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Corticotropin releasing factor type-1 receptor antagonism in the dorsolateral bed nucleus of the stria terminalis disrupts contextually conditioned fear, but not unconditioned fear to a predator odor

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Highlights

- Pre-conditioning ICV and $_{L}$BNST antalarmin disrupts retention of contextual fear
- Pre-conditioning ICV and $_{L}$BNST antalarmin does not affect post-shock freezing
- ICV and $_{L}$BNST antalarmin does not affect unconditioned freezing to TMT predator odor
- $_{L}$BNST antalarmin does not affect responsivity to varying footshock intensities
- $_{L}$BNST CRFr1 receptors are important for long-term contextual fear learning and memory

Abstract

The bed nucleus of the stria terminalis (BNST) plays a critical role in fear and anxiety. The BNST is important for contextual fear learning, but the mechanisms regulating this function remain unclear. One candidate mechanism is corticotropin-releasing-factor (CRF) acting at CRF type 1 receptors (CRFr1s). Yet, there has been little progress in elucidating if CRFr1s in the BNST are involved in different types of fear (conditioned and/or unconditioned). Therefore, the present study investigated the effect of antalarmin, a potent CRFr1 receptor antagonist, injected intracerebroventricularly (ICV) and into the dorsolateral BNST ($_{L}$BNST) during single trial contextual fear conditioning or exposure to the predator odor 2,5-dihydro-2,4,5-trimethylthiazoline (TMT). Neither ICV nor $_{L}$BNST antalarmin disrupted unconditioned freezing to TMT. In contrast, ICV and $_{L}$BNST antalarmin disrupted the retention of contextual fear when tested 24 hours later. Neither ICV nor $_{L}$BNST antalarmin affected baseline or post-shock freezing – indicating antalarmin does not interfere with the early phases of contextual fear acquisition. Antalarmin did not (1) permanently affect the ability to learn and express contextual fear, (2) change responsivity to footshocks, or (3) affect the ability to freeze. Our findings highlight an important role for CRFr1s within the $_{L}$BNST during contextually conditioned fear, but not unconditioned predator odor fear.

Keywords: context fear, predator odor, TMT, fear conditioning, bed nucleus of the stria terminalis, corticotropin releasing factor

1. Introduction

Corticotropin-releasing factor (CRF) is a 41 amino acid neuropeptide widely studied for its role in the neuroendocrine stress response (Bale and Vale, 2004; Kovács, 2013; Smagin et al., 2001; Vale et al., 1981). In addition to the paraventricular nucleus of the hypothalamus, CRF is also expressed in a number of extrahypothalamic structures including the amygdala and bed nucleus of the stria terminalis (BNST) (Makino et al., 1995; Wong et al., 1994). While these two structures have been investigated for their role in conditioned and unconditioned fear and anxiety-like behaviors (Campeau et al., 1991; Walker and Davis, 1997), our understanding of the function of CRF within these areas is continually expanding.

CRF within the BNST, a part of the extended amygdala, has received substantial attention over the last few decades for its function in mediating fear and anxiety-like behaviors (Walker and Davis, 2008; Walker et al., 2003). Recent work has shed light on how the BNST is involved in associative learning using contextual fear conditioning paradigms (Haufler et al., 2013; Nijsen et al., 2001; Poulos et al., 2010; Resstel et al., 2008; Sullivan et al., 2004). In this paradigm, a neutral context (CS) is paired with a footshock (US) to produce a conditioned response (CR), the most studied of which is freezing. Lesions of the BNST disrupt long-term freezing to a context, but not freezing to discrete cues such as tones (LeDoux et al., 1988; Sullivan et al., 2004). Importantly, the BNST may play a significant role in contextual fear learning given that it can compensate for contextual, but not auditory, fear learning when the basolateral amygdala, a structure critical to fear conditioning, is inactivated (Poulos et al., 2010; Zimmerman and Maren, 2011).

