Published in final edited form as:

Neuropharmacology. 2017 July 15; 121: 130–139. doi:10.1016/j.neuropharm.2017.04.039.

Oxytocin receptor neurotransmission in the dorsolateral bed nucleus of the stria terminalis facilitates the acquisition of cued fear in the fear-potentiated startle paradigm in rats

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Abstract

Oxytocin (OT) is a hypothalamic neuropeptide that modulates fear and anxiety-like behaviors. Dorsolateral bed nucleus of the stria terminalis (BNST_{dl}) plays a critical role in the regulation of fear and anxiety, and expresses high levels of OT receptor (OTR). However, the role of OTR neurotransmission within the BNST_{dl} in mediating these behaviors is unknown. Here, we used adult male Sprague-Dawley rats to investigate the role of OTR neurotransmission in the BNST_{dl} in the modulation of the acoustic startle response, as well as in the acquisition and consolidation of conditioned fear using fear potentiated startle (FPS) paradigm. Bilateral intra-BNST_{dl} administration of OT (100 ng) did not affect the acquisition of conditioned fear response. However, intra-BNST_{dl} administration of specific OTR antagonist (OTA), (d(CH₂)₅¹, Tyr(Me)², Thr⁴, Orn⁸, des-Gly-NH₂⁹)-vasotocin, (200 ng), prior to the fear conditioning session, impaired the acquisition of cued fear, without affecting a non-cued fear component of FPS. Neither OTA, nor OT affected baseline startle or shock reactivity during fear conditioning. Therefore, the observed impairment of cued fear after OTA infusion resulted from the specific effect on the formation of cued fear. In contrast to the acquisition, neither OTA nor OT affected the consolidation of FPS, when administered after the completion of fear conditioning session. Taken together, these results reveal the important role of OTR neurotransmission in the BNST_{dl} in the formation of conditioned fear to a discrete cue. This study highlights the role of the BNST_{dl} in learning to discriminate between threatening and safe stimuli.

Keywords

oxytocin; fear; anxiety; startle; learning; BNST

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1. Introduction

Oxytocin (OT) is a hypothalamic neuropeptide that modulates a wide range of social behaviors, as well as fear and anxiety-like behaviors, for review see (Neumann and Slattery, 2016). Although substantial evidence suggests that OT has anxiolytic properties (Bale et al., 2001; Ellenbogen et al., 2014; Ring et al., 2006), the role of OT neurotransmission in the regulation of conditioned fear appears to be more complex and brain site-specific. When applied intracerebroventricularly (ICV) prior to the fear conditioning, OT reduces cued fear expression, but OT impairs cued fear extinction when delivered prior to the extinction training (Toth et al., 2012). In addition, OT in the central amygdala (CeA) (Knobloch et al., 2012) and medial prefrontal cortex (Lahoud and Maroun, 2013) reduces contextual fear expression, but the opposite effect is observed when OT is delivered into the basolateral amygdala (Lahoud and Maroun, 2013), or when OT receptors (OTR) are overexpressed in the lateral septum (Guzman et al., 2013). In the fear potentiated startle (FPS) experiments systemic OT decreases background anxiety, but it has no effect on cued or contextual fear, when administered systemically or ICV (Ayers et al., 2011; Missig et al., 2010). In these experiments, cued fear was measured as a potentiation of the startle amplitude that occurred in a presence of conditioned stimulus (CS⁺). Background anxiety was expressed as a potentiation of the startle amplitude during noise-alone trials (CS⁻) that occurred after the first presentation of CS⁺ (measured in a novel context). Contextual fear was measured as a startle potentiation to noise-only trials (no CS presentation) in the training context.

