total length) and immediately followed by ten 500 ms white noise pips (80 dB, 1-20 kHz) presented  
608 at a frequency of 1 Hz (500 ms inter-pip intervals, 10-s total length). During conditioning the SCS  
609 was paired with a mild unconditioned footshock stimulus (US, 1.0 mA, 2 sec), unless noted  
610 otherwise. Intertrial-intervals (ITI) were always 60 seconds.

611

612 *Experiment 1: Flight behavior in the conditioning vs habituation contexts.*

613 In this experiment we tested if the SCS paradigm could evoke flight-like behavior in rats similar  
614 to what has previously been reported in mice. To this end, behavioral testing consisted of a  
615 habituation session, three conditioning sessions, and a retrieval session. Habituation sessions  
616 consisted of a 3 min pre-SCS baseline period followed by 4 SCS presentations without footshock  
617 in context A. Next, all rats underwent conditioning consisting of a 3 min pre-SCS baseline period  
618 followed by 5 SCS-US presentations in context B for three consecutive days. Multiple days of  
619 conditioning were necessary to drive flight responses (Fadok et al., 2017). Retrieval testing  
620 consisted of a 3-min pre-SCS baseline period followed by 4 SCS-alone presentations in either  
621 context A (habituation) or context B (conditioning). A within-subject design was used such that  
622 half the rats were tested in either the habituation or conditioning context first and later were tested  
623 in either the conditioning or habituation context, respectively, the subsequent day (counter-

624 balanced for test order). There was only one group tested in this experiment using a within-subjects  
625 design ( $n = 8$ ). No animals were excluded.

626

### 627 *Experiment 2: Unsignaled footshock*

628 For this experiment we wanted to determine if flight-like behavior could be driven in other shock-  
629 associated contexts. Behavioral testing for this experiment consisted of a habituation session  
630 (context A), three conditioning sessions (context B), a session where rats either received  
631 unsignaled footshocks (context C), and a final between-subjects retrieval session in context C.  
632 Habituation and conditioning sessions were identical to Experiment 1. For the unsignaled  
633 footshock session, all rats were placed into a novel context C where half were presented a 3-min  
634 pre-stimulus baseline followed by 5 unsignaled footshocks (1.0 mA, 2 sec) with 60-sec ITIs  
635 (Shock), and the other half were merely exposed to the context for an equal amount of time (No-  
636 Shock). The next day all rats were returned to context C where they were presented with 1 SCS-  
637 alone trial following a 3 min baseline period. The groups were as follows: Shock ( $n = 8$ ) and No-  
638 Shock ( $n = 8$ ). No animals were excluded.

639

### 640 *Experiment 3: Context extinction*

641 In this experiment we tested if flight behavior could be diminished by extinguishing contextual  
642 fear. Behavioral testing consisted of habituation, three days of conditioning, a context extinction  
643 session, and a retrieval session. Habituation and conditioning were identical to previous  
644 experiments. For context extinction, half of the rats underwent context extinction by exposing  
645 them to the conditioning context (context B) for 45 min for two consecutive days (EXT) while the  
646 other half were re-exposed to context A for an equal amount of time (No-EXT). The subsequent

647 day all animals were placed back into context B where they underwent a retrieval session  
648 consisting of a 3-min pre-SCS baseline followed by 4 SCS-alone presentations. Groups were as  
649 follows: EXT ( $n = 14$ ) and No-Ext ( $n = 15$ ). Two EXT ( $n = 2$ ) and one No-EXT ( $n = 1$ ) animals  
650 were excluded as statistical outliers (flight ratio  $>2$  SDs  $\pm$  mean).

651

#### 652 *Experiment 4: Reverse and unpaired SCS*

653 This experiment tested both the temporal order of the SCS and the SCS-US relationship on flight  
654 behavioral. Behavioral testing for this experiment was similar to previous experiments in that it  
655 consisted of one day of habituation (context A), three days of conditioning (context B), and a  
656 retrieval session (context C). However, conditioning consisted of either 5 standard SCS-US  
657 pairings (Standard), 5 reversed (noise followed by tone) SCS-US pairings (Reverse), or 5 unpaired  
658 presentations with a 60-sec delay between each SCS and US (Unpaired). Following the final day  
659 of conditioning, all rats were placed back into context B and presented 4 SCS-alone presentations.  
660 Groups are as follows: Standard ( $n = 8$ ), Reverse ( $n = 8$ ), and Unpaired ( $n = 8$ ). No animals were  
661 excluded.

