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# Reversible inactivation of the bed nucleus of the stria terminalis prevents reinstatement but not renewal of extinguished fear

BNST and fear relapse

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T.D.G., J.J.K., and S.M. designed research; T.D.G. and J.J.K. performed research; T.D.G., J.J.K., and S.M. analyzed data; T.D.G. and S.M. wrote the paper.

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**Abstract**

The extinction of conditioned fear is labile. For example, fear to an extinguished conditioned stimulus (CS) returns after presentation of an aversive stimulus ('reinstatement') or a change in context ('renewal'). Substantial research implicates the bed nucleus of the stria terminalis (BNST) in the stress-induced relapse of extinguished behaviors, such as in instrumental drug seeking, but its role in the relapse of extinguished fear responses is not clear. Here, we explored the role of the BNST in both reinstatement and renewal of fear, two forms of relapse that are differentially triggered by stress. In Experiment 1, rats received pairings of an auditory CS and footshock unconditioned stimulus (US) followed by an extinction procedure. After extinction, rats received an unsignaled US to reinstate fear to the extinguished CS. Twenty-four hours later, they were infused with either muscimol or vehicle into the BNST immediately prior to a CS retrieval test. In Experiment 2, rats were conditioned and extinguished in two distinct contexts. Twenty-four hours after extinction, the rats were infused with muscimol, NBQX, or vehicle immediately prior to a CS retrieval test in either the extinction context or a different (but familiar) context. In both experiments, freezing behavior served as the index of conditioned fear. The results revealed that BNST inactivation prevented reinstatement (Experiment 1), but not renewal (Experiment 2), of conditioned freezing to the extinguished CS. Hence, the BNST is critical for the reinstatement of extinguished fear in an aversive context, but not for the contextual retrieval processes that mediate fear renewal.

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**Significance Statement**

Relapse of extinguished fear is a major challenge to clinical treatments of fear-related anxiety disorders (e.g., exposure therapy). Pavlovian fear conditioning and extinction are important models for understanding the behavioral and brain mechanisms underlying fear relapse. Here we explore the role of the bed nucleus of the stria terminalis (BNST) in two different forms of fear relapse in rats: 'reinstatement' and 'renewal'. We find that reversible inactivation of the BNST prevents the reinstatement, but not renewal, of extinguished fear. This reveals a dissociation in the role of the BNST in different forms of relapse, a finding that will serve to enhance the selectivity of neural interventions for anxiety disorders.

## Introduction

Fear relapse plagues clinical interventions for fear-related anxiety disorders (Hooley, 2007; Boschen et al., 2009; Vervliet et al., 2013b). Various factors—such as the nature of the therapeutic intervention, duration of time since treatment, and intervening stress—have been shown to be important in determining the degree of retention of extinguished fear in humans and other animals (Kehoe and Macrae, 1997; Bouton, 2002; Myers and Davis, 2002; Bouton et al., 2006; Hermans et al., 2006; Fitzgerald et al., 2013; Bouton, 2014; Goode and Maren, 2014; Luck and Lipp, 2015). Pavlovian fear conditioning and extinction in rodents provides a clinically relevant model to explore the behavioral and brain mechanisms of relapse. Specifically, fear conditioning in rats is a behavioral procedure through which subjects experience concomitant pairings of a neutral conditioned stimulus (CS), such as a tone, with an aversive unconditioned stimulus (US), such as a footshock (Rescorla, 1988a, 1988b). After fear conditioning, presentation of the CS alone comes to elicit conditioned fear responses (CRs), including freezing behavior (Fendt and Fanselow, 1999; LeDoux, 2000; Maren, 2001). Fear CRs also occur in the place or 'context' in which fear conditioning was experienced (Bouton and King, 1983; Maren et al., 2013).

After conditioning, repeated presentations of the CS in the absence of footshock lead to the extinction of fear (Pavlov, 1927; Chang et al., 2009). It is widely believed that research on fear extinction in rodents can enhance our understanding of exposure therapy in humans (Barad et al., 2005; Milad et al., 2006; Milad et al., 2014; Morrison and Ressler, 2014). Extensive research indicates that extinction training does not necessarily erase fear memory; rather it results in a new 'inhibitory' memory that limits the expression of the fear (Konorski, 1967;

Bouton, 2004; Maren, 2011). Consequently, extinction memories are susceptible to relapse. Two forms of fear relapse have received considerable attention over the years: 'reinstatement' and 'renewal'. Reinstatement of fear occurs when an aversive, unsignaled US is experienced prior to presentation of the extinguished CS (Rescorla and Heth, 1975; Bouton and Bolles, 1979). Reinstatement is most robust in contexts in which reinstating shocks are delivered, although it can also occur in contexts never paired with shock (Westbrook et al., 2002; see also Morris et al., 2005a; Halladay et al., 2012; Goode et al., 2015). This suggests that reinstatement can be mediated by either direct context-US associations (Bouton and King, 1983; Bouton et al., 2006), or though stress states that generalize across contexts (Haroutunian and Riccio, 1977; Morris et al., 2005b; Deschaux et al., 2013). Fear renewal, on the other hand, occurs when a CS is presented outside of its extinction context (Bouton and Bolles, 1979; Polack et al., 2013; Vervliet et al., 2013a). Importantly, renewal does not require that the animal experience the US after extinction. Indeed, direct context-US associations do not mediate renewal (Bouton and Ricker, 1994; Harris et al., 2000; Corcoran and Maren, 2004).

