Bed nucleus of the stria terminalis regulates fear to ambiguous threat signals

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ABSTRACT

Anxiety is characterized by a fear of potential future threats. The bed nucleus of the stria terminalis (BNST) has been implicated in anxiety, but its role in processing ambiguous threats is unclear. Here we examined whether the BNST mediates conditioned freezing to conditioned stimuli (CSs) that poorly predict the onset of aversive unconditioned stimuli (USs) in rats. Reversible inactivation of the BNST selectively reduced freezing to CSs that poorly predicted US onset (e.g., a backward CS that followed the US), but did not affect freezing to forward CSs even when they predicted USs of variable intensity. Importantly, backward (but not forward) CSs selectively increased Fos in the ventral BNST and in BNST-projecting neurons in the infralimbic region of the medial prefrontal cortex (mPFC), but not in the hippocampus or amygdala. These data reveal that BNST circuits process ambiguous threat signals central to the etiology and expression of anxiety.

IMPACT STATEMENT

The bed nucleus of the stria terminalis (BNST) is required for the expression of defensive behavior to uncertain threats, a function that is central to pathological anxiety.

INTRODUCTION

Excessive apprehension about potential future threats, including financial loss, illness, or death, is a defining feature of many anxiety disorders, including generalized anxiety disorder (GAD) (Behar et al., 2009). Anxiety and trauma-related disorders are widespread and costly (Blanco et al., 2011; Comer et al., 2011; Kinley et al., 2011; Salas-Wright et al., 2014; Stein et al., 2017; Wittchen, 2002), and remain difficult to treat (Colvonen et al., 2017; Costello et al., 2014; Iza et al., 2013; Sinnema et al., 2015). Understanding the neural circuits underlying anxiety is important for refining behavioral and pharmacotherapeutic treatments (Berridge, 2018; Deslauriers et al., 2017; Fanselow and Pennington, 2018, 2017; Graham et al., 2014; LeDoux and Daw, 2018; Nees et al., 2015; Pine and LeDoux, 2017; Tye, 2018). Several recent studies have demonstrated changes in the function of the bed nucleus of the stria terminalis (BNST) in individuals with post-traumatic stress disorder (PTSD) and anxiety disorders (Andreescu et al., 2015; Brinkmann et al., 2018; L Brinkmann et al., 2017; Leonie Brinkmann et al., 2017; Buff et al., 2017; Münsterkötter et al., 2015; Rabellino et al., 2018; Straube et al., 2007; Yassa et al., 2012). However, the conditions that recruit the BNST to aversive learning and memory processes believed to underlie anxiety disorders are still not understood (Avery et al., 2016, 2014; Ch'ng et al., 2018; Daniel and Rainnie, 2016; Davis et al., 2010; Fox and Shackman, 2017; Goode and Maren, 2017; Gungor and Paré, 2016; Lebow and Chen, 2016; Perusini and Fanselow, 2015; Shackman and Fox, 2016; Walker et al., 2009; Walker and Davis, 2008).

Early work on this question revealed that BNST lesions in rats impair defensive behaviors evoked by unconditioned threats (Gewirtz et al., 1998). For example, BNST inactivation results in a loss of unconditioned defensive responding to the presence of predator odors (Breitfeld et al., 2015; Fendt et al., 2003; Xu et al., 2012). Additionally, unconditioned increases in the acoustic

25 startle reflex produced by either intracranial administration of corticotropin-releasing factor (CRF)
26 or exposure to bright light require the BNST, whereas fear-potentiated startle to punctate
27 conditioned stimuli (CSs) do not (Walker et al., 2009). However, the involvement of the BNST in
28 defensive responding is not restricted to unconditioned threat: BNST lesions produce deficits in
29 both freezing and corticosterone release elicited by contextual, but not auditory, CSs after
30 Pavlovian fear conditioning in rats (LeDoux et al., 1988; Sullivan et al., 2004). Interestingly,
31 however, it is not stimulus modality that differentiates the effects of BNST lesions on tone and
32 context freezing. BNST lesions impair freezing responses to long-duration (e.g., 10-min) auditory
33 conditioned stimuli (CSs) (Waddell et al., 2006), and spare freezing to short-duration (1-min)
34 contextual stimuli (Hammack et al., 2015). Based on this work, it has been proposed that the BNST
35 is required to organize behavioral and hormonal responses to sustained threats (whether
36 conditioned or unconditioned) (Hammack et al., 2015, 2009; Takahashi, 2014; Waddell et al.,
37 2008, 2006). Of course, the duration of the behavioral responses in these situations is confounded
38 with the duration of the eliciting stimulus. Thus, it has been argued that the BNST mediates
39 sustained defensive *responses* independent of the specific features and durations of the stimuli that
40 evoke them (Davis, 2006; Davis et al., 2010; Walker et al., 2009; Walker and Davis, 2008).

41 Of course, another factor that differentiates discrete tones from contexts, or short- from
42 long-duration stimuli (whether tones or contexts), is the temporal information the stimuli provide
43 about when an aversive event will occur (Goode and Maren, 2017). For example, long-duration
44 CSs or contexts provide relatively poor information about *when* a future US will occur (i.e., the
45 US will occur at some time in the future), whereas short-duration CSs or contexts provide more
46 certainty about the temporal imminence of US onset (i.e., the US will occur soon). We and others
47 have proposed that the BNST may have a critical role in processing temporally ambiguous threats,
48 particularly in mediating defensive responses to threatening stimuli that poorly predict when an

49 aversive event will occur (Goode and Maren, 2017; Lange et al., 2017; Luyck et al., 2018a).
50 Consistent with this view, punctate auditory CSs that are followed by shock at unpredictable
51 latencies yield freezing responses that are sensitive to BNST manipulations [(Lange et al., 2017);
52 also, see (Daldrup et al., 2016)]. This suggests that a crucial parameter that determines the role for
53 the BNST in defensive behavior is neither the duration nor modality of the threat (nor the duration
54 of the elicited defensive response), but rather the information a signal provides about when an
55 aversive event will occur.

56 Here we directly examined this possibility by investigating the role of the BNST in
57 conditioned freezing responses elicited by procedures that equated both the duration and modality
58 of the threat CSs, as well as the total number of unconditioned stimulus (US) presentations, but
59 differed according to the timing of the aversive US in relation to the CS. Specifically, we arranged
60 a brief (10-sec) auditory CS to either precede (forward conditioning, FW) or follow (backward
61 conditioning, BW) a footshock US in rats. Although extensively-trained BW CSs become
62 conditioned inhibitors that dampen responding to other first-order excitatory cues (Andreatta et
63 al., 2012; Ayres et al., 1976; Christianson et al., 2011; Gerber et al., 2014; Moscovitch and
64 LoLordo, 1968; Siegel and Domjan, 1971), minimally-trained BW CSs elicit excitatory
65 conditioned responses that transfer across contexts (Ayres et al., 1987; Barnet and Miller, 1996;
66 Bevins and Ayres, 1992; Chang et al., 2003; Connor et al., 2017; Heth, 1976; Mahoney and Ayres,
67 1976; Prével et al., 2018, 2016; Rescorla, 1968). After conditioning, we examined the effect of
68 pharmacological inactivation of the BNST on freezing to FW or BW CSs. We hypothesized that
69 pharmacological inactivation of the BNST would selectively disrupt fear expression to the BW CS
70 and that the presentation of the BW CS would recruit BNST neurons (as assessed by Fos) in greater
71 numbers than the FW CS. Furthermore, we anticipated that the backward CS would increase the
72 activity of BNST-projecting neurons in afferent brain regions implicated in anxiety states.

73 Consistent with our hypothesis, we found that BNST inactivation reduced freezing to a BW, but
74 not FW, CS. This effect was related to the ambiguity of the BW CS in predicting US onset, because
75 the same outcome was observed when USs occurred randomly with respect to the CS. We also
76 observed that BW CSs selectively increased Fos expression in the BNST and BNST-projecting
77 medial prefrontal cortical (mPFC) neurons. These data reveal that BNST circuits process the
78 expression of defensive behavior in the presence of ambiguous threat signals.
79
80 **RESULTS**
81
82 ***Reversible inactivation of the BNST attenuates fear to backward, not forward, CSs.*** To examine
83 the role of the BNST in threat uncertainty, we reversibly inactivated the BNST during retrieval of
84 fear to either a forward- ("FW"; imminent threat) or backward-trained ("BW"; ambiguous threat)
85 CS. A schematic of the behavioral design is shown in Figure 1A. Representative cannula tracts
86 and histological placements are presented in Figure 1—Figure Supplement 1 and Figure 1—Figure
87 Supplement 2 (respectively). Freezing behavior during the conditioning session is shown in Figure
88 1B. A main effect of conditioning trial was observed (repeated measures: $F_{6,288} = 37.87$, $p <$
89 0.0001). No other significant main effects or interactions were observed for any of the conditioning
90 or drug assignments across the conditioning trials (F's < 0.70, p's > 0.60).
91 One day after conditioning, the animals were infused with NBQX, an AMPA receptor
92 antagonist, to reversibly inactivate the BNST; saline ("VEH") infusions served as a control.
93 Immediately after the infusions, the rats were placed in a novel context and received twelve
94 presentations of the CS [some BW-trained rats received no CS exposure at the test, "NoCS(Test)"].
95 As shown in (Figure 1B), inactivation of the BNST produced a selective decrease in conditioned
96 freezing to the BW CS. Analysis of freezing behavior across the entire session (including the

97 baseline) revealed a main effect of trial (repeated measures: $F_{6,264} = 8.06$, $p < 0.0001$), a significant
98 main effect of CS exposure ($F_{2,44} = 25.38$, $p < 0.0001$), and a significant CS × drug interaction
99 ($F_{2,44} = 3.66$, $p < 0.05$). There were no other main effects or interactions (F's < 1.6, p's > 0.05).
100 Fisher's PLSD indicated that rats in the FW condition exhibited significantly more freezing during
101 the retrieval test than rats in all other groups (p's < 0.05). Importantly, BW-VEH rats exhibited
102 significantly higher levels of freezing than BW-NBQX rats ($p < 0.005$); BW-NBQX rats did not
103 significantly differ from the NoCS(Test) groups.

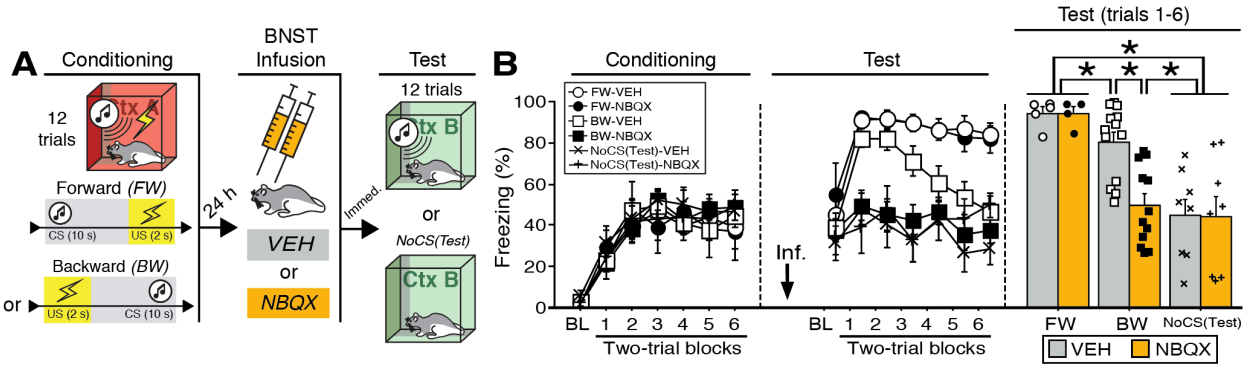

Figure 1. *Reversible inactivation of the BNST attenuates conditioned fear expression to a backward, but not forward, CS.* **(A)** Behavioral schematic. **(B)** Freezing behavior at conditioning and retrieval testing. For conditioning, the left panel depicts mean percentage freezing during the 5-min baseline (BL) and across each conditioning block (each 138-sec block is comprised of two trials; conditioning trials consist of freezing during the 10-sec CS followed by the 58-sec interstimulus interval). For retrieval testing, the center panel shows mean percentage freezing at the 5-min baseline (BL) and across each test block (each 140-sec block is comprised of two trials; trials consist of freezing during the 10-sec CS followed by the 60-sec interstimulus interval). The right panel shows mean percentage freezing during the first half of the test (trials 1-6; corresponding to 420 sec of behavior). All data are represented as means ± s.e.m [FW-VEH (n = 5); FW-NBQX (n = 4); BW-VEH (n = 13); BW-NBQX (n = 12); NoCS(Test)-VEH (n = 8); NoCS(Test)-NBQX (n = 8)]; * = $p < 0.05$.