The role of the BNST in contextually conditioned fear complements a number of studies showing that the BNST is also essential for modulating other fear and anxiety-like behaviors. For

example, lesions of the BNST disrupt the sustained enhancement of startle responses to long-lasting environmental threats (Davis et al., 2010). Both light-enhanced and CRF-enhanced startle, but not fear-potentiated startle to short-duration cues, are blocked by non-selective CRF antagonism in the BNST (Lee and Davis, 1997) and selective CRFr1 antagonism peripherally (Walker et al., 2009b). While overexpression of CRF within the BNST has no effect on unconditioned fear-like behavior (in the elevated plus maze), it disrupts sustained fear as measured by enhanced acoustic startle and decreases CRFr1 expression (Sink et al., 2013b). Taken together, these studies suggest that CRF and CRFr1s in the BNST may play an important role in contextually conditioned, but not unconditioned, fear and anxiety-like behaviors.

However, the BNST *is* important for behavioral and endocrine responses to particular types of unconditioned threats – predator odors (Fendt et al., 2003; Rosen et al., 2015; Walker and Davis, 1997). Predator odors are advantageous for investigating unconditioned fear and anxiety-like behaviors for two reasons. First, although laboratory rats have never encountered the odor, they still exhibit robust defensive responses upon the first exposure. Second, predator odors are ethologically relevant stimuli for rodents relative to foot-shocks. Inactivation of the BNST, but not key nuclei of the amygdala important for fear conditioning (e.g., the CeA and basal nucleus of the amygdala), disrupts freezing to the predator odor 2,5-dihydro-2,4,5-trimethylthiazoline (TMT; (Fendt et al., 2003; Rosen, 2004; Wallace and Rosen, 2001)), a synthesized compound derived from the anal secretions of the red fox. TMT exposure also increases numerous immediate-early genes (Day et al., 2004; Kobayakawa et al., 2007) and CRF mRNA across the extended amygdala (Asok et al., 2013a), in addition to elevating corticosterone secretion (Day et al., 2004). These studies suggest that the BNST, and possibly CRF within the BNST, may modulate unconditioned fear-like behavior to predatory threats. However, the role of

CRF within the BNST during unconditioned predator odor fear to TMT and contextually conditioned fear has not been studied. Therefore, the present study investigated how CRF within the BNST is involved in both conditioned freezing and unconditioned freezing to a predator odor. We evaluated the effects of blocking the anxiogenic CRF type 1 receptor (CRFr1) with a selective CRFr1 antagonist, antalarmin, administered intracerebroventricularly (ICV) or into the dorsolateral BNST ($_{L}$BNST), prior to contextual fear conditioning or exposure to the predator odor TMT.

2. Materials and Methods

2.1 Subjects

Male Sprague-Dawley rats (8 -11 weeks of age) obtained from Harlan breeders (Indianapolis, IN) and weighing between 280-330g were used for all experiments. Rats were maintained on a 12h light/dark cycle (lights on at 7:00 A.M.) at constant temperature with free access to food and water. Following arrival in the animal colony, rats were left undisturbed for seven days prior to the start experimental procedures. Rats were pair-housed in opaque polycarbonate cages with wood shavings for the duration of the study. All animals were handled by the experimenter for 2 consecutive days (~5 min/day) prior to the start of behavioral experiments. All procedures were approved by the University of Delaware's Institutional Animal Care and Use Committee.

2.2 Surgery

Rats were anesthetized with a ketamine/xylazine cocktail (85/15 mg/kg) prior to stereotaxic surgery. For rats that received ICV surgery, a single 26-gauge 5mm guide cannula (Plastics One, Roanoke, VA) was implanted 1mm above the rat's right lateral ventricle using the following coordinates: AP = - 0.1, ML = -1.8, DV = -3.2. For rats that received cannula implanted into the dorsolateral division of the bed nucleus of the stria terminalis ($_{L}$BNST), two 26-gauge guide cannula were angularly implanted using the following coordinates: AP = -0.1 mm, ML = ± 3.8 mm, DV = - 5.4 mm, at a 19º angle. Following surgeries, a dummy cannula that extended 1 mm beyond the tip of the guide cannula was inserted to prevent blockage.