The different roles context plays in reinstatement and renewal suggest that distinct neural circuits mediate them. One brain area that has been implicated in reinstatement is the bed nucleus of the stria terminalis (BNST). In particular, BNST lesions impair the shock-induced reinstatement of fear (Waddell et al., 2006; see also Waddell et al., 2008). Sustained fear responses to conditioned contexts (Sullivan et al., 2004) and long-duration CSs (Waddell et al., 2006) also appear to rely on the BNST (also, see Walker and Davis, 1997). Conversely, BNST manipulations do not affect fear to short-duration CSs paired with shock (LeDoux et al., 1988; Sullivan et al., 2004; Waddell et al., 2006; Zimmerman and Maren, 2011). Coinciding with this evidence, BNST circuitry is also involved in the stress-induced reinstatement of drug seeking.

For example, antagonism of corticotropin-releasing factor receptors within the BNST blocks the reinstatement of cocaine seeking following footshock exposure (Erb and Stewart, 1999). Similarly, pharmacological inactivation of the BNST prevents the stress-induced reinstatement of cocaine seeking following systemic administration of the anxiogenic drug, yohimbine (Buffalari and See, 2011). Collectively, these data suggest that the BNST may have a selective role in forms of relapse that depend on stress and/or contextual fear, such as in reinstatement. To explore this question, we examined the consequences of reversibly inactivating the BNST in the expression of both the reinstatement and renewal of fear after extinction in rats. We hypothesized that BNST inactivation would attenuate the reinstatement, but not renewal, of extinguished fear.

## Materials and Methods

### *Subjects*

All subjects were adult (200-250 g) male Long-Evans (Blue Spruce) rats from Harlan Laboratories (Indianapolis, IN, USA). Upon arrival, rats were individually housed in clear plastic cages on a rotating cage rack (Animal Care Systems). Group assignments for behavioral training were randomized for cage position on the racks. Rats were given free access to standard rodent chow and water. Sawdust served as bedding for the rats (bedding was changed once a week). Behavioral experiments took place on separate days from the cage changings. Rats were kept on a fixed light/dark cycle, with rats experiencing fourteen hours of light (starting at 7:00 a.m.) followed by ten hours of darkness each day. All handling, surgeries, and behavioral testing occurred during the light hours of the light/dark cycle. The experimenters handled each rat for one minute per day for five days prior to the start of surgeries. Additionally, rats were habituated

to the infusion procedures and to the infusion room prior to behavioral training. The Texas A&M University Institutional Animal Care and Use Committee approved all experimental procedures.

*Surgery*

Rats were anesthetized with an intraperitoneal (i.p.) injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and treated with atropine methyl nitrate (0.4 mg/kg, i.p.). After the induction of anesthesia, each rat's head was shaved and they were placed in a stereotaxic frame (David Kopf Instruments). The scalp was incised and the skull was leveled with bregma and lambda in the same horizontal plane. Small holes were drilled in the skull and steel guide cannulas (26 gauge, 8 mm; Small Parts) were lowered into the BNST (0 mm A/P, +/- 2.7 mm M/L, -5.9 mm ventral to dura). Guide cannulas were angled at 10° to limit penetration of the lateral ventricles. Three stainless steel screws were affixed to the skull and the entire skull surface was covered with dental cement to secure the cannulas to the skull. Stainless steel obturators (30 gauge, 9 mm; Small Parts) were placed inside each cannula and changed every two days prior to behavioral testing. Rats were given a single bacon-flavored Rimadyl tablet (2 mg/tablet; Bio-Serv) following surgery. The rats were allowed at least one week to recover from surgery before behavioral testing.