104 Given that freezing to the BW CS in the VEH-treated animals was maximal in the first half
105 of the test, a separate factorial ANOVA was performed on the average percentage of freezing
106 during trials 1-6 (Figure 1B). For this period, there was a main effect of CS treatment ($F_{2,44}$ =
107 18.61, $p < 0.0001$) as well as a CS treatment × drug interaction ($F_{2,44} = 3.81$, $p < 0.05$); there was
108 no main effect of drug across all groups ($F < 3.00$, $p > 0.05$). Fisher's PLSD revealed significant
109 differences in NBQX- and VEH-treated animals in the BW group ($p < 0.0005$). Additionally, rats

110 in the NoCS(Test) groups differed from all others (p's < 0.0005) except those in the BW-NBQX
111 group, indicating selective reduction of CS-elicited freezing in the BW-NBQX animals. It has been
112 suggested that the BNST mediates sustained but not acute fear responses (Davis, 2006; Davis et
113 al., 2010; Walker et al., 2009; Walker and Davis, 2008). However, it is notable that in this
114 experiment BNST inactivation selectively attenuated conditioned freezing to the backward CS,
115 which produced less sustained freezing during the retrieval test compared to the FW CS.

116 The different effects of BNST inactivation on freezing to FW and BW CSs may have been
117 related to differences in memory strength between the two CSs (reflected in overall levels of
118 freezing to each CS across the retrieval test). Indeed, to generate conditioned freezing to a
119 backward CS we used more conditioning trials than typical in our laboratory. Of course, this may
120 have resulted in moderate overtraining of the FW CS, which might have rendered the FW response
121 less sensitive to disruption. To examine this possibility, we also examined the effects of BNST
122 inactivation of FW and BW conditioning after five conditioning trials (Figure 1—Figure
123 Supplement 3). The behavioral design for the five-trial experiment is shown in Figure 1—Figure
124 Supplement 3A; cannula placements for this experiment are shown in Figure 1—Figure
125 Supplement 4.

126 During the 5-trial conditioning procedure (Figure 1—Figure Supplement 3B), animals
127 exhibited reliable increases in freezing across the session (repeated measures: $F_{5,115} = 12.154$, $p <$
128 0.0001; there were no main effects of conditioning procedure or drug treatment and no interaction
129 of these variables: F's < 2.3, p's > 0.15). After infusions of NBQX or VEH, animals were tested
130 to the CS in a familiar context that was distinct from the conditioning context. As shown in Figure
131 1—Figure Supplement 3B, BNST inactivation did not affect conditioned freezing to either the FW
132 or BW CS. An ANOVA revealed a main effect of trial (repeated measures [including baseline
133 freezing]: $F_{5,105} = 22.118$, $p < 0.0001$), a main effect of conditioning procedure ($F_{1,21} = 15.930$, p

134 < 0.001), and a trial × conditioning procedure interaction (repeated measures: $F_{5,105} = 12.346$, $p <$
135 0.0001). A separate ANOVA performed on trials 1-5 (excluding baseline; Figure 1—Figure
136 Supplement 3B) revealed a significant main effect of conditioning procedure ($F_{1,21} = 21.585$, $p =$
137 0.0001; there was no main effect of drug and no interaction: F's < 0.15, p's > 0.7). Thus, the failure
138 of BNST inactivation to impair conditioned freezing to the FW CS in the 12-trial procedure does
139 not appear to be due to a ceiling effect; freezing to the FW CS in the 5-trial procedure was not
140 maximal and remained insensitive to BNST inactivation. That said, the absence of an effect of
141 BNST inactivation on freezing to the BW CS in this experiment may be due to a floor effect insofar
142 as the 5-trial procedure did not yield conditional significant freezing to the CS (at least freezing
143 that exceeded the pre-CS baseline). Together, these data indicate that the BNST is required for the
144 expression of conditioned freezing to an excitatory BW, but not FW, CS.

145 One index that might reveal differences in the ability of the CS to predict the US is the US-
146 evoked response itself. Footshock USs elicit an unconditioned response (UR) that includes
147 vocalization, autonomic adjustments, and burst of locomotor activity (Bali and Jaggi, 2015;
148 Fanselow, 1994). To better understand the mechanisms of forward and backward conditioning on
149 behavior, we examined shock-evoked activity bursts (Kunwar et al., 2015; Zelikowsky et al., 2018)
150 during the conditioning session in a separate cohort of animals (Figure 1—Figure Supplement 5).
151 Fear conditioning resulted in robust freezing in both groups of animals (Figure 1—Figure
152 Supplement 5A). An ANOVA revealed a significant main effect of trial (repeated measures: $F_{6,180}$
153 $= 67.02$, $p < 0.0001$) with no differences between levels of freezing in FW or BW animals (no
154 other main effects and no interactions: F's < 1.80, p's > 0.15). In contrast, shock-induced activity
155 differed in FW and BW animals (Figure 1—Figure Supplement 5C). All animals exhibited a
156 reliable decrease in shock-induced activity across the conditioning session ($F_{5,150} = 9.59$, $p <$
157 0.0001) and the rate of decline in activity was similar in the two groups (no trial × group

158 interaction: $F < 1.70$, $p > 0.09$), but the overall level of shock-induced activity was significantly
159 lower in the FW animals (main effect of group: $F_{1,30} = 9.59$, $p < 0.05$). For comparison, two-tailed
160 unpaired *t*-test of mean levels of activity during the 5-min baseline revealed no significant
161 difference between FW and BW rats (Figure 1—Figure Supplement 5B; $t < 0.30$; $p > 0.75$). Hence,
162 USs that were not signaled by a forward CS evoked greater activity bursts than those that were,
163 which may suggest greater regulation of the shock-induced activity when the US is preceded by a
164 temporally predictive cue.
165
166 ***Inactivation of the BNST disrupts freezing to a discrete CS associated with random USs.*** The
167 differential effect of BNST inactivation on freezing to a FW or BW CS suggests that the BNST
168 regulates defensive behavior to stimuli that poorly signal US onset. However, the BW CS does not
169 completely lack temporal information about US onset; the BW conditioning procedure may have
170 resulted in forward trace conditioning to the CS, which always preceded the next US by 60-sec
171 (after trial 1). To address this possibility, a separate cohort of rats was implanted with cannulas in
172 the BNST and submitted to one of three conditioning procedures (Figure 2). In the first group,
173 animals were conditioned to a backward CS (identical to 12-trial experiment above; "BW"). In the
174 second group, animals received CS presentations followed by randomized onset of the US
175 ("RANDOM"). In the third control group, animals were conditioned using the RANDOM
176 procedure but in the absence of any CS ["NoCS(Cond)"]. A summary of the behavioral design is
177 shown in Figure 2A. Cannula placements for these animals are shown in Fig. 2—Figure
178 Supplement 1.
179 The percentage of freezing during fear conditioning for the RANDOM, BW, and
180 NoCS(Cond) procedures is shown in Figure 2B. For conditioning, an ANOVA revealed a main
181 effect of trial (repeated measures: $F_{6,204} = 85.209$, $p < 0.0001$), insofar as all groups reliably

increased their freezing across the conditioning trials. There was no main effect of conditioning procedure ($F < 3.10$, $p > 0.05$) and no main effect or interactions with regard to future drug or vehicle assignments (F's < 1.00, p's > 0.30).

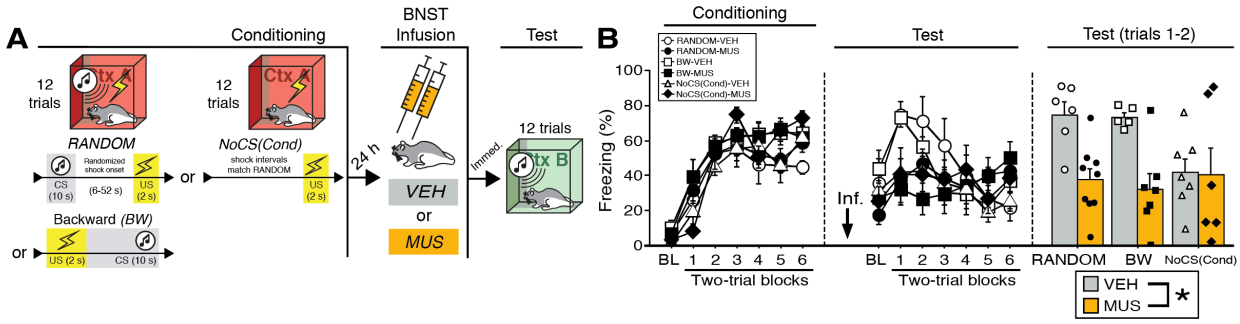

Figure 2. *BNST inactivation attenuates freezing to a discrete CS paired with random onset of shock.* **(A)** Behavioral schematic. **(B)** Freezing behavior during conditioning and retrieval testing. For conditioning, the left panel depicts mean percentage freezing during the 5-min baseline (BL) and across each conditioning block (each block is comprised of two trials; trials consist of freezing during the 10-sec CS followed by the 58-sec interval for the BW animals, whereas blocks for the other groups include equivalent time periods which include the CS [if present] and inter-CS intervals [excluding the duration of the shock]). For retrieval testing, the center panel shows mean percentage freezing at the 5-min baseline (BL) and across each test block (each 140-sec block is comprised of two trials; trials consist of freezing during the 10-sec CS followed by the 60-sec interval). The right panel shows mean percentage freezing across the first two test trials (after BL; corresponding to 140 sec of behavior). All data are represented as means ± s.e.m [RANDOM-VEH (n = 6); RANDOM-MUS (n = 9); BW-VEH (n = 5); BW-MUS (n = 7); NoCS(Cond)-VEH (n = 7); NoCS(Cond)-VEH (n = 6)].

As shown in Figure 2B, inactivation of the BNST produced deficits in freezing during retrieval testing in the BW and RANDOM groups, but did not affect freezing in the NoCS(Cond) control. This was confirmed in an ANOVA that revealed a significant drug × trial interaction (repeated measures: $F_{6,204} = 7.91$, $p < 0.0001$). A significant main effect of trial (repeated measures: $F_{6,204} = 7.80$, $p < 0.0001$) indicated significant changes in freezing across the entire test amongst all groups. No other significant main effects or interactions were detected for this test (F's < 1.80, p's > 0.05). Similar to the aforementioned experiments, freezing was maximal in the early test trials, so a separate ANOVA was performed on mean responding during the first two trials of testing (Figure 2B). These analyses revealed a significant main effect of drug ($F_{1,34} = 6.217$, $p <$ 0.05), indicating a disruption of freezing in drug-treated animals; no other significant main effects

195 or interactions were detected for these trials (F's < 3.00, p's > 0.07). Excluding NoCS(Cond)
196 animals, a main effect of drug treatment was also identified for these trials ($F_{1,23}$ = 28.115, p <
197 0.0001). Additionally, we ran an ANOVA solely on baseline freezing. MUS-treated animals
198 exhibited a trending, but nonsignificant (F < 4.1, p > 0.05), decrease in freezing during the baseline
199 [no other main effect or interact was detected: (F's < 1.00, p's > 0.30)]. While baseline freezing
200 may impact CS-responding, subtracting baseline responding from CS-elicited freezing (of the first
201 two trials for RANDOM and BW animals) did not eliminate a main effect a drug-treatment ($F_{1,23}$
202 = 5.877, p < 0.05). Thus, these data replicate the effects of BNST inactivation on BW CS-elicited
203 freezing as seen in our first experiment and extend these results to show that the BNST mediates
204 freezing to CSs that signal uncertain latency of shock onset.
205
206 ***Temporary inactivation of the BNST does not eliminate fear to a forward CS that is paired with***
207 ***a US of variable intensity.*** To determine whether the BNST is involved in other conditioning
208 procedures imbued with outcome (but not temporal) uncertainty, we examined whether freezing
209 to a FW CS that is paired with a US of variable intensity is also BNST-dependent. In this case, rats
210 received forward fear conditioning with either a fixed ("FIXED") or variable ("VARIABLE") US
211 intensity (Figure 3). A schematic of the behavioral design is shown in Figure 3A; cannula
212 placements are illustrated in Figure 3—Figure Supplement 1. During conditioning (Figure 3B), a
213 repeated measures ANOVA revealed a main effect of trial (repeated measures: $F_{6,168}$ = 68.15, p <
214 0.0001), with no main effect of drug or conditioning procedure, and no interactions (F's < 1.50,
215 p's > 0.20).
216 Twenty-four hours after conditioning and immediately before a test to the CS in a novel
217 context (Figure 3B), the animals were infused with either muscimol ("MUS") to reversibly
218 inactivate the BNST; saline ("VEH") infusions served as a control. As shown in Figure 3B, BNST

219 inactivation did not affect freezing to a FW CS paired with either a fixed or variable US. During
220 retrieval testing, there was a main effect of trial (repeated measures: $F_{6,156} = 24.31$, $p < 0.0001$),
221 however no other main effects or interactions were detected (F's < 2.70, p's > 0.15). Although
222 average baseline freezing was highest in VARIABLE-VEH animals, an ANOVA of baseline
223 freezing did not reveal any main effects or interactions (F's < 3.60, p's > 0.05). To equate for
224 differences in pre-CS freezing in the experimental groups, baseline freezing was subtracted from
225 CS-elicited freezing (Figure 3C). This did not reveal an effect of BNST inactivation on freezing;
226 there were no significant main effects or interactions in the ANOVA (F's < 0.15, p's > 0.70).
227 Although BNST inactivation trended towards reducing generalized contextual freezing in this
228 experiment, it did not eliminate CS-elicited freezing to the temporally predictable cue, regardless
229 of the variable magnitude of the US.