2.3 Drug Preparation and Delivery

The selective CRFr1 receptor antagonist antalarmin hydrochloride (Sigma, St. Louis, MO) was used for all experiments (Zorrilla et al., 2002b). Antalarmin was dissolved in dimethyl sulfoxide (DMSO), as a vehicle, for all experiments. For ICV experiments, rats received either 3 µL of DMSO vehicle or 3 µL of DMSO vehicle containing 20 µg antalarmin. This dose was selected because it was in range with previous ICV and peripheral studies (Deak et al., 1999; Zorrilla et al., 2002a; Zorrilla et al., 2002b). For BNST infusions, rats received either 0.2 µL of DMSO vehicle or 0.2 µL of DMSO containing antalarmin. Three doses were tested. Antalarmin was dissolved in DMSO to a final concentration of either 10 µg/µL (for 2 µg dose), 1 µg/µL (for 0.2 µg dose), or 0.01 µg/µL (for 0.02 µg dose). These doses were selected because the BNST is part of the extended amygdala and other studies have used a similar dose range for antalarmin infused into the amygdala (Vicentini et al., 2014; Wellman et al., 2013).

Antalarmin or vehicle was administered 30 minutes prior to fear conditioning, TMT exposure, or shock responsivity testing using an electronic infusion pump (Harvard Apparatus,

Holliston, MA). One µL Hamilton syringes were connected to polyethylene tubing, and capped with a cannula injector that extended 1mm below the end of the guide cannula. Solutions were infused at a rate of 1 µL/min for ICV and 0.2 µL/min for BNST. The vehicle and administration time point were chosen based off of previous studies evaluating the pharmacokinetic profile of antalarmin (Sanghvi et al., 2009).

2.4 Contextual Fear Conditioning

Contextual fear conditioning was conducted in four identical Plexiglas/metal chambers (25cm x 31cm x 32cm) containing metal grid floors (19 stainless steel bars, 0.5 cm in diameter, and 1.25 cm apart). All groups were counterbalanced within and across days. For conditioning, each animal was placed in the chamber for 180s (baseline freezing measurement), followed by a single 1s 1.5mA shock, followed by a 300s shock-free period (post-shock freezing measurement). Twenty-four hours later, animals were returned to the same chamber and tested for freezing to the context for 300s (retention freezing measurement). All chambers were cleaned with a 5% ammonium hydroxide solution between sessions. A camera positioned at the top of each chamber recorded behavior for each animal and transmitted the signal to a computer running FreezeFrame software (Actimetrics, Wilmette, IL). Freezeframe was configured to score freezing as 0.75s bouts without changes in pixel luminance and then verified offline by an experimenter (see Asok et al. 2014).

2.5 Contextual Fear Re-training in an Alternate Context

Rats were re-trained in a different environment without any drug and under identical contextual fear conditioning parameters (e.g., 180s baseline, a single 1s 1.5 mA shock, 300s

measure of post-shock freezing, and 300s retention test) in an alternate context four rectangular Plexiglas chambers (16.5 cm × 12.1 cm × 21.6 cm) with metal grid floors (9 stainless steel bars, 4 mm in diameter, and 1 cm apart) inside a fume hood. All chambers were cleaned with a 70% ethanol solution between sessions.

2.6 Shock Responsivity Testing

Eight identical SR Lab ventilated startle chambers (San Diego Instruments, San Diego, CA) with clear Plexiglas cylinders (8.6 cm in diameter and 20 cm in length) were used for shock responsivity testing similar to our previous studies (Thompson et al., 2004). Eight currents were tested (0 mA, 0.1 mA, 0.4 mA, 0.8 mA, 1.2 mA, 1.5 mA, 1.8 mA, and 2.1 mA) and responsivity was measured by an accelerometer attached to the bottom of the chamber and quantified as arbitrary units (AU; (Thompson et al., 2004)). Subjects were acclimated for 300s, then presented with a shock every 120s. Each current was presented twice in ascending intensity. Given that contextual fear conditioning was conducted using alternating current (AC) shock generators and our startle chambers used direct current (DC) shock generators, we adjusted the DC current levels to match the AC current levels animals were trained under for contextual fear conditioning. For DC currents, this was achieved by computing the root mean square (RMS) of the AC voltage. All rats were trained in the dark with white noise (65 dB) in the background.

2.7 Predator Odor Exposure

Subjects were tested for freezing to the synthetic predator odor 2, 5-dihydro-2, 4, 5-trimethylthiazoline (TMT) 30 minutes after administration antalarmin. The context was the same as that used for Contextual Fear Re-training (see above). Procedures for TMT exposure were

identical to Asok et al. (2013a). Briefly, rats were acclimated to the context 10 minutes/day for three days (baseline) prior to TMT exposure on day four. Rats were exposed to 300 µmoles of TMT. One hundred and fifty µmole/19.4µl was pipetted on two pieces of filter paper taped to opposite walls of the chamber. This amount of TMT was used because it consistently produces robust freezing and CRF expression in the $_{L}$BNST (Asok et al., 2013a; Wallace and Rosen, 2000). Freezing was scored by Freezeframe similar to Asok et al. (2013a).