*Behavioral Apparatus*

Behavioral testing was conducted in two distinct rooms within the laboratory ("Wellborn" and "University"). Each testing room contained eight identical conditioning chambers (Med Associates) fabricated with aluminum (sidewalls) and Plexiglas (rear wall, ceiling, and front cage door) walls (30 x 24 x 21 cm). The conditioning chambers were housed in

external sound-attenuating cabinets. The floor of each chamber consisted of nineteen stainless steel rods (4 mm in diameter); each rod was spaced 1.5 cm apart (center to center). Each chamber was equipped with a speaker to provide the CS. As described for each context (see below), a 15-W house light provided ambient lighting and a cabinet fan provided background noise for each chamber (~70 dB). The grid floors of each chamber were connected to a shock source and solid-state grid scrambler to deliver the footshock US (Med Associates). Each chamber rested on a load-cell platform that detected chamber displacement in response to each animal's movement. Load-cell activity values (ranging from -10 V to +10 V) were acquired during all behavioral phases and digitized at 5 Hz with Threshold Activity Software (Med Associates). Load-cell output was transformed offline to values ranging from 0 to 100 (higher values indicate more displacement of the cage). A bout of freezing was scored if the absolute values of load-cell activity were ≤10 for 1 sec or more (see Maren, 1998). The number of 1-sec bins of freezing was divided by the total number of bins in each observation period (typically a 30-sec or 1-min period after each trial) to yield the percentage of time each animal was freezing.

Distinct contexts were created through the use of different odors and visual cues. For Experiment 1, conditioning and extinction occurred in Context A; these chambers were located in the "Wellborn" test room in the laboratory. For Context A, the cage walls were wiped with acetic acid (1.5%) and a small volume was placed in the trays underneath the grid floor. The houselights were extinguished, but the overhead fluorescent room lights were illuminated. Rats were transported to Context A in white containers and the cabinet doors enclosing the conditioning chambers were open during testing. The reinstatement and test session occurred in Context B; these chambers were located in the "University" test room in the laboratory. For Context B, ammonium hydroxide (1%) was used to wipe the cage walls and a small volume was

placed in the trays underneath the grid floors. The overhead fluorescent room lights remained off (red room lights provided overhead illumination); the houselights within each testing chamber were illuminated. Rats were transported to Context B in black containers and the cabinet doors enclosing the conditioning chambers were closed during testing. Cabinet fans were turned on for both Context A and B in Experiment 1. For Experiment 2, conditioning was conducted Context A as described above, whereas extinction and renewal testing utilized Contexts B and C (per group assignments; cabinet fans were turned off for Contexts B and C in Experiment 2). For Context C ("Wellborn" room), ethanol (70%) was used to wipe the cage walls and a small volume was placed in the trays beneath the grid floor. The houselights were illuminated and the overhead lights were extinguished; a thin black Plexiglas sheet covered the grid floor for Context C. Cabinet doors enclosing the chambers were open during testing in Context C. Rats were transported to Context C in white five-gallon buckets; a layer of sawdust was placed in each bucket and changed out for each squad of animals.

*Behavioral Procedures*

*Experiment 1: Effects of BNST inactivation on the expression of reinstatement.* An illustration of the behavioral paradigm for Experiment 1 is shown in Figure 1. Prior to behavioral testing, thirty-two rats were randomly assigned to groups that would receive intracranial infusions of either muscimol ('MUS', a selective $GABA_A$ receptor agonist; $n = 16$) or vehicle ('VEH', physiological saline; $n = 16$) prior to retrieval testing. MUS rats received a total of 0.3 µg of muscimol (1.0 µg/µl in 0.3 µl) per hemisphere. VEH rats were infused with 0.3 µl of physiological saline per hemisphere. All infusions occurred over one minute at a rate of 0.3 µl/min. Within each drug condition, rats were also randomly assigned to receive reinstatement

shock in either the test context (Context B) or the extinction context (Context A). Rats did not differ at test based on the context in which the reinstatement shock was delivered [$Fs < 1$]; therefore we collapsed rats across this condition.

On Day 1, rats were transported to Context A for fear conditioning. Three minutes after placement in the chambers, rats received five auditory CS (10 sec, 2 kHz, 80 dB)-footshock US (2 sec, 1 mA) pairings (US onset occurred upon CS offset; 70-sec intertrial intervals, ITIs). After the final conditioning trial, rats remained in the chambers for one minute before being returned to their home cages. Twenty-four hours later, rats underwent the first of two extinction sessions. During these sessions, they were returned to the conditioning context (Context A) and, after three minutes, rats were presented with forty-five CS-alone trials (40-sec ITIs). After the final CS presentation, the rats remained in the chambers for three minutes before being returned to their home cages. The second extinction session was identical to the first and occurred on the following day. Twenty-four hours after the final extinction session, rats underwent a reinstating shock session. First, rats were exposed to either Context A or B for four minutes in the absence of the CS or US; rats were returned to their homecages after this experience. Two hours later, rats were brought back to the laboratory and were placed in the other context (A or B; per group assignments) for a reinstating shock in that context. For the reinstating shock session, rats received a single, unsignaled footshock (1 sec, 0.4 mA) after three minutes in chambers. Rats remained in the chambers for one minute after shock offset. Lastly, on Day 5, and immediately prior to retrieval testing, the rats were infused with muscimol or vehicle and transported to Context B to assess fear to the extinguished CS. Ten minutes after placement in Context B, the rats received five CS-only presentations (40-sec ITIs). Rats remained in the testing chambers for three minutes following the final CS presentation.