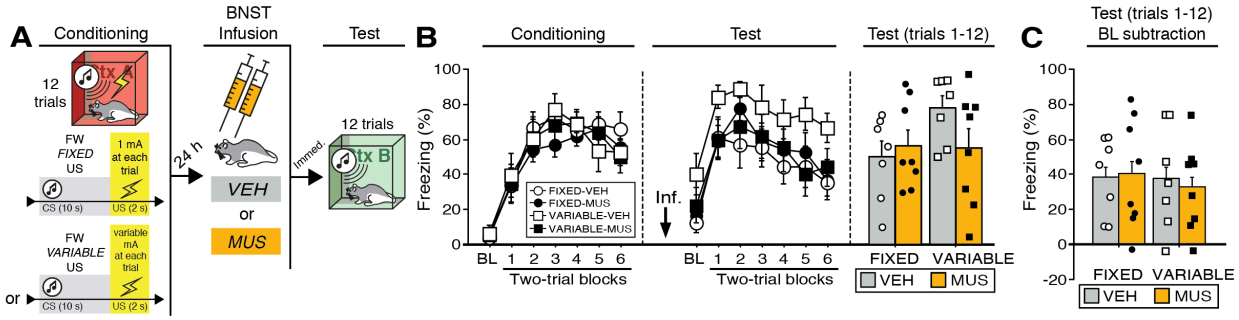

Figure 3. *Temporary inactivation of the BNST does not prevent conditioned fear expression to a forward CS paired with a US of fixed or variable intensity.* **(A)** Behavioral schematic. **(B)** Freezing behavior during conditioning and retrieval testing. For conditioning, the left panel depicts mean percentage freezing during the 5-min baseline (BL) and across each conditioning block (each 138-sec block is comprised of two trials; trials consist of freezing during the 10-sec CS followed by the 58-sec post-shock interval). For retrieval testing, the center panel shows mean percentage freezing at the 5-min baseline (BL) and across each test block (each 140-sec block is comprised of two trials; trials consist of freezing during the 10-sec CS followed by the 60-sec interval). The right panel shows mean percentage freezing across all test trials (after BL; corresponding to 840 sec of behavior). **(C)** Freezing percentages across all twelve trials, with BL levels of freezing subtracted from these values. All data are represented as means ± s.e.m [FIXED-VEH (n = 7); FIXED-MUS (n = 8); VARIABLE-VEH (n = 7); VARIABLE-MUS (n = 8)].

230 ***Backward CSs selectively increase Fos expression in the ventral BNST.*** To further examine the
231 role of the BNST in the expression of conditioned fear, we quantified Fos expression in multiple

232 subregions of the BNST following the presentation of either a FW or BW CS during a shock-free
233 retrieval test (Figure 4). The behavioral design is summarized in Figure 4A. Four experimental
234 groups were compared: rats conditioned and tested to a forward CS ("FW"), rats conditioned and
235 tested to a backward CS ("BW"), rats conditioned to a forward or backward CS but not receiving
236 a CS at test ["NoCS(Test)"], and animals that were conditioned but not tested ("NoTest").
237 Conditioning (Figure 4B) was similar to previous experiments (main effect of trial; repeated
238 measures: $F_{6,258} = 77.346$, $p < 0.0001$; no other main effects or interactions: F's < 1.30, p's > 0.30).
239 As shown in Figure 4B, the groups differed in their levels of conditioned freezing during the
240 retrieval test. ANOVA revealed a main effect of trial (repeated measures: $F_{6,216} = 15.54$, $p <$
241 0.0001), conditioning procedure ($F_{2,36} = 11.42$, $p < 0.0001$), and a procedure × trial interaction
242 ($F_{6,216} = 3.65$, $p < 0.0001$) (includes baseline). Post-hoc analyses revealed that FW ($p < 0.0001$)
243 and BW ($p < 0.005$) rats exhibited significantly higher levels of freezing behavior than NoCS(Test)
244 rats. Similarly, an ANOVA of average freezing across the test trials (without baseline) revealed a
245 main effect of group ($F_{2,36} = 13.76$, $p < 0.0001$). Post-hoc comparisons revealed significant
246 differences between FW vs. BW ($p < 0.05$) and FW vs. NoCS(Test) rats ($p < 0.0001$), as well as
247 BW vs. NoCS(Test) animals ($p < 0.005$).

248 Ninety minutes after the retrieval test, the animals were sacrificed for Fos
249 immunohistochemistry. Fos-positive nuclei were counted in three BNST subregions (Figure 5A):
250 "ovBNST" (Fos counts targeting the oval nucleus of the BNST), "dBNST" (counts in an area
251 containing the dorsal region of the anteromedial BNST), and "vBNST" [Fos counts in a region
252 containing the BNST's anterolateral, fusiform, and anteromedial (ventral) nuclei; refer to
253 (Swanson, 2003)]. As shown in Figure 5B, the average number of Fos-positive nuclei for each of
254 these regions differed in rats undergoing FW or BW conditioning in the ovBNST and vBNST, but
255 not the dBNST. In the vBNST, an ANOVA revealed a main effect of group ($F_{3,43} = 11.41$, $p <$

256 0.0001). Fisher's PLSD revealed that BW rats exhibited significantly higher levels of Fos

257 compared to FW ($p < 0.0005$), NoCS(Test) ($p < 0.001$), and NoTest ($p < 0.0001$) rats. Additionally,

258 NoCS(Test) rats exhibited significantly greater Fos expression than NoTest rats ($p < 0.05$). In the

259 ovBNST, an ANOVA revealed a main effect of group ($F_{3,43} = 3.26$, $p < 0.05$). Post-hoc analyses

260 revealed that NoTest rats exhibited significantly higher Fos levels compared to FW ($p < 0.05$) and

261 No-CS ($p < 0.01$) rats. In dBNST, there was no effect of conditioning procedure on Fos expression

262 (factorial ANOVA: $F < 2.60$, $p > 0.07$). Together, these data indicate that presentation of a BW

263 CS increases Fos expression in the vBNST. Moreover, exposure to the temporally predictive FW

264 CS (which elicited the highest levels of fear) was associated with low levels of Fos in the BNST.

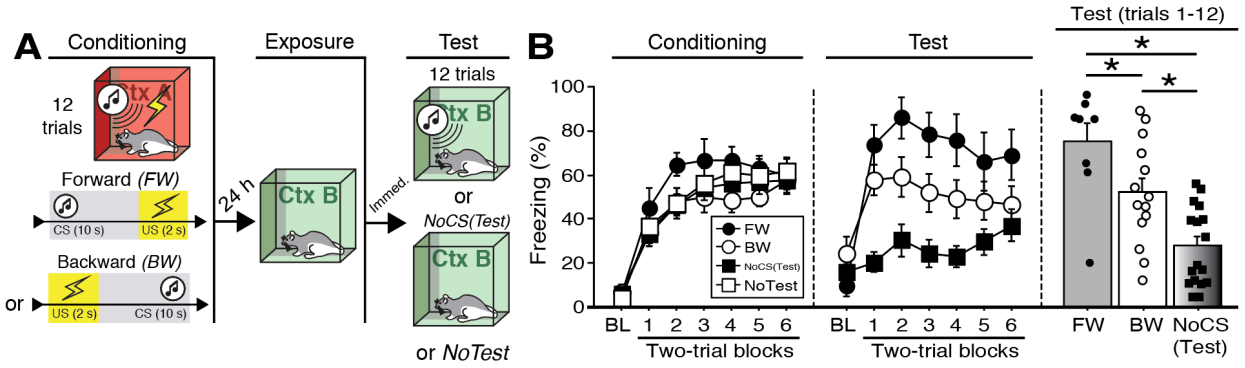

Figure 4. *CS-evoked freezing in rats utilized for Fos analyses.* **(A)** Behavioral schematic. **(B)** Freezing during conditioning and retrieval testing. For conditioning, the left panel depicts mean percentage freezing during the 5-min baseline (BL) and across each conditioning block (each block is comprised of two trials; conditioning trials consist of freezing during the 10-sec CS followed by the 58-sec interstimulus interval). For retrieval testing, the center panel shows mean percentage freezing at the 5-min baseline (BL) and across each test block (each block is comprised of two trials; trials consist of freezing during the 10-sec CS followed by the 60-sec interval). The right panel shows mean percentage freezing after BL. Animals were sacrificed for Fos analyses 90 min after trial 1. All data are represented as means ± s.e.m [FW ($n = 8$); BW ($n = 14$); NoCS(Test) ($n = 17$); NoTest ($n = 8$)]; * = $p < 0.05$.

265

266 ***Backward CSs selectively increase Fos expression in mPFC afferents of the BNST.*** The BNST

267 receives input from many areas involved in the regulation of fear (Fox and Shackman, 2017; Goode

268 and Maren, 2017; Lebow and Chen, 2016). Therefore, we used a functional tracing procedure to

269 quantify Fos expression in neurons targeting the BNST. Specifically, rats were injected with a

retrograde tracer (CTb-488) into the BNST (prior to conditioning) so that we could quantify Fos expression in BNST-projecting neurons in its major limbic and cortical afferents. We quantified Fos in the infralimbic (IL) and prelimbic (PL) regions of the medial prefrontal cortex (mPFC), the basolateral amygdala (BLA), and the ventral hippocampus (HPC). The behavioral data for these animals are shown in Figure 4.

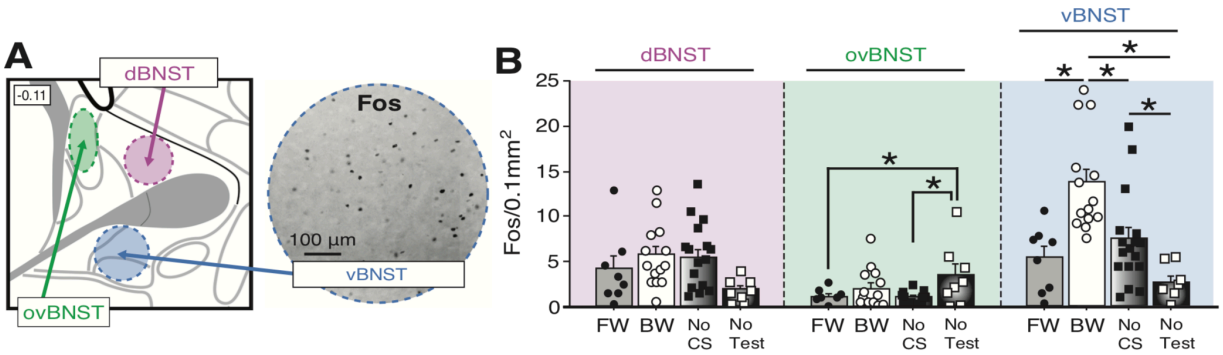

Figure 5. *Fos expression in the BNST following exposure to a temporally predictable or uncertain CS.* **(A)** Schematic depicting regions counted within the BNST (left panel). The right panel shows example of Fos expression in the ventral BNST (vBNST). **(B)** Mean Fos-positive cells per 0.1 mm^2 for each of the quantified regions. All data are represented as means ± s.e.m [FW (n = 8); BW (n = 14); NoCS(Test) (n = 17); NoTest (n = 8)]; * = $p < 0.05$.