2.8 Cannula Verification (Figure 1)

For ICV infusions, cannula placement was verified at both the time of surgery and following behavioral testing. During surgery, sterile physiological saline was drawn into polyethylene tubing connected to an injector extending 1mm beyond the tip of the guide cannula. After achieving the targeted coordinates, a stop was removed from the tube to allow a small amount of saline to enter the lateral ventricle via gravity flow. Given that saline will not perfuse into the ventricles if the injector tip is not within the ventricle, we appropriately adjusted the cannula depth as needed in those instances. Additionally, following behavioral testing, the rat was anesthetized, infused with 1 µL of Indian ink, rapidly decapitated, and the brain removed. The brain was sagittally sliced at the midline with a razor blade and the presence of Indian ink in the ventricles was visually confirmed.

For $_{L}$BNST infusions, cannula placement was verified post-mortem. Following decapitation, brains were flash frozen in isopentane and stored at -80ºC until slicing. Brains were cut on a cryostat and sections corresponding to the cannula site were stored at -80ºC until staining. All brains were post-fixed in a 4% paraformaldehyde solution (pH 7.2) prior to staining with cresyl violet. Brain images were captured via a Dage CCD video camera and captured

sections were overlain against corresponding sections in the Paxinos and Watson rat brain atlas (Paxinos and Watson, 2007). Accurate cannula placements were defined as within ~1.5 mm of target. This criterion was set given that the injector extended 1mm beyond the cannula tip and to account for a ~ 1mm diffusion diameter (0.5mm radius) of the drug (See Figure 1 for cannula placement).

2.9 Statistical Analyses

For contextual fear conditioning, a group X testing phase (i.e., baseline, post-shock, and retention test) repeated measures ANOVA was used. Following significant main-effects and interactions, Fisher's LSD (for ICV) or a Dunnett's test (for $_{L}$BNST dose response analysis) was used. For TMT induced freezing with only two groups, a group X testing phase (i.e., baseline and TMT exposure) two-way ANOVA was used for both ICV and BNST infusions. For shock responsivity testing, the startle amplitude score for each animal was averaged across both presentations of the same current. Subsequently, a group X shock intensity paired samples t-test was used. An *a priori* criteria was set to remove any animal that (1) had improperly placed or clogged cannula or (2) exhibited freezing scores above or below 2 *S.D.* of the group mean at the retention test, similar to our previous studies (Asok et al., 2013b; Schreiber et al., 2014). The final numbers of animals included in analyses are listed below.

3. Results

3.1 Pre-training ICV CRFr1 antagonism disrupts retention of contextual fear, but not unconditioned predator odor fear (Figures 2A, 2B, and 2C)

We investigated whether antagonizing CRFr1 receptors throughout the entire brain affected contextual fear learning (context-shock US) or freezing to the unconditioned predator odor TMT (US). Intracerebroventricular antalarmin prior to fear conditioning disrupted freezing 24 hours later at the retention test, but did not affect baseline (context) or post-shock (context-US acquisition) freezing. Intracerebroventricular antalarmin had no effect on freezing to the unconditioned predator odor TMT even at very high doses. These results were confirmed statistically below.

For contextual fear conditioning, 24 subjects were included in analyses ($n_{\text{surgery control}} = 8$, $n_{20ug} = 8$, and $n_{\text{vehicle control}} = 8$). One animal from each group was removed as an outlier. A 3 x 3 repeated measures ANOVA revealed a main effect of group ($F(2, 21) = 3.88$, $p < .05$), a main effect of testing phase ($F(1, 21) = 31.66$, $p < .001$) and a significant group X testing phase interaction ($F(2, 21) = 4.75$, $p < .05$). Groups did not differ at baseline or during the post-shock testing phases (p's > .05), but significantly differed at the retention test ($F(2, 21) = 11.68$, $p < .001$). The 20 µg antalarmin group exhibited significantly lower freezing relative to the vehicle controls and surgery controls (p's < .05; Figure 2A). Importantly, vehicle controls (which received DMSO) did not statistically differ from surgery controls during the retention test ($p > .05$).