*Experiment 2: Effects of BNST inactivation on the expression of renewal.* Refer to Figure 1 for an illustration of the behavioral paradigm used for Experiment 2. Seventy-six rats were randomly assigned to drug ('DRUG' or 'VEH') and testing ('DIFF' or 'SAME') conditions. Immediately prior to renewal testing, DRUG rats were infused with either 0.3 µg of muscimol (1.0 µg/µl in 0.3 µl) per hemisphere (identical to Experiment 1) or 3.0 µg of 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX; 10.0 µg/µl in 0.3 µl) per hemisphere. NBQX is a potent α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor and kainate receptor antagonist. We found no difference in the effects of NBQX or muscimol on conditional freezing at test [$Fs < 0.1$]; therefore, we collapsed DRUG rats across this condition. Rats assigned to VEH were infused with 0.3 µl of physiological saline. As in Experiment 1, all infusions were delivered at 0.3 µl/min for one minute. Rats assigned to the SAME condition were tested to the extinguished CS in the extinction context, whereas rats assigned to the DIFF condition experienced the extinguished CS outside of the extinction context (but in a familiar context).

On Day 1, all rats were conditioned with five CS-US pairings (CS = 10 sec, 2 kHz, 80 dB auditory tone; US = 2 sec, 1 mA footshock) in Context A (procedure identical to Experiment 1). Twenty-four hours later (Day 2), rats were first exposed for thirty-five minutes to the context (either Context B or Context C) that was not hosting extinction; this insured that exposure to all contexts was counterbalanced. Three hours later, the rats were extinguished in the alternate context (either Context B or Context C; counterbalanced by group). Three minutes after placement in the extinction context, the rats received forty-five CS-only presentations (40-sec ITIs); the rats remained in the chambers for three minutes after the final CS presentation. Twenty-four hours later, and immediately prior to renewal testing, rats were infused with either

drug (muscimol or NBQX) or vehicle and transported to the appropriate test context (which was either the same as or different from the extinction context). Responding at test was not affected by whether the renewal context was B or C [$Fs < 0.1$]; the data are collapsed across this condition. Three minutes after placement in the test context, rats received five CS-only presentations (40-sec ITIs). Rats remained in the testing chamber for three minutes after the final CS-only presentation.

*Intracranial infusions*

Rats were transported in five-gallon buckets to a procedure room within the colony for drug infusions. The obturators were removed from the guide cannulas and stainless steel injection needles (33 gauge, 9 mm; Small Parts; extending 1 mm beyond the end of the guide) were inserted. Each injector was attached to polyethylene tubing (PE-20; Braintree Scientific), which in turn was connected to a gastight 10 µl syringe (Hamilton, Co.). Syringes were mounted in an infusion pump (KD Scientific). After insertion of the injectors, the rats were returned to the buckets where they remained unrestrained during the infusion procedure. After the infusion, the injection needles remained in the guide cannulas for one minute before being removed; clean obturators were inserted into the guides and the rats were returned to the buckets and transported to the conditioning chambers.

*Histological Procedures*

Within one week after the final retention test, the rats were overdosed with sodium pentobarbital (Fatal Plus; 100 mg/ml, 0.5 ml, i.p.) and perfused. Transcardial perfusions were performed with physiological saline followed by 10% formalin solution. Brains were removed

from the skull and stored in 10% formalin for twenty-four hours at 4° C followed by 30% sucrose-formalin for at least three days before sectioning. Brain tissue was flash frozen with dry ice and sectioned at 40 µm on a cryostat (Leica Microsystems) at -20° C. Sections were wet-mounted to microscope slides and stained with 0.25% thionin to identify cannula tracts and to localize injection sites in the tissue. Photomicrographs of the sections (10× magnification) were captured and digitized using a Leica MZFLIII microscope. Figure 2 shows a representative coronal section from a rat with injector tips localized to the BNST.

*Data Analyses*

Freezing served as the index of fear for all behavioral analyses. All data were submitted to analysis of variance (ANOVA). Post-hoc comparisons (Fisher's Protected LSD test) on individual group means were calculated after a significant omnibus *F* ratio in the ANOVA; alpha was set at 0.05. Rats were excluded from the analyses if they failed to extinguish by the final extinction session (mean freezing >50%) or if mean pre-CS freezing during the retrieval test was >50%. Based on these criteria, 10 rats were excluded from Experiment 2. Unless noted otherwise, freezing data (as a percentage of total time spent immobile) were analyzed across the trials as described for Figure 4 and Figure 5.