A representative image of CTb infusion into the BNST is shown in Figure 6A and an illustration of the largest and smallest CTb spread of injection included in the analyses is shown in Figure 6B. Approximate microinjection sites for CTb for all animals are shown in Figure 6—Figure Supplement 1. Representative Fos and CTb co-labeling is shown in Figure 6C. As shown in Figure 6D, there were reliable differences in CTb labeling among the BNST afferents we quantified. An ANOVA revealed a significant main effect of region ($F_{3,116} = 42.34$, $p < 0.0001$). Post-hoc comparisons indicated that the number of CTb-positive cells in the HPC were significantly higher than that in all other regions ($p < 0.0001$, per comparison); IL and BLA exhibited significantly greater CTb counts compared to PL ($p < 0.0001$, per comparison). CTb counts in each region did not differ by behavioral condition (F's < 0.5, p's > 0.75) and were

collapsed for Figure 6D. These data indicate extensive connectivity of the PFC, BLA, and HPC with the BNST.

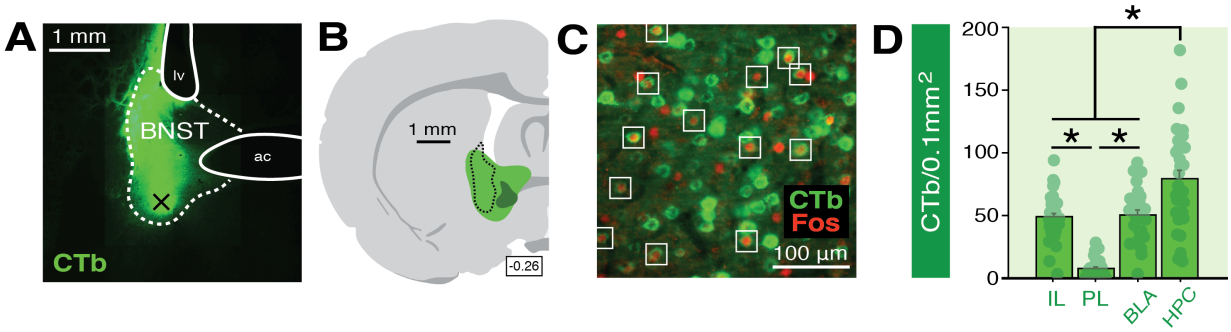

Figure 6. *Functional tracing in afferents targeting the BNST.* **(A)** Coronal section (10×) showing representative fluorescence of a CTb infusion (green) into the BNST (dotted outline; "ac" = anterior commissure, "lv" = lateral ventricle). Black "X" denotes approximate lowest point of the infusion (as an example of how injection sites are documented in Figure 6—Figure Supplement 1). **(B)** Coronal schematic (-0.26 mm from bregma) showing the approximate largest (green) and smallest (dark green) areas of CTb spread in the BNST for animals included in the analyses; the black dotted outline represents the extent of spread of CTb in the BNST in the image shown in panel **A**. **(C)** Example CTb-positive (green) and Fos-positive (red; nuclei) cells in a coronal section (40 µm) of the IL; open white squares denote double-labeled cells. **(D)** Mean number of BNST-targeting CTb-positive cells (per 0.1 mm^2) for each of the quantified regions (shows FW, BW, and NoTest animals corresponding to Figure 8). All data are represented as means ± s.e.m (for each region, $n = 30$); * = $p < 0.05$.

As shown in Figure 7A, the number of Fos-positive nuclei in the IL, PL, HPC, and BLA differed among the behavioral groups. Factorial ANOVA of Fos counts in IL revealed a main effect of group ($F_{2,27} = 8.55$, $p < 0.005$). Post-hoc comparisons for IL revealed significant differences between BW vs. NoTest rats ($p < 0.0005$) and FW vs. NoTest rats ($p < 0.05$). In PL, a main effect of group was also identified ($F_{2,27} = 6.79$, $p < 0.005$). Post-hoc analyses indicated that BW rats exhibited significantly more Fos in PL as compared to NoTest animals ($p < 0.001$). For BLA, a main effect of group was detected (factorial ANOVA: $F_{2,27} = 8.10$, $p < 0.005$). Post-hoc analyses revealed that FW and BW rats (which did not significantly differ) exhibited significantly more Fos in BLA as compared to No Test rats ($p < 0.01$, FW vs. NoTest; $p < 0.001$, BW vs. NoTest). In HPC, an ANOVA revealed a significant main effect of group ($F_{2,27} = 4.28$, $p < 0.05$). Post-hoc comparisons indicated that FW and BW rats (again, which did not significantly differ)

299 had significantly more Fos expression in HPC as compared to NoTest animals ($p < 0.05$ for FW
300 vs. NoTest and BW vs. NoTest). These data indicate that conditioned freezing to forward or
301 backward CSs increased the number of Fos-positive neurons in the PFC, BLA, and HPC.

302 To quantify the fraction of BNST-projecting neurons within the PFC, BLA, and HPC that
303 were activated by a FW or BW CS, we calculated a ratio of Fos-positive to CTb-positive nuclei in
304 each afferent region ("Fos-CTb%"; Figure 7B). Interestingly, within the IL, animals in the BW
305 group exhibited the highest fraction of double-labeled cells. An ANOVA revealed a significant
306 main effect of group ($F_{2,27} = 14.22$, $p < 0.0001$). Post-hoc comparisons revealed significant
307 differences between BW and FW rats ($p < 0.005$) as well as BW and NoTest animals ($p < 0.0001$).
308 In contrast, there were no differences between FW and BW rats in any of the other regions
309 quantified. In PL, there was not a significant main effect of group ($F < 1.90$, $p > 0.17$) and no
310 group effects were observed in the BLA ($F < 2.0$, $p > 0.15$). In the HPC, rats in the FW and BW
311 groups exhibited greater numbers of double-labeled cells than those in the NoTest group ($F_{2,27} =$
312 7.82, $p < 0.01$). Additionally, we examined whether freezing significantly correlates with any of
313 the aforementioned Fos measures; gross measures of freezing percentages (trials 1-12) for FW and
314 BW rats did not significantly correlate with Fos levels across the multiple regions (data not shown
315 in figures), suggesting that the presence or absence of the fear CS was more predictive of activity
316 in these circuits than freezing performance *per se*. Nonetheless, these data indicate that conditioned
317 freezing (in both FW and BW rats) is associated with increased activity in BNST-targeting cells
318 of HPC, and that BW CSs selectively increase Fos in BNST-projecting neurons in IL. Hence, IL
319 projections to the BNST may regulate the effects of BW CSs on BNST Fos expression and
320 freezing.

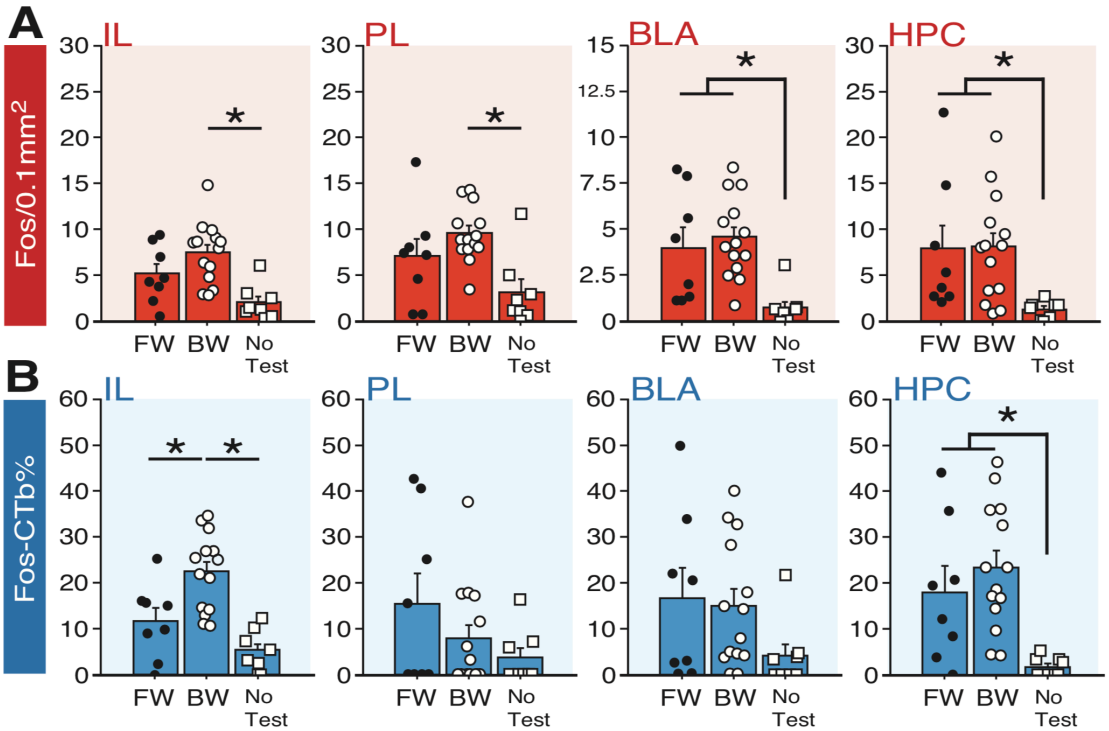

Figure 7. *Fos expression in BNST-targeting cells of the prefrontal cortex, amygdala, and hippocampus following exposure to a forward or backward CS.* **(A)** Mean number of Fos-positive cells (per 0.1 mm^2) for each of the quantified regions. **(B)** Mean percentage (per 0.1 mm^2) of Fos-positive and CTb-positive cells divided by the total number of CTb-positive cells for each region. All data are represented as means ± s.e.m [FW (n = 8); BW (n = 14); NoTest (n = 8)]; * = $p < 0.05$.

DISCUSSION

In the present study, we demonstrate that the BNST is critical for processing ambiguous threats, specifically discrete auditory CSs that are poor predictors of when aversive footshocks will occur. Reversible inactivation of the BNST inactivation disrupted CS-elicited freezing to a backward CS, but did not prevent freezing to a CS that reliably signaled the imminent onset of shock, even when that CS signaled an unpredictable intensity of the US. Moreover, pharmacological blockade of the BNST attenuated fear to both a backward CS and a CS that was trained with random US onset. This data suggests that uncertain timing of shock is a critical factor in recruiting the BNST to conditioned fear. Moreover, BNST inactivation did not affect freezing to a forward CS that was

331 paired with a US that varied in intensity, suggesting that contributions of the BNST to conditioned
332 fear may depend on the type of uncertainty imbued by the CS. Interestingly, freezing to the
333 backward CS was less sustained than that to the forward CS, suggesting that neither long-duration
334 stimuli nor sustained freezing responses are required to recruit the BNST. Finally, although both
335 forward and backward CSs increased Fos expression in the hippocampus, BNST, amygdala, and
336 mPFC, only backward CSs selectively increased Fos expression in both the ventral BNST and
337 BNST-projecting neurons in the infralimbic cortex. Collectively, these data reveal that the BNST
338 and its mPFC afferents may mediate defensive responding to threats that signal uncertain or remote
339 onset of shock.

340 Unpredictable threats come in many forms [including uncertain timing, magnitude or type,
341 or probability of threat: (Bennett et al., 2018; Daldrup et al., 2015; Davies and Craske, 2015;
342 McHugh et al., 2015; McNally et al., 2011; Schroijen et al., 2016; Seidenbecher et al., 2016)];
343 these subtypes of uncertainty may have dissociable and cumulative contributions to anxiety. In
344 particular, uncertainty about the timing of the onset of aversive events has been linked to measures
345 of anxiety in humans and other animals (Amadi et al., 2017; Bennett et al., 2018; Shankman et al.,
346 2011). Interestingly, ambiguous threats (of varying types and degrees) are associated with
347 increases or changes in regional cerebral blood flow and functional connectivity in the primate
348 BNST (Alvarez et al., 2015, 2011; Brinkmann et al., 2018; L Brinkmann et al., 2017; Leonie
349 Brinkmann et al., 2017; Buff et al., 2017; Choi et al., 2014, 2012; Coaster et al., 2011; Fox et al.,
350 2018; Grupe et al., 2013; Herrmann et al., 2016; Kalin et al., 2008, 2005, Klumpers et al., 2017,
351 2015; McMenamin et al., 2014; Meyer et al., 2018; Mobbs et al., 2010; Münsterkötter et al., 2015;
352 Naaz et al., 2019; Pedersen et al., 2018, 2017; Schlund et al., 2013; Sladky et al., 2018; Somerville
353 et al., 2010; Torrisi et al., 2018). In turn, BNST lesions or circuit inactivations reduce defensive
354 responding to contextual cues that poorly signal the imminence of the aversive US [(Asok et al.,

355 2018; Davis and Walker, 2014; Goode et al., 2015; Hammack et al., 2015; Luyten et al., 2011;
356 Poulos et al., 2010; Sullivan et al., 2004; Waddell et al., 2006; Zimmerman and Maren, 2011); but,
357 see (Luyck et al., 2018b)]. Consistent with the possibility that the BNST mediates conditioned fear
358 to temporally uncertain threats, we observed significant and selective decreases in the expression
359 of defensive freezing when we inactivated the BNST in the presence of BW CSs or CSs trained
360 with randomized USs. Conversely, cues that predicted imminent shock were not affected by BNST
361 inactivation. Additionally, we observed significant differences in shock reactivity during forward
362 and backward conditioning, insofar as rats exhibited more robust shock-elicited activity bursts than
363 during forward conditioning. These data may suggest that conditioning in the presence of
364 predictive cues may reduce the magnitude of the unconditioned response, in a way that is not
365 present for the backward CS.