For TMT exposure, 16 subjects were used ($n_{20\mu g} = 5$, and $n_{\text{vehicle control}} = 11$). A two-way ANOVA contrasting groups (vehicle and 20 µg antalarmin) across the measured testing phases (acclimation and TMT exposure) revealed no main effect of group during acclimation ($F(1,14) = .878$, $p > .05$) and, more importantly, no effect of group during TMT testing ($F(1,14) = .162$, $p > .05$; Figure 2B). Pairwise comparisons confirmed these results. Similarly, a high-dose of

antalarmin (100 µg) did not affect freezing to TMT, confirming that the lack of an effect of antalarmin on TMT-induced freezing was not a result of ineffective dosage ($p > .05$; Figure 2C).

3.2 Pre-training $_{L}$BNST CRFr1 antagonism disrupts retention of contextual fear, but not unconditioned predator odor fear (Figures 3A, 3B, and Supplementary Figure 1)

In addition to ICV infusion, we investigated if selective CRFr1 antagonism within the $_{L}$BNST affected contextual fear learning or freezing to the predator odor TMT. CRFr1 antagonism at a dose of 2 µg prior to training disrupted freezing during the retention test, but did not affect freezing at baseline or post-shock testing phases. However, a 2ug dose did not affect unconditioned freezing to the predator odor TMT. Additionally, $_{L}$BNST CRFr1 antagonism did not permanently disrupt animal's ability to re-learn context-fear (i.e., no permanent cellular damage). These results were confirmed statistically below.

For contextual fear conditioning, 47 subjects were included in the final analysis ($n_{vehicle\ control} = 12$, $n_{0.02µg} = 11$, $n_{0.2µg} = 12$, $n_{2µg} = 12$). Five animals were excluded for improperly placed cannula and as statistical outliers (two from the 0.02 µg group, one from the 0.2 µg group, and two from the 2 µg group). A 4 x 3 repeated measures ANOVA revealed a significant main effect of testing phase ($F(2,86) = 161.22$, $p < .001$), no main effect of group $F(3,43) = 1.056$, $p > .05$) , but a significant group X testing phase interaction ($F(6,86) = 2.38$, $p < .05$). An ANOVA showed that groups did not differ at baseline or during the post-shock testing phases (p's > .05), but significantly differed during the retention test ($F(3,43) = 3.41$, $p < .05$). A post-hoc Dunnett's test revealed that 0.02 µg did not significantly differ from vehicle controls ($p > .05$), 0.2 µg marginally differed from vehicle controls ($p = .08$), and 2 µg significantly differed from vehicle controls ($p < .01$; Figure 3A).

Animals were re-trained in an alternate context to examine if antalarmin permanently disrupted rats' ability to learn contextual fear ($n_{vehicle\ control}$ = 8, $n_{0.02ug}$ = 11, $n_{0.2ug}$ = 12, n_{2ug} = 8). Eight animals were lost due to clogged cannula (4 in the vehicle control group and 4 in the 2 µg group). We detected a main effect of testing phase ($F(2, 70) = 89.572$, $p < .001$), but no main effect of group $F(3, 35) = .304$, $p > .05$, and no group by testing phase interaction ($F(6, 70) = 0.546$, $p > .05$); Supplementary Figure 1).

Finally, we examined the effects of $_{L}$BNST CRFr1 antagonism on freezing to TMT ($n_{vehicle\ control}$ = 4, n_{2ug} = 6). Two animals from the vehicle control group were excluded due to improper cannula placements. A two-way ANOVA contrasting group (vehicle control and 2 µg) by testing phase (baseline and TMT exposure) revealed no main effect of group at acclimation ($F(1, 8) = 2.497$, $p > .05$) or at the TMT exposure session ($F(1, 8) = 0.326$, $p > .05$; Figure 3B).

3.3 $_{L}$BNST CRFr1 antagonism does not affect shock responsivity (Figure 4 and Supplementary Table 1)

Finally, given that antalarmin did not affect freezing during the post-shock testing phase (i.e., context-shock US acquisition) or to the unconditioned predator odor TMT, we wanted to investigate whether the effect of antalarmin on the retention of contextual fear was produced by changes in processing the shock-US, rather than associative context-US learning. Similar to the lack of an effect with antalarmin on freezing to the unconditioned predator odor TMT, antalarmin infused into the $_{L}$BNST did not affect animals' ability to respond to the shock-US. These results were confirmed statistically below.