## Results

*Histology*

Injection sites within the BNST are illustrated in Figure 3. For Experiment 1 (Figure 3A), sixteen injectors terminated within the anterior lateral division of the BNST (which includes the

anterolateral area, juxtacapsular nucleus, oval nucleus, and rhomboid nucleus), four were localized to the anterior medial BNST (which includes the anterodorsal area and central core of the anterodorsal area), five were localized to the anterior ventral BNST (which includes the anteroventral area, dorsolateral nucleus, dorsomedial nucleus, fusiform nucleus, magnocellular nucleus, subcommissural zone, and ventral nucleus), and nine were located in the anterior commissure within the anterior BNST (refer to Swanson, 1998). This yielded the following groups for the final analyses: MUS, $n = 7$; VEH, $n = 10$. Cannulas missed their targets in fifteen animals; these animals (MUS, $n = 9$; VEH, $n = 6$) were analyzed separately to determine whether off-target drug infusions affected reinstatement.

For Experiment 2, forty-five animals received bilateral injectors within the BNST. Of these animals, ten subjects were excluded based on the behavioral criteria described above, yielding the following groups: DRUG/DIFF, $n = 11$; DRUG/SAME, $n = 6$; VEH/DIFF, $n = 8$; VEH/SAME, $n = 10$. Accordingly, seventy injection sites from thirty-five animals are illustrated in Figure 3B. Thirteen injectors were localized to the anterior lateral division of the BNST, four terminated within the anterior medial division, twenty-two terminated within the anterior ventral division, five terminated in the anterior commissure within the anterior BNST, and twenty-six terminated within the posterior division of the BNST (which includes the interfascicular nucleus, principal nucleus, and transverse nucleus).

*Experiment 1: BNST inactivation prevents reinstatement of fear to an extinguished CS*

Rats exhibited reliable fear conditioning to the auditory CS (Figure 4). This impression was confirmed in an ANOVA by a significant main effect of trial [$F_{(5,75)} = 9.1$; $p < 0.0001$[a]]; freezing behavior increased across the conditioning session and there were no group differences

on this measure [*Fs* < 1]. Over the next two days, all rats were extinguished to the CS in Context A. During the first extinction session (Figure 4), there was a significant main effect of trial [$F_{(10,150)} = 10.6$; $p < 0.0001$[b]] as freezing behavior decreased over the course of the extinction session; there were no group differences in extinction rate or magnitude [*Fs* < 1]. During the second extinction session (Figure 4), there again was a significant main effect of trial [$F_{(10,150)} = 7.9$; $p < 0.0001$[c]] reflecting decreases in freezing behavior over the course of the session; again, there were no group differences in extinction rate or magnitude [*Fs* < 1]. On Day 4, all rats received an unsignaled footshock to reinstate fear to the extinguished CS (Figure 4). Freezing behavior reliably increased after footshock. This impression was confirmed in an ANOVA that revealed a significant main effect of trial for freezing across the pre- and post-shock periods [$F_{(1,15)} = 49.5$; $p < 0.0001$[d]]. Shock-induced increases in fear on Day 4 were similar across drug and context conditions [*Fs* < 2].

Twenty-four hours after the reinstatement shock, rats were infused with muscimol or vehicle into the BNST and immediately placed in Context B for a CS retrieval test. During the ten-minute baseline prior to the first CS presentation, vehicle-treated rats exhibited significantly greater levels of freezing than muscimol-treated animals (VEH: 31.108% ± 5.644%; MUS: 3.968% ± 1.409%). This was confirmed in the ANOVA by a significant main effect of drug across baseline freezing [$F_{(1,50)} = 15.423$; $p = 0.0013$[e]]. In addition, and as shown in Figure 4, vehicle-treated rats exhibited significantly greater levels of fear to the extinguished CS than MUS animals. This impression was confirmed in the ANOVA by a main effect of drug across all testing trials [$F_{(1,90)} = 14.446$; $p < 0.0017$[f]]. There was a significant main effect of trial [$F_{(6,90)} = 5.737$; $p < 0.0001$[g]] insofar as freezing behavior increased on average after presentation of the CS. Freezing to the CS during the retrieval test in vehicle-treated rats was significantly greater

than during the final block of extinction, indicating successful reinstatement of extinguished fear [$F_{(1,9)} = 14.607$; $p < 0.0041$[h]]. Importantly, reinstatement impairments were only obtained in rats with cannula placements in the BNST. Muscimol infusion in rats with off-target placements that missed the BNST exhibited normal reinstatement and did not differ from controls during either the baseline or CS-periods [*Fs* < 0.5]. Overall, these data reveal that BNST inactivation reduced both contextual freezing and the reinstatement of fear to an extinguished CS.