366 A critical finding in the present study is that the BNST mediates conditioned freezing to
367 ambiguous short-duration discrete CSs. This contrasts with prior work suggesting that the BNST
368 only mediates freezing behavior to long-duration cues or contexts (Hammack et al., 2015; Waddell
369 et al., 2006). However, recent data in other paradigms suggest a role for the BNST in the processing
370 of responses to relatively brief threat stimuli (Brinkmann et al., 2018; Choi et al., 2014; Haufler et
371 al., 2013; Kinnison et al., 2012; Kiyokawa et al., 2015; Klumpers et al., 2017; Luyck et al., 2018b,
372 2018a; Naaz et al., 2019). Together, these data suggest that stimulus duration is not necessarily a
373 determinant of BNST involvement. Similarly, it does not appear that response duration is a
374 determinant of BNST in threat reactions. In the present work, conditioned freezing to the backward
375 CS was generally shorter-lived than that to the forward CS, but was more sensitive to BNST
376 inactivation than freezing to the forward CS. One might conclude from this observation that the
377 BNST mediates weak (but not strong) freezing responses. However, we and others have shown
378 that BNST lesions or inactivation reduce the expression of freezing even when those levels are

379 high (Goode et al., 2015; Hammack et al., 2015). Hence, the BNST mediates fear to cues that
380 signal ambiguous or remote threat, independent of the duration of the antecedent stimuli or
381 behavioral responses associated with the threat (Goode and Maren, 2017).

382 Interestingly, it has been suggested that fear to a backward CS relies on associations
383 between the CS and the conditioning context (Chang et al., 2003), which more strongly predicts
384 the US. That is, extinction of fear to the conditioning context reduces fear to a BW, but not FW,
385 CS. Thus, it is possible that the BNST mediates fear expression to a backward CS by retrieving a
386 memory of the context-US association. Indeed, considerable work indicates that BNST is involved
387 in the expression of contextual fear (Davis and Walker, 2014; Goode et al., 2015; Hammack et al.,
388 2015; Luyten et al., 2011; Sullivan et al., 2004; Waddell et al., 2006; Zimmerman and Maren,
389 2011). Increases in immediate early gene expression, metabolic activity, and changes in
390 electrophysiological firing patterns have been observed in the BNST in response to direct exposure
391 to conditioned contextual cues (Campeau et al., 1997; Daldrup et al., 2016; Jennings et al., 2013;
392 Lemos et al., 2010; Luyten et al., 2012; Marcinkiewcz et al., 2016). If the backward CS functions
393 similarly to a conditioned context, then one might expect Fos activity to be elevated in the BNST
394 of animals exposed to the backward CS. Indeed, we observed significantly more Fos expression
395 in the BNST in response to the backward compared to the forward CS. The backward CS-elicited
396 Fos expression was specific to vBNST, insofar as backward and forward rats did not differ in
397 overall levels of Fos in dBNST or ovBNST. Similarly, others have shown elevated Fos expression,
398 in regions in or near vBNST after stress or conditioned fear-related behavior [(Besnard et al., 2019;
399 Sterrenburg et al., 2012; Verma et al., 2018); also, see (Radley and Johnson, 2018; Radley and
400 Sawchenko, 2011)].

401 Of course, another possibility is that the backward conditioning procedure yielded forward
402 trace conditioning after the first trial (Burman et al., 2014; Marchand et al., 2004; Raybuck and

403 Lattal, 2014; Tipps et al., 2014). Although there has been considerable work on the neural
404 mechanisms of trace conditioning (Raybuck and Lattal, 2014), a role for the BNST in this form of
405 learning has not yet been established. Because trace conditioning also degrades the temporal
406 imminence of the US following the CS, it might be expected to require the BNST. Consistent with
407 this possibility, we found that inactivation of the BNST reduced fear in the presence of a cue was
408 conditioned with randomized onset of shock after the CS. In either event, the BNST appears to be
409 recruited by stimuli, whether cues or contexts, that signal remote or ambiguous onset of shock.

410 The amygdala, PFC, and hippocampus have strong connections with the BNST (Canteras
411 and Swanson, 1992; deCampo and Fudge, 2013; Dong et al., 2001; Glangetas et al., 2017; Johnson
412 et al., 2018; McDonald et al., 1999; Reichard et al., 2017; Reynolds and Zahm, 2005; Torrisi et
413 al., 2015; Vertes, 2004; Weller and Smith, 1982; Wood et al., 2018). In line with previous work,
414 the expression of freezing (whether elicited by a forward or, in our case, backward CS) increased
415 the number of Fos-positive neurons in these regions (Herry et al., 2008; Jin and Maren, 2015;
416 Knapska and Maren, 2009; Lemos et al., 2010; Wang et al., 2016). In addition, we found that
417 forward and backward CSs increased Fos expression in BNST-targeting cells of the HPC (with
418 similar, but trending, effects within the BLA). HPC and BLA projections to the BNST are thought
419 to regulate hypothalamic-pituitary-adrenal (HPA) axis activity (Crestani et al., 2013; Forray and
420 Gysling, 2004; Zhu et al., 2001), and these afferents may be engaged in high-fear states to mobilize
421 corticosterone release, for example [also, see (Kim et al., 2013)]. However, unlike the HPC and
422 BLA, we observed a greater number of Fos-positive cells in the IL in response to a backward CS
423 relative to forward CS. This suggests a unique role for BNST-projecting neurons of the IL [which
424 appear to be glutamatergic: (Crowley et al., 2016; Glangetas et al., 2017)] in processing excitatory
425 backwards CSs. There is growing evidence for a regulatory role for prefrontal connections to the
426 BNST in stress and anxiety-like behavior (Fox et al., 2010; Glangetas et al., 2017, 2013; Johnson

427 et al., 2018; Kinnison et al., 2012; Motzkin et al., 2015; Naaz et al., 2019; Radley et al., 2009), the
428 present data adds to this work by suggesting that the IL may be involved in generating freezing
429 and/or stress responding to the backward CS. This would stand in contrast to considerable data
430 indicating that the IL is involved in the inhibition of freezing behavior after extinction, for example
431 (Milad and Quirk, 2002; Quirk and Mueller, 2008). However, the fact that the backward CS elicits
432 less fear (or more extinguishable fear) could suggest this circuit is active, in part, to gate or regulate
433 fear expression. Indeed, there is evidence for a role of the infralimbic cortex in regulating
434 discrimination of conditioned excitors and inhibitors (Sangha et al., 2014). An alternative view is
435 that the IL is involved in processing contextual information conveyed by the hippocampus
436 (Corcoran and Quirk, 2007; Marek et al., 2018; Zelikowsky et al., 2013) during threat processing.
437 To the extent that that backward CSs elicit fear via CS-(context-US) associations (Chang et al.,
438 2003), it is possible that the IL is involved in retrieving CS-context associations.

439 In conclusion, we demonstrate a novel role for the BNST and its circuits in processing
440 discrete cues, including excitatory backward CSs and randomized CSs. Specifically, BNST
441 inactivation impaired conditioned freezing to a backward, but not forward, auditory CS that
442 differed only in their temporal relationship to a footshock US. This reveals that neither stimulus
443 modality nor duration (or response duration) are the critical parameters driving BNST involvement
444 in defensive behavior. We further show that backward CSs increase the number of Fos-positive
445 neurons in the vBNST and in BNST-projecting neurons in the infralimbic cortex. This suggests a
446 novel role for mPFC projections to the BNST in processing ambiguous threats, possibly of the
447 aversive contexts to which backward CSs are associated. These findings may help in our
448 understanding of the broader contributions of the BNST to motivated behaviors that depend on
449 uncertainty, such as during BNST-dependent triggers of fear and drug relapse (Aston-Jones and
450 Harris, 2004; Avery et al., 2016; Goode et al., 2018; Goode and Maren, 2018; Harris and Winder,

2018; Miles et al., 2018; Silberman and Winder, 2013; Stamatakis et al., 2014). In total, these experiments provide new insight into the antecedents for BNST-dependent defensive behavior and highlight behavioral procedures to explore these processes.

MATERIALS AND METHODS

Subjects. All experiments used adult (200-240 g upon arrival; n = 285, before exclusions) male Long-Evans rats (Envigo; Indianapolis, IN). Rats were housed in a climate-controlled vivarium and kept on a fixed light/dark cycle (lights on starting at 7:00 AM and off at 9:00 PM; experiments took place during the light phase of the cycle). Rats were individually housed in clear plastic cages (with bedding consisting of wood shavings; changed weekly) on a rotating cage rack. Group assignments for behavioral testing was randomized for cage position on the racks. Animals had access to standard rodent chow and water *ad libitum*. Animals were handled by the experimenter(s) (~30 sec/day) for five consecutive days prior to the start of any surgeries or behavior. All procedures were in accordance with the US National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Texas A&M University Institutional Animal Care and Use Committee.

Apparatuses. All behavioral testing occurred within distinct rooms in the laboratory. Each behavioral room housed eight identical rodent conditioning chambers (30 cm × 24 cm × 21 cm; MED Associates, Inc.). Each chamber was housed in a larger, external sound-attenuating cabinet. Rear walls, ceilings, and the front doors of the testing chambers were made of Plexiglas, while their sidewalls were made of aluminum. Grid floors of the chambers were comprised of nineteen stainless steel bars (4 mm in diameter), and spaced 1.5 cm apart (center to center). The grid floors

475 were attached to an electric shock source and a solid-state grid scrambler for delivery of the US
476 (MED Associates, Inc.). A speaker attached to each chamber was used to deliver the auditory CS.
477 As needed for each context, the chambers were equipped with 15 W house lights, and small fans
478 were embedded in the cabinets (providing background noise of ~70 dB). An aluminum pan was
479 inserted beneath the grid floor to collect animal waste. A small camera was attached to the top of
480 the cabinet for video monitoring of behavior.

481 Measurements of freezing were performed using an automated and unbiased system
482 (Maren, 1998). Specifically, each behavioral testing chamber rested on a load-cell platform that
483 was sensitive to cage displacement due to the animal's movements. During behavioral testing,
484 load-cell activity values (ranging from -10 to +10 V) were collected and digitized at 5 Hz using
485 Threshold Activity Software (MED Associates, Inc.). Offline conversions of the load-cell activity
486 values were performed to generate absolute values ranging from 0 to 100; lower values indicate
487 minimal cage displacement, which coincided with freezing behaviors in the chambers.
488 Accordingly, freezing bouts were defined as absolute values of ≤10 for 1 s or more. The percentage
489 of freezing behavior during the pre-CS baseline, the CS, and the inter-trial intervals was computed
490 for each behavioral session. Shock reactivity was analyzed by directly reporting the absolute
491 values generated by the Threshold Activity Software (i.e., larger values indicated more movement
492 in the cage).