662

#### 663 *Experiment 5: Contextual fear potentiation*

664 For this experiment we wanted to determine if the context dependence of flight behavior could be  
665 accounted for by fear potentiated startle. This experiment is designed similar to Experiment 2  
666 which consisted of habituation (context A), three days of conditioning (context B), unsignaled  
667 footshock (context C), and retrieval (context C). However, following habituation, rats were either  
668 conditioned with 5 SCS-US pairings (SCS-US) or received 5 unsignaled footshocks (US-alone) in  
669 context B for three consecutive days. After conditioning, each of these groups were placed in a

670 novel context C where half received unsignaled footshocks (Shock) and half were merely exposed  
671 to the context for the same amount of time (No-Shock), thus creating a 2x2 design. On the final  
672 day, all rats were placed in context C and presented 10 SCS-alone trials after a 3-min baseline.  
673 Groups were as follows: SCS-US/Shock ( $n = 8$ ), SCS-US/No-Shock ( $n = 8$ ), US-alone/Shock ( $n$   
674 = 8), and US-alone/No-Shock ( $n = 8$ ). No animals were excluded.

675

#### 676 *Experiment 6: Muscimol inactivation of the CeA and BNST*

677 In Experiment 6 we tested if flight behavior that depended on contextual fear could be blocked by  
678 inactivating regions necessary for the expression of context fear. Rats were first chronically  
679 implanted with bilateral cannula targeting either the CeA or BNST. One week after surgery, all  
680 rats underwent habituation (context A) and three days of conditioning (context B) as previously  
681 described. Immediately prior to retrieval testing, rats received 0.3- $\mu$ l microinfusions of either the  
682 GABA<sub>A</sub> agonist muscimol (0.1  $\mu$ g / $\mu$ l) or saline at a rate of 0.3  $\mu$ l/min and infusions needles stayed  
683 in-place for at least 2 minutes post-infusion. They were then placed into transport boxes and moved  
684 to the behavioral testing room for retrieval testing took place as previously described with a 3-min  
685 baseline period and 4 SCS-alone trials. Groups were as follows: CeA ( $n = 14$ ), BNST ( $n = 14$ ),  
686 SAL ( $n = 16$ ).

687

688 **Surgery.** Rats were anesthetized with isoflurane (5% induction, ~2% maintenance), the top of  
689 their heads were shaven, and they were placed in a stereotaxic mount (Kopf Instruments, Tujunga,  
690 CA). A small incision was made with a scalpel, fascia lining the skull was scrubbed away with  
691 cotton swabs, and the scalp was retracted with forceps. The skull was leveled horizontally before  
692 burr holes were drilled above either the BNST or CeA. Four additional holes were made anteriorly

693 and posteriorly (two each) for skull screws. After skull screws were placed, two stainless-steel  
694 cannulas (26 gauge, 8mm; Plastics One) were lowered into either the CeA (target coordinates; ML:  
695 4.0, AP: -2.0, DV: -8.0 ) or the BNST (target coordinates; ML: 1.5, AP: 0.0, DV: -6.5). Cannula  
696 targeting the BNST were inserted at a 10° angle to avoid rupturing the ventricle. Thus, angled  
697 coordinates used during stereotaxic surgery targeting the BNST were as follows: ML: 3.13, AP:  
698 0.0, DV: -6.19 (ML: medial-lateral, AP: anterior-posterior, DV: dorsal-ventral). All coordinates  
699 are in reference to the skull surface at bregma. Cannula were then affixed to the skull with dental  
700 acrylic and a stainless-steel dummy (30 gauge, 9 mm; Plastics One) was inserted into the guide  
701 cannula. Rats were allowed to recover for ~1 week after surgery before behavioral testing.