*Experiment 2: BNST inactivation does not alter the expression of fear renewal*

As shown in Figure 5, rats exhibited reliable fear conditioning. This impression was confirmed in the ANOVA by a significant main effect of trial [$F_{(5,155)} = 31.\ 2$; $p < 0.0001$[i]]; freezing behavior increased over the course of conditioning and the groups did not differ from one another [*Fs* < 1]. Twenty-four hours later, rats significantly reduced their fear across extinction trials (Figure 5; main effect of trial [$F_{(10,310)} = 50.6$; $p < 0.0001$[j]]). Extinction of fear on Day 2 was similar across group assignments [*Fs* < 1].

On Day 3, rats were infused with either drug or vehicle immediately before receiving a retrieval test in the extinction context (SAME) or in the context in which extinction did not occur (DIFF). As indicated in Figure 5, both VEH- and DRUG-treated rats exhibited robust fear renewal in the DIFF context relative to the low levels of freezing expressed by rats in the SAME context. BNST inactivation did not attenuate renewal of fear to the extinguished CS. This impression was confirmed in the ANOVA by a main effect of context [$F_{(1,186)} = 12.843$; $p = 0.0011$[k]] such that rats in the DIFF condition exhibited significantly more freezing across test trials than those in the SAME condition (regardless of drug condition). A significant trial x context interaction [$F_{(6,186)} = 2.647$; $p = 0.0173$[l]] indicated that DIFF rats exhibited greater

freezing after CS onset as compared to SAME rats. Moreover, DIFF (but not SAME [$Fs < 0.5$]) rats exhibited significantly more freezing across test trials relative to the final block of extinction [$F_{(1,18)} = 16.005$; $p = 0.0008^{m}$], indicating robust renewal of fear. Hence, BNST inactivation did not impair either the renewal or expression of fear to an extinguished auditory CS.

**Discussion**

The current study reveals the novel finding that the BNST plays a specific role in the shock-induced reinstatement of extinguished fear; BNST inactivation did not affect the renewal of extinguished fear that accompanies a change in context. Deficits in the reinstatement of extinguished fear were paralleled by reductions in the expression of contextual freezing after BNST inactivation. These results are consistent with an earlier report revealing that neurotoxic lesions of the BNST impair the shock-induced reinstatement of extinguished fear (Waddell et al., 2006). Additionally, our work parallels findings revealing that the BNST is necessary for shock-induced reinstatement of extinguished drug-seeking behavior (Erb and Stewart, 1999; Erb et al., 2001; see also Leri et al., 2002; Buffalari and See, 2011). Collectively, these data suggest that the BNST has a critical role in the relapse of extinguished behaviors caused by the experience of aversive stimuli (for reviews, see Smith and Aston-Jones, 2008; Silberman and Winder, 2013; Stamatakis et al., 2014).

Although BNST inactivation impaired fear reinstatement, it did not affect fear renewal despite the fact that the extent of relapse was similar between experiments. This reveals that deficits in reinstatement are not due to impairments in the expression of freezing *per se*. Indeed, this pattern of results is consistent with other reports indicating that the BNST has a selective

328 role in the expression of fear to contextual compared to discrete CSs (LeDoux et al., 1988;
329 Walker and Davis, 1997; Sullivan et al., 2004; Waddell et al., 2006; Waddell et al., 2008;
330 Zimmerman and Maren, 2011; Sink et al., 2013; see also Duvarci et al., 2009; Haufler et al.,
331 2013). Importantly, pre-training lesions of the BNST do not disrupt the acquisition of
332 conditioned fear to discrete CSs (LeDoux et al., 1988; Waddell et al., 2006), nor do post-training
333 lesions of the BNST affect expression of conditioned fear to discrete CSs (Sullivan et al., 2004).
334 However, BNST lesions attenuate the expression of fear to shock-associated contexts (Sullivan
335 et al., 2004) and attenuate the expression of fear responses to long-duration CSs (i.e., 10-min
336 tones paired with shock) (Waddell et al., 2006). Additionally, Sullivan et al. (2004) reported that
337 rats with BNST lesions exhibited blunted corticosterone responding during exposure to a
338 conditioned context (see also Resstel et al., 2008). Hence, it is believed that BNST inactivation
339 prevents reinstatement by reducing the expression of contextual fear, which is thought to be
340 essential for the reinstatement effect (Bouton and Bolles, 1979; Westbrook et al., 2002; Bouton
341 et al., 2006; Waddell et al., 2006).