493 Unique contexts (A and B) were used for conditioning retrieval testing. Chamber
494 assignments were unique to each context and group assignments were counterbalanced across test
495 chambers when possible. For each experiment, context A was assigned to one of the behavioral
496 rooms, and B the other. For context A, the test chamber was wiped down with an acetic acid
497 solution (3%) and a small amount was poured in the pans beneath the grid floors. The cage lights
498 were turned on, while the chamber fans were turned off. The cabinet doors were closed. The

499 behavioral room was illuminated with dim red light. Animals were transported to and from the
500 chambers using white plastic transport boxes. For context B, an ammonium hydroxide solution
501 (1%) was used to wipe down and scent the chambers, black Plexiglas panels were placed over the
502 grid floors, the cage lights were turned off, the chamber fans were turned on, and the cabinet doors
503 remained open. The behavioral room was illuminated with white light (red room lights were turned
504 off). Rats were transported to and from the context using black plastic transport boxes that included
505 a layer of clean bedding.
506
507 **Surgeries.** For animals receiving intracranial microinfusions into the BNST, rats were transported
508 to the surgical suite and deeply anesthetized using isoflurane (5% for induction, 1-2% for
509 maintenance) (Acca et al., 2017; Goode et al., 2015; Nagaya et al., 2015; Zimmerman and Maren,
510 2011). Rats were then secured in a stereotaxic frame (Kopf Instruments). Hair on top of the
511 rodent's head was shaved, povidone-iodine was applied, and a small incision was made in the scalp
512 to expose the skull. Bregma and lambda were aligned on a horizontal plane, and small holes were
513 drilled in the skull for the placement of anchoring screws and bilateral stainless-steel guide
514 cannulas (26 gauge, 8 mm from the bottom of their plastic pedestals; Small Parts). The cannulas
515 were inserted into the BNST at the following coordinates: -0.15 mm posterior to bregma, ±2.65
516 mm lateral to the midline, and -5.85 mm dorsal to dura (guide cannulas were angled at 10° with
517 their needles directed at the midline). Dental cement was used to secure the cannulas to the screws
518 and stainless steel obturators (33 gauge, 9 mm; Small Parts) were inserted into the guide cannulas.
519 Animals were given at least one week to recover prior to the onset of behavioral training.
520 For rats injected with cholera toxin subunit B (CTb) conjugated with Alexa Fluor-488
521 (CTb-488; ThermoFisher Scientific) in the BNST, the surgical procedures were identical to those
522 described above. A single small hole was drilled into the skull to allow for the insertion of a glass

523 micropipette. Injection tips were backfilled with mineral oil and secured in the injector; CTb-488
524 was then drawn up into the injector immediately before use. Rats received unilateral CTb-488
525 infusions into either the left or right hemisphere (group assignments were randomized for sites of
526 CTb-488 infusion): -0.15 mm posterior to bregma, ±2.65 mm lateral to the midline, and -6.50 mm
527 dorsal to dura (the pipette was angled at 10° with the tip directed at the midline). CTb-488 (5.0
528 mg/µl; total volume of 0.25 µl) was microinfused into the brain using a Nanoject II auto-nanoliter
529 injector (Drummond Scientific Co.) secured to the arms of the stereotaxic frame. For the infusion
530 process, 50 nl (25 nl/s) of CTb-488 was infused once per min for 5 min to achieve 0.25 µl of the
531 total infusion (the injection needle was left in the brain for 5 additional minutes to allow for
532 diffusion of CTb-488). Following the infusion procedures, the incision was closed with sutures
533 and the animals were returned to their homecages where they recovered for ~10 days.
534
535 **Intracranial infusions.** Prior to behavioral testing, the animals were acclimated to the process of
536 intracranial infusions. On two occasions, the animals were transported from the vivarium to the
537 infusion room in 5-gallon buckets containing a layer of bedding; the experimenter(s) removed the
538 obturators and replaced them with clean ones. On the day of infusions, animals (in predetermined
539 squads of four to eight rats; representing all drug/behavioral groups as possible) were transported
540 to the laboratory, obturators were removed, and injectors were inserted into the guides. Stainless
541 steel injectors (33 gauge, Small Parts; 9-mm extending 1 mm beyond the end of the guide cannula)
542 were connected to polyethylene tubing (PE-20; Braintree Scientific); the other end of the tubing
543 was connected to gastight 10 µl syringes (Hamilton, Co.). The syringes were mounted to an
544 infusion pump (KD Scientific, Inc.). For the experiments shown in Figure 1 and Figure 1—Figure
545 Supplement 3, the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor
546 antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) was used

547 to reversibly inactivate the BNST (Adami et al., 2017; Davis and Walker, 2014; Goode et al., 2015;
548 Zimmerman and Maren, 2011). NBQX disodium salt hydrate (Sigma Life Sciences) was dissolved
549 in saline to a concentration of 10.0 μg/μl ("NBQX"); physiological saline served as the vehicle
550 ("VEH" for all experiments). For the experiments shown in Figure 2 and 3, the γ-aminobutyric
551 acid $(GABA)_A$ receptor agonist muscimol was used to reversibly inactivate the BNST (Bangasser
552 et al., 2005; Breitfeld et al., 2015; Buffalari and See, 2011; Fendt et al., 2003; Goode et al., 2015;
553 Markham et al., 2009; Pina et al., 2015; Sajdyk et al., 2008; Xu et al., 2012). Muscimol (Sigma-
554 Aldrich) was dissolved in physiological saline to a concentration of 0.1 μg/μl ("MUS" for Figure
555 3 and 5); physiological saline served as the vehicle ("VEH"). For the representative image showing
556 drug spread in the BNST (Figure 1—Figure Supplement 1), muscimol TMR-X conjugate (Thermo
557 Fisher Scientific) was dissolved in physiological saline to a concentration of 0.1 μg/μl and used
558 for infusions. For all of the aforementioned experiments, drug or vehicle was drawn into the
559 injectors (immediately prior to the infusions) and a total volume of 0.275 μl/hemisphere of drug
560 or vehicle was infused at a rate of 0.275 μl/min; injectors were left in the cannulas for 1 min
561 following the infusions to allow for diffusion. Once the injectors were removed, clean obturators
562 were inserted into the guides.
563
564 **Behavioral procedures and exclusions.** Overviews of each behavioral experiment are provided
565 in the figures. The conditioned stimulus (CS) for all experiments was an auditory tone (80 dB, 2
566 kHz, 10 sec), which was paired with the unconditioned stimulus (US, footshock; 1.0 mA, 2 sec).
567 For the variable shock intensity experiment (Figure 4), US intensity varied as described.
568 *FW/BW/NoCS(Test) BNST inactivation (twelve training trials).* In a 3 × 2 design, animals
569 (n = 64, prior to exclusions) were randomly assigned to receive a CS at test [either a forward
570 ("FW")- or backward ("BW")-trained CS] or no CS at test [animals were trained to a BW CS;

571 “No-CS”], and NBQX (“NBQX”) or vehicle infusions (“VEH”). Of these rats, seven were
572 excluded due to off-target cannulas, three additional rats were excluded due to illness, and four
573 more were excluded due to a technical error during infusions that resulted in no drug or vehicle to
574 be infused (fourteen total exclusions). This resulted in the following (final) group numbers (shown
575 in data/figures): FW-VEH (n = 5); FW-NBQX (n = 4); BW-VEH (n = 13); BW-NBQX (n = 12);
576 No-CS-VEH (n = 8); No-CS-NBQX (n = 8). For conditioning, animals (in squads of eight rats)
577 were transported from the vivarium to context A; FW and BW procedures were run in alternating
578 squads of animals. Drug and vehicle assignments were counterbalanced for position in the
579 chambers. For FW conditioning, were placed in the context for 5 min before the delivery of twelve
580 CS-US trials (CS offset immediately preceded US onset; 70-sec intertrial interval). After the final
581 conditioning trial, the animals remained in the chamber for 1 min before being returned to their
582 homecages (the entire conditioning session consisted of 19 min total; for both FW and BW
583 conditioning). For BW conditioning, all aspects of conditioning were identical to the FW training
584 except the order of the CS and US was reversed. That is, after a 5-min baseline period, the animals
585 received 12 US-CS trials (the onset of the CS occurred immediately after the offset of the US; 70-
586 sec intertrial intervals). After conditioning, rats were returned to their homecages.

587 Twenty-four hours after conditioning, animals (in squads of four) were infused with NBQX
588 or VEH into the BNST immediately before being placed in context B. After 5 min of acclimation
589 to the context, FW and BW animals (intermixed in each squad) received twelve presentations of
590 the CS in the absence of the US (70-sec intertrial interval). The rats remained in the chambers for
591 1 min following the final CS (19 min session, in total). For rats not receiving the CS at test, the
592 animals remained in the test context for 19 min without presentation of the CS or US. We alternated
593 running squads that received the CS and those that did not after the infusions. Following the test,
594 animals were returned to their homecages.

595 *FW/BW BNST inactivation (five training trials).* In a 2 × 2 design, animals (*n* = 28, prior
596 to exclusions) were randomly assigned to receive forward ("FW") or backward ("BW")
597 conditioning using five training trials, and NBQX ("NBQX") or vehicle infusions ("VEH"). Of
598 these rats, three were excluded due to off-target cannulas. This resulted in the following (final)
599 group numbers (shown in data/figures): FW-NBQX (*n* = 6); FW-VEH (*n* = 6); BW-NBQX (*n* =
600 6); BW-VEH (*n* = 7). At the start of behavior, rats (in squads of seven) were transported from the
601 vivarium and placed in context A. We alternated squads that received forward or backward
602 conditioning. For forward conditioning, rats were given 3 min of acclimation to the context prior
603 to the onset of five CS-US trials. Rats remained in the chamber for 1 min following the final trial
604 (530 sec total conditioning session). For backward conditioning, rats were placed in context a for
605 a 3-min baseline (530 sec session total), after which they received five US-CS trials. Rats were
606 returned to their homecages after conditioning. Twenty-four hours after conditioning, the animals
607 were placed in context B in the absence of the CS or US for 530 sec to acclimate them to the test
608 context. After this acclimation session, rats were again returned to their homecages.

609 Forty-eight hours after conditioning, the animals received NBQX or VEH infusions
610 (identical to above) immediately prior to retrieval testing. FW and BW animals were intermixed
611 in each squad. Immediately after the infusions, animals were placed in context B for a retrieval
612 test to assess freezing to the CS. The test (530 sec in total) consisted of a 3-min baseline period
613 followed by five CS-alone presentations (70-s intertrial interval). Rats were returned to their
614 homecages following the test session.

615 *RANDOM/BW/NoCS(Cond) BNST inactivation.* In a 3 × 2 design, rats (*n* = 70, prior to
616 exclusions) were randomly assigned to receive backward conditioning ("BW"; identical to the 12-
617 trial BW conditioning described above), randomized CS/US trials in which the duration of the
618 intervals between CS offset and US onset vary on each trial ("RANDOM"), or US-only contextual

619 conditioning [shock intervals matched the RANDOM group; "NoCS(Cond)"]. Of these groups,
620 animals were randomly assigned to receive muscimol ("MUS") or vehicle ("VEH") infusions
621 immediately prior to retrieval. For this experiment, 30 animals were excluded due to off-target
622 cannulas. This resulted in the following (final) group numbers (shown in data/figures): BW-VEH
623 (n = 5); BW-MUS (n = 7); RANDOM-VEH (n = 6); RANDOM-MUS (n = 9); NoCS(Cond)-VEH
624 (n = 7); NoCS(Cond)-MUS (n = 6).

625 For conditioning, animals (in squads of five to eight rats; counterbalanced for the type of
626 conditioning) were transported to context A and underwent BW, RANDOM, or NoCS(Cond)
627 training. Drug and vehicle assignments were counterbalanced for the conditioning procedure and
628 chamber placements for each squad. For BW animals, the conditioning parameters matched those
629 used for the 12-trial experiment described above. For RANDOM animals, and after the 5-min
630 baseline, CS presentations matched the 60 s interstimulus intervals used for the 12-trial FW
631 paradigms (noted above); US onset occurred at the following intervals after offset of each CS at
632 each trial: 26 s, 52 s, 10 s, 40 s, 16 s, 52 s, 14 s, 50 s, 18 s, 36 s, 48 s, 6 s (the entire session last 19
633 min). For NoCS(Cond) animals, rats experienced the same protocol as the RANDOM group, but
634 no auditory CS occurred. Rats were returned to their homecages following conditioning.

635 Twenty-four hours later, animals (in squads of four to six) were transported to the
636 laboratory to receive pre-retrieval infusions of MUS or VEH (counterbalanced in each squad).
637 BW, RANDOM, and NoCS(Cond) animals were intermixed in each squad. Immediately after the
638 intracranial infusions, and after a 5-min baseline in context B, each squad of rats received twelve
639 presentations of the CS in the absence of the US. Each CS presentation was separated by 70 sec
640 intertrial intervals, with the entire test lasting 19 min. Rats were returned to their homecages
641 following the test session.