702

703 **Drug microinfusions.** The day of retrieval testing rats were placed into 5-gallon white buckets  
704 and moved into a room adjacent to the vivarium for microinfusions. Dummy cannula internals  
705 were removed and a stainless-steel injector (33 gauge, 9mm; Plastics One) connected to  
706 polyethylene tubing was inserted into the guide cannula. Polyethylene tubing was connected to 10-  
707 µl Hamilton syringes that were mounted in an infusion pump (Kd Scientific). Muscimol was  
708 diluted to a concentration of 0.1 µg/µl in sterile saline. Infusions were made a rate of 0.3 µl/min  
709 for 1 min and the injectors were left in place for 2 min post-infusion to allow for adequate diffusion.  
710 Each infusion was verified by movement of an air bubble that separated the drug or sterile saline  
711 from distilled water within the polyethylene tubing. Clean dummy internals were inserted into each  
712 guide cannula after infusions. All infusions were made ~5 min prior to behavioral testing.

713

714 **Histology.** Twenty-four hours after retrieval testing animals were sacrificed to confirm cannula  
715 placement. Animals were overdosed with sodium pentobarbital (Fatal Plus, 100 mg/ml, 0.7 ml),

716 transcidentally perfused with ice-cold saline and fixed with 10% physiological formalin. Perfused  
717 brains were placed in physiological formalin for 14-24 hours before being moved to a 30% sucrose  
718 solution for a minimum of three days. After three days, or until brains had sunk in 30% sucrose,  
719 all brains were frozen and sectioned at  $-20^{\circ}$  on a cryostat at a thickness of 40  $\mu\text{m}$ . Sections were  
720 mounted onto gelatin subbed slides, thionin stained (0.25%) to better visualize cannula placement,  
721 cover-slipped with Permount (Fisher Scientific), and then imaged on a wide-field stereoscope.

722 A subset of animals infused with fluorescent muscimol to verify drug spread. These  
723 animals were overdosed with sodium pentobarbital and infused with 0.3  $\mu\text{l}$  of fluorescent  
724 muscimol (BODIPY TMR-X conjugate; Thermo Fisher Scientific) at a rate of 0.3  $\mu\text{l}/\text{min}$ . A rest  
725 period of 2 minutes was given post-infusion and then animals were immediately sacrificed. Non-  
726 perfused brains were placed in a physiological formalin solution for 14-24 hours before being  
727 placed in a 30% sucrose-formalin solution for a minimum of three days. These brains were also  
728 sectioned at  $-20^{\circ}$  on a cryostat at a thickness of 40  $\mu\text{m}$ . Sections were then mounted onto subbed  
729 slides, coverslipped with fluoromount (Diagnostic Bio-systems), and imaged on a fluorescent  
730 microscope at 10x resolution. Hits were confirmed by verifying that the tip of the infusion needles  
731 was within the CeA or BNST. Only animals that had bilaterally confirmed placements were  
732 included in statistical analyses. Thus, animals in which the tip of either one or both cannulas were  
733 outside of the CeA or BNST were excluded from analyses.

734

735 **Statistical analyses.** All freezing and raw threshold data were analyzed offline by custom written  
736 Python and MATLAB scripts before eventual statistical testing in Statview software. All data were  
737 submitted to repeated or factorial analysis of variance (ANOVA) as described for each experiment.  
738 Fisher's protected least significant difference (PLSD) test was used for *post hoc* comparisons of



739 group means following a significant omnibus  $F$  ratio in the ANOVA ( $\alpha$  was set at 0.05). No  
740 statistical methods were used to predetermine group sizes (group sizes were selected based on prior  
741 work and what is common for the field). Sex was included as a biological variable for all statistical  
742 comparisons. Data distributions were assumed to be normal, but these were not formally tested.  
743 All data are represented as means  $\pm$  S.E.M.

744

#### 745 **ACKNOWLEDGEMENTS**

746 Supported by grants from the National Institutes of Health (R01MH065961 and R01MH117852  
747 to S.M.).

748

#### 749 **ADDITIONAL INFORMATION**

##### 750 **Competing interests**

751 The authors declare no competing interests.

752

##### 753 **Author contributions**

754 M.T. and S.M. designed experiments; M.T., N.W., I.H., K.R., R.R., and C.O. performed the  
755 experiments; M.T. and N.W. analyzed data; M.T. and S.M. wrote the manuscript.

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