Fourteen subjects were included in shock responsivity analyses (n_{2ug} = 8 and $n_{\text{vehicle control}}$ = 6). Two animals were excluded due to clogged cannula. A 2 x 8 paired samples t-test contrasting groups (vehicle and 2 µg antalarmin) across the measured shock intensities (0 mA, 0.1 mA, 0.4 mA, 0.8 mA, 1.2 mA, 1.5 mA, 1.8 mA, and 2.1 mA) revealed no main effect of group ($F(1, 12) = 1.87$, $p > .05$, a main effect of shock intensity ($F(7, 84) = 46.60$, $p < .001$), but no group by shock intensity interaction ($F(7, 84)=0.83$, $p > .05$; Figure 4). Given that the 2 µg group appeared as if it may be responding *more* to specific shock intensities (.08mA, 1.2mA, and 1.5 mA) than the vehicle controls in the graphed data, we conducted exploratory t-tests to confirm groups did not differ. Rats did not differ at 0.8mA ($t(12) = 1.80$, $p > .05$), 1.2mA ($t(12) = 1.77$, $p > .05$), or 1.5mA ($t(12) = 1.53$, $p > .05$; Supplementary Table 1).

4. Discussion

In the present study we examined the role of CRF type 1 receptors during two different types of fear – contextually conditioned fear and unconditioned fear to the predator odor TMT. Antagonism of CRFr1s globally within brain (ICV) and selectively in the dorsolateral BNST with antalarmin disrupted the retention of contextual fear, but had no effect on freezing to the predator odor TMT. Antalarmin also did not change foot-shock responsivity, indicating that behavior to the shock itself was unaffected. Our results highlight a unique role for CRFr1s in the dorsolateral BNST for modulating contextual fear learning and not behavior to the shock per se and freezing behavior elicited immediately following the shock. Furthermore, we also show that CRFr1s are not important for modulating behavior to unconditioned threats that qualitatively differ (e.g., shock-US or predator odor-US). Broadly, our findings point to a diverging role for CRFr1s in the dorsolateral BNST during conditioned and unconditioned fear-like behaviors –

suggesting that CRFr1s in the dorsolateral BNST selectively modulate fear acquisition and possibly consolidation, but not unconditioned freezing to a predator odor.

The BNST is known to play a crucial role in fear to discrete cues of long-duration (i.e., cues being tones and lights (Walker et al., 2009a)) and recent work has shown that contextual stimuli may be processed in a similar way (Radke, 2009; Sullivan et al., 2004), but only when presented for a long duration before receiving a shock (Hammack et al., 2015). Thus, the BNST's role in processing aversive stimuli may be constrained along a temporal domain rather than a cue-specific (e.g., tones and lights) domain – a phenomenon that our study may be tapping into given that animals received three minutes of context exposure prior to shock. Importantly, we found that antalarmin in the BNST only affected freezing during the five-minute retention test 24 hours after conditioning. CRFr1s were not important for (1) freezing during the five minute post-shock period (i.e., a measure of acquisition (Fanselow, 1980, 1986)) or (2) the ten minutes of TMT exposure (i.e., an unconditioned stimulus that qualitatively differed from the shock). The lack of an effect of antalarmin on freezing to a shock-US or a predator odor-US provides an important insight. That is, behavior to unconditioned stimuli in general is not regulated by CRFr1s in the dorsolateral BNST. Furthermore, although contextual fear acquisition (i.e., post-shock freezing) was unaffected, it is still unclear how CRFr1s in the dorsolateral BNST may regulate learning. Our data point to a role in memory consolidation, but future experiments that antagonize CRFr1s at specific time-points after contextual fear learning and prior to retention testing are needed to more fully assess the role of CRFr1s during different phases of memory.