642 *FIXED/VARIABLE BNST inactivation.* In a 2 × 2 design, rats (*n* = 31, prior to exclusions)
643 were randomly assigned to receive forward conditioning with consistent ("FIXED") or variable
644 magnitudes of the US ("VARIABLE"); muscimol ("MUS") or vehicle ("VEH") infusions were
645 made prior to retrieval testing. For FIXED animals, US intensity was 1 mA at each trial. For
646 VARIABLE animals, shock intensity (in mA) varied across trials in this order (mean = 1 mA):
647 0.5, 1.8, 0.4, 1.6, 1.4, 0.3, 0.5, 1.8, 0.4, 1.6, 1.4, 0.3. In this experiment, one rat was excluded due
648 to off-target cannulas. This resulted in the following (final) group numbers (shown in data/figures):
649 FIXED-VEH (*n* = 7); FIXED-MUS (*n* = 8); VARIABLE-VEH (*n* = 7); VARIABLE-MUS (*n* =
650 8).

651 For conditioning, animals (in squads of seven to eight) were transported to context A
652 (squads alternated between FIXED and VARIABLE paradigms). The rats were given 5 min to
653 acclimate to the context before the onset of 12 CS-US pairings (70-sec intertrial intervals). Rats
654 remained in the chambers for 1 min following the final CS, with the entire conditioning session
655 lasting 19 min. Rats were returned to their homecages following conditioning.

656 Twenty-four hours later, rats (in squads of seven to eight) received intracranial infusions
657 of MUS or VEH into the BNST immediately before being placed in context B for retrieval testing.
658 Rats were given a 5-min baseline period before the onset of twelve CS-alone presentations (70-
659 sec intertrial intervals). Rats remained in the chambers for 1 min following the final CS, with the
660 entire test session lasting 19 min. Rats were returned to their homecages following the test.

661 *FW/BW Fos-CTb.* Animals (*n* = 60, before exclusions) were randomly assigned to receive
662 a forward ("FW")- or backward ("BW")-trained CS at testing, or no CS retrieval at test
663 ["NoCS(Test)"]. Note that the No-CS group consists of animals that were trained to either a FW
664 or BW CS. Additionally, a group of BW-trained animals remained in their homecages ("NoTest")
665 during the final test and were sacrificed alongside the other groups. Twelve rats were excluded for

666 either excessive or off-target infusion of CTb-488 outside the borders of the BNST or an absence
667 of CTb-488 labeling in the ventral BNST. One additional rat was excluded due to a technical issue
668 that resulted in the loss of tissue at the level of the prefrontal cortex. This resulted in the following
669 (final) group numbers (shown in data/figures): FW (n = 8); BW (n = 14); No-CS [n = 17 (BW-
670 trained: n = 9; FW-trained: n = 8)]; NoTest (n = 8). For behavioral training, rats (in squads of six
671 to eight; with all groups intermixed) were transported to the laboratory and placed in context B to
672 acclimate for 5 min (no CS or US) and then returned to their homecages. Later that day, the animals
673 were transported to context A where they received twelve trials of either FW or BW fear
674 conditioning, which was identical to the other aforementioned procedures. We alternated FW and
675 BW conditioning squads. After conditioning, the rats were returned to their homecages. Twenty-
676 four hours after conditioning, all rats were exposed to context B for 20 min in the absence of the
677 CS or US to acclimate them to the test context.

678 Forty-eight hours later, FW, BW, and No-CS rats were transported to context B to receive
679 a retrieval test to the CS or were merely placed in the context (No-CS condition). Squads alternated
680 between CS and No-CS test. For rats undergoing CS retrieval, FW and BW animals (intermixed
681 in each squad) received CS trials as described previously. Rats were perfused 90 min following
682 the first CS of the test, in groups of three to four. For No-CS rats (with FW- and BW-trained
683 animals intermixed), animals were exposed to context B in the absence of the CS or US. No-CS
684 rats were perfused 95 min after being placed in the test context, in groups of three to four. NoTest
685 rats were perfused alongside groups of FW, BW, and No-CS rats. Rats were returned to their
686 homecages after testing and prior to the perfusions.

687 *FW/BW intra-shock reactivity.* Rats (n = 32, no exclusions) were randomly assigned to
688 receive forward ("FW") or backward ("BW") conditioning. No rats were excluded from this
689 experiment (no infusions occurred; n = 16, per group); only data from the conditioning session are

690 shown. Animals (in squads of eight) were transported to context A for either FW or BW
691 conditioning. Parameters for FW and BW conditioning were identical to the procedures for the
692 previous 12-trial experiments. We alternated FW and BW squads. Rats were returned to their
693 homecages following training.
694
695 **Histological procedures.** At the conclusion of behavioral testing, cannula-implanted animals were
696 overdosed with sodium pentobarbital (Fatal Plus; 100 mg/ml, 0.5 ml, i.p.). Transcardial perfusions
697 were then performed using chilled physiological saline followed by 10% formalin. Brains were
698 extracted and stored in 10% formalin for 24 hr at 4° C; brains were transferred to a 30% sucrose-
699 formalin solution for three or more days (at 4° C) before sectioning. Brains were flash frozen with
700 crushed dry ice and coronal sections (40 μm) containing the BNST were collected using a cryostat
701 (Leica Microsystems) at -20° C. The tissue was wet-mounted to gelatin-subbed microscope slides
702 and stained with 0.25% thionin prior to adding glass coverslips secured with mounting medium
703 (Permount, Sigma). To further examine the spread of drug, a subset of animals was infused
704 (identical to the aforementioned infusion parameters) with fluorescent muscimol (1.0 μg/μl;
705 EverFluor TMR-X conjugate, Setareh Biotech) before being sacrificed (these animals were not
706 perfused) and having brains dissected (40 μm; brains were stored in 30% sucrose solution at 4° C
707 until sectioning). These tissues were wet-mounted to slides and aqueous mounting medium
708 (Fluoromount; Sigma-Aldrich) was used to secure glass coverslips.
709 For CTb-injected animals, post-behavior perfusions mirrored the aforementioned
710 procedures. For sectioning, coronal sections (which included regions of the prefrontal cortex,
711 BNST, basolateral amygdala, and ventral hippocampus) were collected into well plates containing
712 phosphate-buffered saline (1× PBS) and stored in the dark at 4° C until immunohistochemistry

713 could be performed. For localization of the CTb-injection site, separate sections at the level of the
714 BNST were wet-mounted and coverslipped using Fluoromount mounting medium.
715
716 **Immunohistochemistry.** Immunohistochemistry for Fos was performed on free-floating brain
717 tissue similar to prior reports (Jin and Maren, 2015; Marek et al., 2018; Orsini et al., 2011; Wang
718 et al., 2016). For sections containing the BNST, Fos was stained using the following procedures
719 [all steps were performed at room temperature (~20° C) on a shaker, unless stated otherwise; rinses
720 were brief (~20 sec)]. The tissue was first rinsed in 1× tris-buffered saline (TBS; 7.4 pH), and then
721 incubated in 0.3% H_2O_2 (in TBS) for 15 min, followed by rinses (×3) in TBS. Slices were
722 transferred to primary antibody [rabbit anti-c-fos, 1:10,000 in 1× TBS containing Tween 20
723 (TBST); Millipore] and incubated overnight. Sections were then rinsed (×3) in TBS before
724 incubating in secondary antibody for 1 hr (biotinylated goat anti-rabbit, 1:1,000 in TBST; Jackson
725 Immunoresearch). Sections were rinsed (×3) again in TBS. The slices were transferred to wells
726 containing avidin biotin complex (ABC, 1:1,000 in TBST; Vector Labs) for 45 min. The tissues
727 were again rinsed (×3) in TBS. Tissue was transferred to wells containing 3,3′ diaminobenzidine
728 [(DAB) 5% stock, 1:200], nickel ammonium sulfate (5% stock, 1:10), and 30% H_2O_2 (1:2,000) in
729 TBS for 10 min to generate purple/black nuclear chromophore products. After another rinse (×3)
730 in TBS, the tissue was subsequently wet-mounted to slides and secured with coverslips using
731 Permount mounting medium.
732 For sections containing the prefrontal cortex, amygdala, and hippocampus (Fos-CTb
733 experiment), Fos was stained using the following procedures [unless stated otherwise, each step
734 occurred at room temperature (~20° C) on a shaker (and away from excess light)]. First, the tissue
735 was rinsed (10 min; ×2) in 1× TBS, followed by a 10 min wash in 1× TBST. The tissue was
736 incubated in 10% normal donkey serum (NDS; in TBST) for 1 hr. The slices were then rinsed (5

737 min; ×2) in TBST. Sections were transferred to primary antibody [goat anti-c-fos, 1:2,000 in 3%
738 NDS (in TBST); Santa Cruz Biotechnology] and incubated on a rotator in the dark for 72 hr at 4°
739 C. The tissue was rinsed (10 min; ×3) in TBST before incubating in secondary antibody
740 [biotinylated donkey anti-goat, 1:200 in 3% NDS (in TBST); Santa Cruz Biotechnology] for 2 hr.
741 Slices were then rinsed (10 min; ×3) in TBST. Sections were transferred to wells containing
742 streptavidin (Alexa-Fluor 594-conjugate, 1:500 in 3% NDS (in TBST); Thermo Fisher Scientific]
743 for 1 hr. Tissue was rinsed (10 min; ×3) in TBS before being wet-mounted to slides and secured
744 with coverslips using Fluoromount mounting medium.
745
746 **Image analyses.** All imaging and counting procedures (for all regions) were performed with the
747 experimenter(s) blind to the group assignments of the animals. For thionin-stained coronal tissue,
748 photomicrographs of cannula in the BNST were generated (10× magnification) using a Leica
749 microscope (MZFLIII) and Leica Firecam software. For animals infused with fluorescent
750 muscimol into the BNST, infusion sites were imaged (10× magnification) using a Zeiss
751 microscope and Axio Imager 2 software (Zen Pro 2012). For CTb-injected animals, BNST images
752 were generated (10× magnification) using the same Zeiss microscope and software.
753 For Fos analyses in BNST, brightfield images of BNST [in BNST regions ranging from
754 approximately -0.00 to -0.50 mm posterior to bregma of the skull, and from both left and right
755 hemispheres (randomized for site of CTb-injection)] were generated (10× magnification) using a
756 Zeiss microscope and Axio Imager 2 software (Zen Pro 2012). ImageJ software (Schneider et al.,
757 2012) was used to count cells. Counts were confined to the following areas of interest: (1)
758 "ovBNST" [an area of 0.217 mm × 0.558 mm (oval); targeting the oval nucleus of the BNST], (2)
759 "dBNST" [0.372 mm^2 (circle); targeting the dorsal and medial subregions of the anterior BNST,
760 including the anteromedial area of the BNST (dorsal to the anterior commissure)], and (3)

761 "vBNST" [0.434 mm^2 (circle); targeting the ventral regions (ventral to the anterior commissure)
762 of the anterior BNST, which includes the ventral portion of the anteromedial area, the anterolateral
763 area, and the fusiform nucleus of the BNST] (Swanson, 2003). For each of these regions, three to
764 six images were quantified and averaged for each animal (Fos levels were standardized to 0.1
765 mm^2).

766 For Fos-CTb analyses in the prefrontal cortex, amygdala, and hippocampus, images (10×
767 magnification) of Fos and CTb expression were generated using a Zeiss microscope and Axio
768 Imager 2 software (Zen Pro 2012). Cell counts were analyzed using ImageJ software. Images were
769 generated and analyzed only for the hemisphere injected with CTb. Counts were confined to the
770 following areas of interest: (1) "IL" [an area of 0.805 mm × 0.217 mm (rectangle); targeting deep
771 and superficial layers of the infralimbic cortex (approximately +2.0 to +3.2 mm anterior to bregma
772 of the skull)], (2) "PL" [1.115 mm × 0.217 mm (rectangle); targeting deep and superficial layers
773 of the prelimbic cortex (approximately +2.0 to +3.2 mm anterior to bregma of the skull)], (3)
774 "BLA" [0.434 mm^2 (circle); targeting the basolateral amygdala (approximately -1.8 to -3.5 mm
775 posterior to bregma of the skull)], and (4) "HPC" [0.496 mm × 0.217 mm (rectangle); targeting
776 the ventral subiculum but may include some CA1 cells of the ventral hippocampus (approximately
777 within -4.7 to -7.0 mm posterior to bregma of the skull)] (Swanson, 2003). For each of these
778 regions, three to six images were quantified and averaged for each animal (all counts and
779 percentages were normalized to 0.1 mm^2).