342 A key finding in the present study is that BNST inactivation did not affect fear renewal.
343 Unlike reinstatement, however, renewal does not require contextual fear. Indeed, fear renewal
344 can be obtained in contexts that have never hosted shock (e.g., “ABC” or “AAB” renewal; see
345 Bouton and Bolles 1979; Bouton and Ricker 1994; Harris et al., 2000; Westbrook et al., 2002;
346 Corcoran and Maren, 2004; Holmes and Westbrook 2013; Jin and Maren, 2015). Renewal also
347 occurs in shock-associated contexts that have themselves undergone extinction and no longer
348 support contextual fear (e.g., “ABA” renewal; see Bouton and King 1983; Vansteenwegen et al.,
349 2005; Effting and Kindt 2007; Knox et al., 2012; Polack et al., 2013; Holmes and Westbrook,
350 2014). These findings support the idea that renewal depends not on direct context-US

associations, but on a contextual retrieval process that informs the animal of what a CS means in a particular context (Bouton and Bolles, 1979; Bouton and King, 1983; Bouton and Peck, 1989; Holland, 1992; Bouton, 1993; Bouton and Ricker, 1994; Harris et al., 2000; Bouton et al., 2006; Ji and Maren, 2007; Maren et al., 2013; Vervliet et al., 2013b; Delamater and Westbrook, 2014). Importantly, the present results strengthen this view insofar as renewal of fear was immune to BNST inactivation (a manipulation that impairs contextual fear). Considerable work now reveals that this contextual retrieval process depends on a circuit involving the amygdala, hippocampus, and prefrontal cortex (Corcoran and Maren, 2004; Ji and Maren, 2005; Ji and Maren, 2007; Herry et al., 2008; Knapska and Maren, 2009; Orsini et al., 2011; Zelikowsky et al., 2012; Orsini et al., 2013; Maren, 2014; Jin and Maren, 2015).

An important issue that has yet to be resolved is which subregions of the BNST contribute to the effects we have observed in the present study. Indeed, the BNST is heterogeneous in structure and different subregions within the BNST appear to make unique contributions to fear and anxiety (Walter et al., 1991; Dong et al., 2001b; Dong and Swanson, 2003; Dong and Swanson, 2004; Choi et al., 2007; Jennings et al., 2013; Kim et al., 2013). For example, Kim et al. (2013) showed that photostimulation of the oval nucleus of the BNST resulted in anxiety-related behaviors, while photostimulation of the anterodorsal region of the BNST resulted in anxiolytic behaviors. Jennings et al. (2013) demonstrated that photostimulation of glutamatergic projections of the ventral BNST to the ventral tegmental area (VTA) produced increases in anxiety, whereas photostimulation of GABAergic BNST projections to the VTA was anxiolytic. In the present study, cannula placements terminated primarily within the anterior portion of the BNST (particularly the anterior lateral and anterior ventral divisions of the BNST), though several rats received infusions within the posterior division of the BNST in Experiment 2.

Spread of drug likely affected multiple BNST nuclei in these areas. Circuit-selective chemo- or optogenetic techniques would help clarify the specific BNST subregions contributing to fear reinstatement (see Sparta et al., 2013).

In humans and other animals, the BNST shares connections with several important emotion-regulating regions in the brain, including the amygdala, dorsal raphe nucleus, hippocampus, hypothalamus, nucleus accumbens, prefrontal cortex, and ventral tegmental area (Swanson and Cowan, 1977; Weller and Smith, 1982; Phelix et al., 1992; Sun and Cassell, 1993; Dong et al., 2001a, 2001b; Dong and Swanson, 2003; Dong and Swanson, 2004a, 2004b; Jalabert et al., 2009; Crestani et al., 2013; Avery et al., 2014; Roman et al., 2014; Krüger et al., 2015). Not surprisingly, the BNST has been implicated in various depression- and anxiety-related behaviors (for reviews, see Walker and Davis, 2008; Hammack et al., 2009; Walker et al., 2009; Davis et al., 2010; Hammack et al., 2010; Hammack et al., 2012; McElligott et al., 2013; Adhikari, 2014; Kash et al., 2015). Importantly, the BNST modulates hypothalamic-pituitary-adrenal axis activity, including corticosterone release, via its connections with the hypothalamic paraventricular nucleus (Cullinan et al., 1993; Herman et al., 1994; Sullivan et al., 2004; Choi et al., 2007; Crestani et al., 2013). Corticosterone release is correlated with both the acquisition and expression of conditioned fear (Campeau et al., 1997; Pugh et al., 1997; Cordero et al., 1998; Roozendaal et al., 2006; Marchand et al., 2007). Hence, BNST lesions might influence reinstatement by limiting the modulatory effects of corticosterone on fear expression to an extinguished CS. Alternatively, the BNST is positioned to directly influence freezing behavior via its projections to the amygdala and periaqueductal gray (Dong et al., 2001b; Dong and Swanson, 2003; Fendt et al., 2003; Dong and Swanson, 2004a, 2004b; Asok et al., 2013). In this way, the BNST might directly drive reinstatement of fear to an extinguished CS by driving

amygdaloid and periaqueductal gray circuits involved in fear expression. In either case, BNST-mediated modulation of contextual fear might summate with fear to the extinguished CS to yield reinstatement.