CRFr1s in the dorsolateral BNST are modulated by local (residing within the BNST) and distal (afferents from the CeA) CRF release, with dense innervations arising from the CeA

(Sakanaka et al., 1986; Swanson et al., 1983). While CRF in the CeA and CRF receptors in the BLA are important for fear memory consolidation, we speculate that the CeA may provide a critical CRF signal to CRFr1s in the $_{L}$BNST that modulates the formation of long-term contextual fear memories. This hypothesis is supported by studies showing that CRF knockdown in the CeA (1) disrupts the consolidation of long-duration contextual fear memories in a time-limited manner (Pitts and Takahashi, 2011; Pitts et al., 2009) and also (2) sensitizes CRFr1s in the BNST (Regev et al., 2012).

However, another lateral BNST subdivision, the ventrolateral BNST, also receives CRF projections from the CeA (Pomrenze et al., 2015; Sakanaka et al., 1986). Both the dorsolateral (Dabrowska et al., 2015) and ventrolateral BNST synthesize CRF (Asok et al., 2013a; Gray and Magnuson, 1992) and contain CRFr1s (Van Pett et al., 2000). While the ventrolateral BNST may play a greater role in modulating overall HPA/glucocortiocid-activity (Choi et al., 2007), the selective role of CRFr1s in the ventrolateral BNST in acquisition and retention of contextually conditioned fear or unconditioned predator-odor fear remains unknown. Neurotoxic lesions of the central nucleus of the amygdala, which presumably disrupt CeA CRF pathways to both BNST subdivisions, have no effect on TMT-induced freezing, but do disrupt the acquisition and retention of contextual fear (Rosen, 2004). Furthermore, inactivation of the dorsolateral BNST has no effect on TMT induced freezing (Fendt et al., 2003), but disruption of noradrenergic activity in the ventrolateral BNST does (Fendt et al., 2005). We targeted the dorsolateral BNST because of its well-studied role in fear conditioning and startle (cf. (Davis et al., 2010; Sullivan et al., 2004)), but it is quite possible that CRFr1s in the ventrolateral BNST may contribute to contextually conditioned (activated by CRF released from local and distal CeA CRF inputs) and

unconditioned predator odor (activated by CRF from local CRF input) fear and anxiety-like behaviors.

CRF administration increases numerous fear and anxiety-like behaviors (for reviews see; Bale and Vale, 2004; Seckler, Kalin, and Reul, 2005) and CRF antagonists block many of these effects (Bale and Vale, 2004). Whereas CRFs primary receptors, the type 1 and type 2 receptors, have opposing roles in fear and anxiety (Bale and Vale, 2004; Takahashi, 2001), blocking CRFr1s produces anxiolytic effects. The importance of CRF within the (extended) amygdala has been highlighted by recent studies showing that (1) non-selective CRF receptor antagonism in the basolateral amygdala (BLA; i.e., a major local amygdala subregion that contains many CRFr1s) disrupts the consolidation of inhibitory avoidance memories (Roozendaal et al., 2002), (2) non-selective CRF blockade in the BNST disrupts CRF-induced freezing (Nijsen et al., 2001), and (3) knockdown of CRF in the central nucleus of the amygdala (CeA; i.e., the CRF synthesizing region in the amygdala that sends CRF to both the BLA and BNST) disrupts the consolidation of contextual fear (Pitts and Takahashi, 2011; Pitts et al., 2009). While we did not evaluate the function of CRFr2s, our findings expand on these studies to show that CRFr1s in the dorsolateral BNST are necessary for the retention of contextual fear memories.

Contextually conditioned defensive responses are thought to rely on CRF (Radulovic et al., 1999) and corticosterone (CORT) signaling (Pugh et al., 1997), but unconditioned predator odor responses may not be regulated by CRF and CORT – an important distinction shown by a number of studies (for review see (Rosen, 2004; Rosen et al., 2015)). This is puzzling given that both types of threat (1) increase CORT (Cordero et al., 1998; Day et al., 2004), (2) increase CRF in the CeA and BNST (Asok et al., 2013a; Lehner et al., 2008), and (3) CORT alone increases CRF expression in the CeA and BNST (Makino et al., 1994a, b). Lesions of the BNST also

disrupt both types fear (Fendt et al., 2003; Sullivan et al., 2004). However, chronic CORT only affects contextually conditioned fear (Skórzewska et al., 2006; Thompson et al., 2004), not unconditioned fear to TMT (Rosen et al., 2008). Additionally, CRF overexpression (which reduces CRFr1 expression) in the BNST does not affect unconditioned fear in the elevated plus maze (Sink et al., 2013b). While CRFr1 antagonism (peripherally and in the BNST) disrupts conditioned fear (Deak et al., 1999; Kalin and Takahashi, 1990; Nijsen et al., 2001), our work adds an important piece to this puzzle by showing that CRFr1s centrally (within the dorsolateral BNST) are important for contextually conditioned fear and not unconditioned predator fear. However, other areas such as the olfactory bulb and amygdalo-piriform transition area may play greater a role in modulating CRF and glucocorticoid activity to predator odors than previously known (Kondoh et al., 2016).