780

781 **Statistics.** All data were submitted to repeated or factorial analysis of variance (ANOVA) or two-
782 tailed *t*-tests as described for each experiment. Fisher's protected least significant difference
783 (PLSD) test was used for *post hoc* comparisons of group means following a significant omnibus *F*
784 ratio in the ANOVA (α was set at 0.05). No statistical methods were used to predetermine group

785 sizes (group sizes were selected based on prior work and what is common for the field). Data
786 distributions were assumed to be normal, but these were not formally tested. Unless stated
787 otherwise, all data are represented as means ± s.e.m.
788

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795

796 **ADDITIONAL INFORMATION**
797 **Competing interests**
798 The authors declare no competing interests.
799

800 **Author contributions**
801 T.D.G. and S.M. designed the research; T.D.G., R.R., G.M.A., and O.W.M. performed the
802 research; T.D.G., R.R., G.M.A., O.W.M., and S.M. analyzed data; T.D.G. and S.M. wrote the
803 paper.

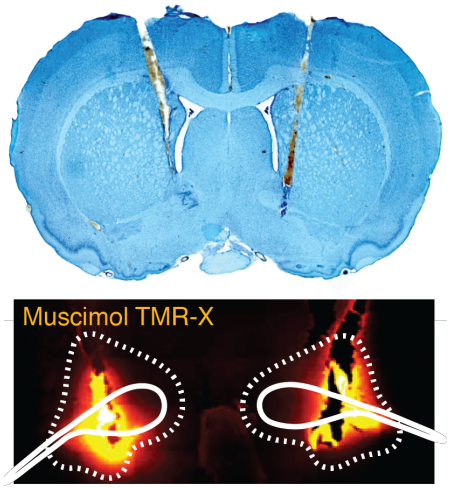

Figure 1—Figure Supplement 1
Representative bilateral cannula placements in the BNST. Photomicrograph (10×) of a thionin-stained coronal section depicting representative cannula tracts in the BNST (top panel). Fluorescent image (gold filter) of a coronal section (10×) showing spread of drug in BNST (BNST outlined in dotted line) (bottom panel).

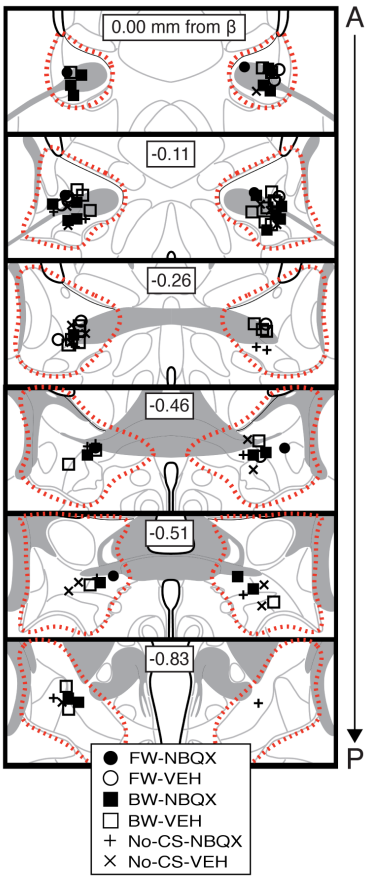

Figure 1—Figure Supplement 2
Bilateral cannula placements for BNST microinfusions. Schematic depicting cannula placements for Figure 1. Symbols (split by each group) correspond to injector tips (approximate borders of BNST are shown by red dotted outline; approximate borders of BNST are shown by red dotted outline).

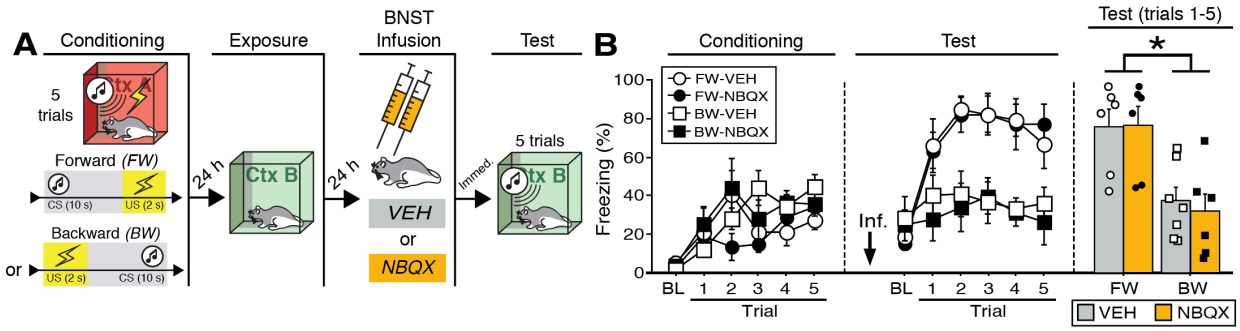

Figure 1—Figure Supplement 3

Effects of BNST inactivation on freezing to a forward vs. backward CS trained with five trials. **(A)** Behavioral schematic. **(B)** Freezing behavior during conditioning and retrieval testing. For conditioning, the left panel depicts mean percentage freezing during the 3-min baseline (BL) and across each conditioning block (each 138-sec block is comprised of two trials; conditioning trials consist of freezing during the 10-sec CS followed by the 58-sec interstimulus interval). For retrieval testing, the center panel shows mean percentage freezing at the 3-min baseline (BL) and across each test trial (each trial consists of freezing during the 10-sec CS followed by the 60-sec interstimulus interval). The right panel shows mean percentage freezing after baseline (trials 1-5; corresponding to 350 sec of behavior). All data are represented as means ± s.e.m [FW-VEH (n = 6); FW-NBQX (n = 6); BW-VEH (n = 7)]; BW-NBQX (n = 6)]; * = $p < 0.05$.

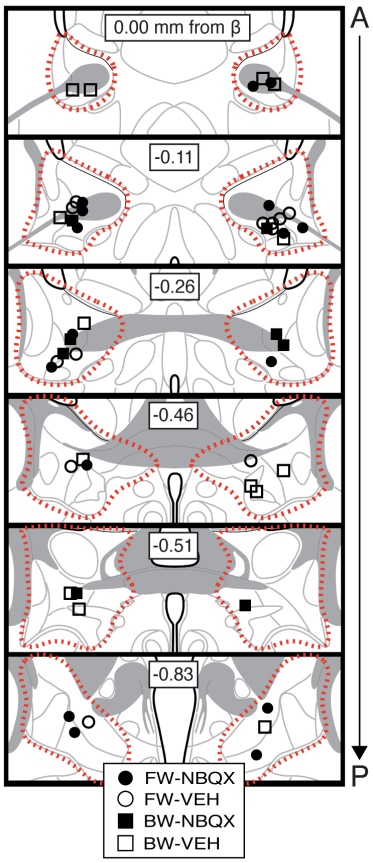

Figure 1—Figure Supplement 4
Bilateral cannula placements for BNST microinfusions. Schematic depicting cannula placements for Figure 1—Figure Supplement 3. Symbols (split by each group) correspond to injector tips (approximate borders of BNST are shown by red dotted outline).

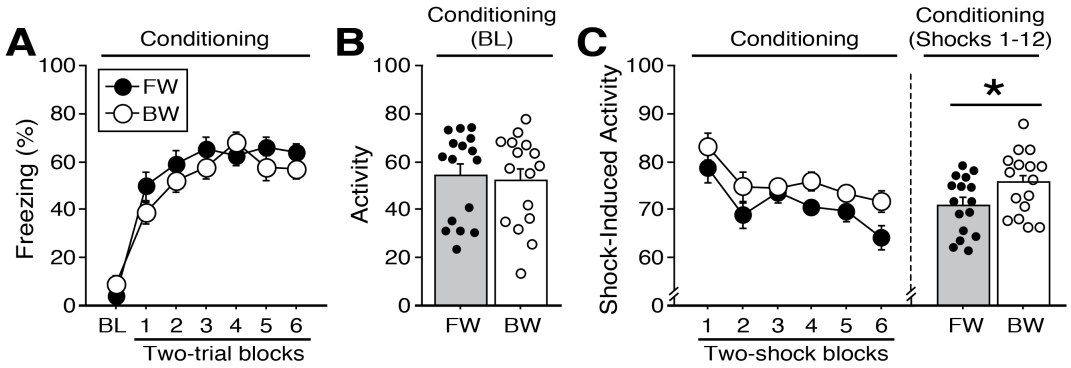

Figure 1—Figure Supplement 5. *Shock-induced activity during conditioning to a forward vs. backward CS.* **(A)** Mean percentage of freezing at baseline (BL; 5-min) and across conditioning blocks (each 138-sec block is comprised of two trials; trials consist of freezing during the 10-sec CS followed by the 58-sec interstimulus interval). **(B)** Mean activity values across the 5-min BL (no shock present). **(C)** The left panel shows mean shock-induced activity (during shocks) at conditioning [averaged into 4-sec (two-shock) blocks]. The right panel shows mean shock-induced activity across all trials. All data are represented as means ± s.e.m [FW (n = 16); BW (n = 16)]; * = $p < 0.05$.

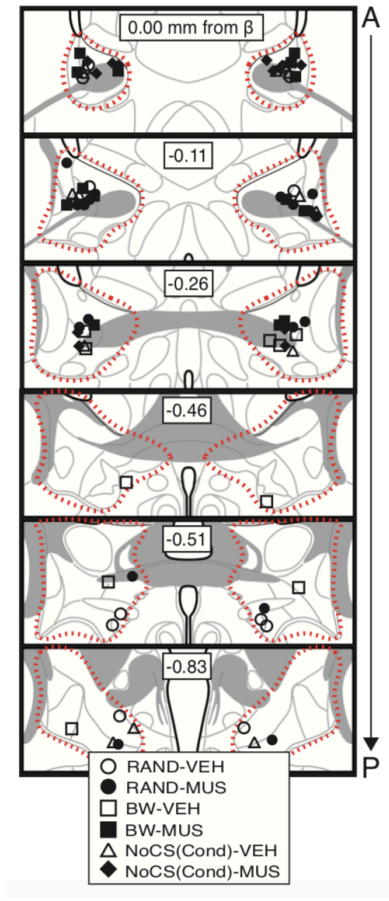

Figure 2—Figure Supplement 1
Bilateral cannula placements for BNST microinfusions. Schematic depicting cannula placements for Figure 2. Symbols (split by each group) correspond to injector tips (approximate borders of BNST are shown by red dotted outline).

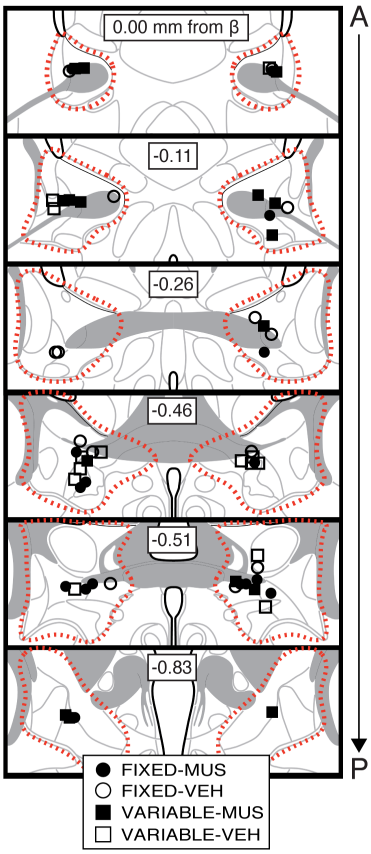

Figure 3—Figure Supplement 1
Bilateral cannula placements for BNST microinfusions. Schematic depicting cannula placements for Figure 3. Symbols (split by each group) correspond to injector tips (approximate borders of BNST are shown by red dotted outline).

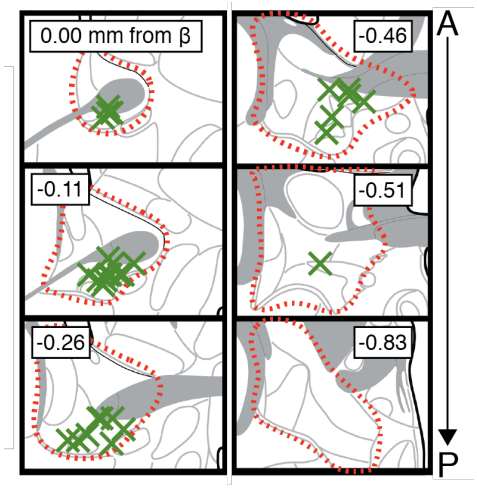

Figure 6—Figure Supplement 1
CTb injection sites in BNST. Approximation of the most ventral and centermost sites of unilateral microinjection of CTb (green X's) for all animals shown in Figure 6 and 7 (red outline approximates borders of BNST).

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