In conclusion, the present results reveal that distinct neural circuits mediate different forms of fear relapse. Here we show that the BNST is especially important for reinstatement, a form of relapse produced by the exposure of animals to aversive stimuli. Hence, selective manipulations of the BNST may be particularly effective in preventing fear relapse in aversive contexts. Ultimately, appreciating the circumstances that give rise to the return of fear will help in isolating circuit-specific therapies for combating fear relapse.

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**Legends**

*Figure 1*

Experimental designs are read from left to right. Each phase of behavior is separated by 24 hrs, however infusions occurred immediately prior to testing in both Experiment 1 and 2. A, B, C = experimental contexts; T = tone CS; + = US; - = no US.

*Figure 2*

Representative photomicrograph of a thionin-stained coronal section (40 μm) from the brain of a rat with injector tips terminating within the bed nucleus of the stria terminalis.

*Figure 3*

Illustration of cannula placements sites in the bed nucleus of the stria terminalis. Placements are shown for all rats included in the final analyses for Experiment 1 (**A**) and for Experiment 2 (**B**). Adapted from Swanson (1998). Distances shown are relative to bregma.

*Figure 4*

Pharmacological inactivation of the BNST prevents reinstatement (Experiment 1). ('Conditioning'), Mean (± SEM) percentage of freezing during the 3-min baseline ('BL') and in the 60-sec inter-stimulus interval following each CS-US pairing. ('Extinction [1st Session]'), Mean (±SEM) percentage freezing during a 3-min baseline ('BL') and across nine extinction blocks. Each block represents average responding during the 30-sec post-CS intervals after five extinction trials. The rats remained in the chambers for 150 sec after the final CS presentation

('P'). ('Extinction [2nd Session]'), Mean (± SEM) percentage freezing for the second day of extinction training (trials are equivalent to the first extinction day). ('Shock'), Mean (± SEM) percentage freezing during the 3-min baseline period before unsignaled footshock and during the 1-min post-shock period. ('Test for Reinstatement'), Mean (± SEM) percentage freezing during the final three minutes of the baseline period immediately prior to CS onset and during five 30-sec inter-stimulus intervals after each test trial; the rats remained in the chambers for 150 sec after the final CS.

*Figure 5*

Pharmacological inactivation of the BNST does not prevent renewal (Experiment 2). ('Conditioning'), Mean percentage of freezing during the 3-min baseline ('BL') and in the 60-sec inter-stimulus interval following each CS-US pairing. ('Extinction'), Mean (± SEM) percentage freezing during a 3-min baseline ('BL') and across 9 extinction blocks. Each block represents average responding during the 30-sec post-CS intervals after five extinction trials. The rats remained in the chambers for 150 sec after the final CS presentation ('P'). ('Test for Renewal'), Mean (± SEM) percentage freezing during the 3-min baseline period immediately prior to CS onset and during five 30-sec inter-stimulus intervals after each test trial; the rats remained in the chambers for 150 sec after the final CS.

**Table 1**

*Statistical Table*

| In-text letter | Data structure | Type of test | Power |
|---|---|---|---|
| a | Normal Distribution | One-way repeated measures ANOVA (conditioning trials) | 1.000 |
| b | Normal Distribution | One-way repeated measures ANOVA (extinction trials [Day 2]) | 1.000 |
| c | Normal Distribution | One-way repeated measures ANOVA (extinction trials [Day 3]) | 1.000 |
| d | Normal Distribution | One-way repeated measures ANOVA (reinstating shock trials) | 1.000 |
| e | Normal Distribution | One-way repeated measures ANOVA (infusion group across baseline at test) | 0.968 |
| f | Normal Distribution | One-way repeated measures ANOVA (infusion group across testing) | 0.957 |
| g | Normal Distribution | One-way repeated measures ANOVA (test trials) | 0.993 |
| h | Normal Distribution | One-way repeated measures ANOVA (final extinction block vs. mean responding at test for VEH animals) | 0.935 |
| i | Normal Distribution | One-way repeated measures ANOVA (conditioning trials) | 1.000 |
| j | Normal Distribution | One-way repeated measures ANOVA (extinction trials) | 1.000 |
| k | Normal Distribution | One-way repeated measures ANOVA (renewal group across testing) | 0.951 |
| l | Normal Distribution | Two-way repeated measures ANOVA (testing trials x renewal group) | 0.858 |
| m | Normal Distribution | One-way repeated measures ANOVA (final extinction block vs. mean responding at test for DIFF animals) | 0.977 |