The present study expands on previous reports that have evaluated the role of CRF and CRFr1s in the BNST during fear and anxiety-like behaviors. Other studies have found that systemic, intra-BNST, or ICV CRFr1 antagonism alone (without additional CRF or other peptides; (Sink et al., 2013a) has no effect on fear-potentiated startle to short-duration cues, only long-duration cues (e.g., contexts, 8 min. tones, etc.; cf. (Walker et al., 2009a)). Systemic CRFr1 antagonism with antalarmin either before fear conditioning or before a retention test disrupted conditioning or expression of contextual fear (Deak et al., 1999; Radulovic et al., 1999). Non-selective CRFr1 antagonism in the BNST disrupts contextual fear conditioning (Nijsen et al., 2001). Our study is an important addition in that we found an effect of CRFr1 antagonism on long-term contextual fear memories (using a single-trial paradigm), but not predator-odor induced fear-like behavior.

Given its connectivity with core amygdala structures, the BNST is ideally situated to control both behavioral and endocrine function (Schulkin et al., 2005) under situations of sustained threat. CRFr1s within the $_{L}$BNST regulate the retention of contextually conditioned fear, but not unconditioned fear to either a shock or a predator odor. Future studies should examine the role of local CRF and CeA CRF projections during discrete phases of contextual fear learning using optogenetic and chemogenetic approaches (Gafford and Ressler, 2015).

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Conflicts of Interest

None of the authors have conflicts of interest.

Contributors

All authors have seen and approved the final version of the submitted manuscript. The article is the authors' original work, and has not received prior publication and is not under consideration for publication elsewhere. Arun Asok conducted the studies with the help of undergraduate students acknowledged in the acknowledgement section. All three authors contributed to the conception of the research, the data analysis, and writing of the manuscript.

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Figure Captions

Figure 1. Representative $_{L}$BNST Cannula Placements. (A) Brain section stained with cresyl violet showing cannula tracks above the $_{L}$BNST. (B) Brain drawing showing the $_{L}$BNST highlighted in dark grey with black arrows. (C) Each black dot represents a histologically confirmed injection site. The anterior (+) and posterior (-) millimeter distances from Bregma are located in the top right of each image. Brain drawings were adapted from the atlas of Paxinos and Watson, (Paxinos and Watson, 2007).

Figure 2. ICV CRFr1 antagonism prior to contextual fear conditioning disrupted fear retention 24 hours later. (A) ICV CRFr1 antagonism at a dose of 20 µg antalarmin disrupted the retention of contextual fear, but had no effect on baseline or post-shock freezing. (B) ICV CRFr1 antagonism had no effect on freezing to the predator odor TMT. (C) ICV CRFr1 antagonism at a 100 µg antalarmin dose (5x greater than what reduced contextual fear) did not affect freezing to TMT. Y-axis is the mean % freezing. Error bars are ± S.E.M, *$p < .05$.

Figure 3. $_{L}$BNST CRFr1 antagonism prior to contextual fear conditioning disrupted fear retention 24 hours later. (A) $_{L}$BNST CRFr1 antagonism dose-dependently disrupted the retention of contextual fear, but had no effect on baseline or post-shock freezing. (B) $_{L}$BNST CRFr1 antagonism had no effect on freezing to TMT. Y-axis is the mean % freezing. Error bars are ± S.E.M, *$p < .05$.

Figure 4. $_{L}$BNST CRFr1 antagonism did not affect responsivity to varying foot-shock intensities. $_{L}$BNST CRFr1 antagonism at a dose of 2 µg (a dose that disrupted the retention of contextual fear) did not affect animal's ability to respond to foot-shocks even at the same intensity used for contextual fear conditioning. Y-axis is mean movement of accelerometer in arbitrary units. Error bars are ± S.E.M.