Dorso-lateral bed nucleus of the stria terminalis (BNST_{dl}) is a key brain area translating stress into sustained anxiety (Dabrowska et al., 2013; Daniel and Rainnie, 2016; Davis et al., 2010; Sparta et al., 2013). While involvement of the BNST in the light- (Walker and Davis, 1997) or corticotropin-releasing factor (CRF) potentiated startle (Lee and Davis, 1997) is well documented, the role of the BNST in the modulation of cued fear is less apparent. BNST lesions disrupt expression of contextual fear (Sullivan et al., 2004), as well as conditioned fear response to long-lasting cues (Davis et al., 2010), but not short, discrete cues (Gewirtz et al., 1998; Hitchcock and Davis, 1991; LeDoux et al., 1988). However, most of the initial reports stem from lesion studies, whereas accumulating evidence from cell-type specific manipulations supports the notion that the BNST might also be involved in the conditioned fear response to discrete cues, for review see (Gungor and Pare, 2016). The BNST has one of the highest expression levels of OTR (Dabrowska et al., 2011; Dumais et al., 2013; Tribollet et al., 1988; Veinante and Freund-Mercier, 1997) and receives dense OT inputs from the paraventricular nucleus of the hypothalamus (Dabrowska et al., 2011; Knobloch et al., 2012), yet the role of OTR neurotransmission in the BNST in the regulation of fear and anxiety is unknown.

Here, we examined the effects of OT and OTR antagonist administration into the BNST_{dl} on the acoustic startle response (ASR), as well as on the acquisition and consolidation of cued and non-cued fear using FPS paradigm. Using *in vivo* pharmacological approach we demonstrate for the first time that OTR neurotransmission in the BNST_{dl} facilitates the acquisition, but not consolidation, of conditioned fear to a discrete cue.

2. Material and methods

2.1 Animals

Adult male Sprague-Dawley rats aged 44–48 days old and weighing 175–199 g were purchased from ENVIGO (IL). The rats were housed in groups of three on a 12 h light/dark cycle (light 7 a.m. to 7 p.m.) with free access to water and food. The rats were allowed to adapt to this environment for one week before the experiments began. A total of 97 animals were used in these experiments. All experimental procedures were approved by the Institutional Animal Care and Use Committees at Rosalind Franklin University of Medicine and Science, and were performed in accordance with the US National Institutes of Health guidelines.

2.2 Drugs

OT (H-2510, Bachem Inc., CA) and the selective OTR antagonist (OTA, H-2908, Bachem Inc., CA), (d(CH₂)₅¹, Tyr(Me), Thr⁴, Orn⁸, des-Gly-NH₂⁹)-vasotocin (Manning et al., 2012) were stored in –80 Celsius degrees freezer and diluted in artificial cerebrospinal fluid (ACSF, pH = 7.4) before an experiment.

2.3 Surgery

Rats weighing between 230–270 g were deeply anesthetized with mix of isoflurane and oxygen and placed in a stereotaxic frame (Model 900; Kopf, CA). Ketoprofen was used as an analgesic (5 mg kg^{–1}, subcutaneous; Zoetis Inc., MI). Rats were bilaterally implanted with guide cannula (22-gauge, 7 mm length; Plastics One, Roanoke, VA) aimed at the BNST_{dl} using the following stereotaxic coordinates (15° coronal angle, from bregma, AP: +0.1 mm; ML: ±3.4 mm; DV: –5.25 mm). Stereotaxic coordinates were based on the rat brain atlas (Paxinos and Watson, 2007) and adapted from the BNST_{dl} coordinates we have published before (Dabrowska et al., 2016). Guide cannulae were positioned 2 mm above the BNST_{dl} and fixed with dental cement bonded to stainless steel screws inserted into the surface of the skull. A dummy cap (7 mm length; Plastics One, Roanoke, VA) was inserted into the guide cannula to maintain it free of obstructions. Following surgery, the rats were allowed to recover for 4–7 days, during which they were handled and their cannulas checked daily to habituate them to the injection procedure.

2.4 Drug administration

OT (100 ng), OTA (200 ng), or ACSF (all in volume of 0.5 µl per side) were injected bilaterally into the BNST_{dl} through microinjector (28-gauge, 7 mm length; Plastics One, Roanoke, VA). Injections were made at a rate of 0.25 µl min^{–1} using microinjection pump (Harvard Apparatus, MA), connected via polyethylene tubing (PE-20) to Hamilton syringes. After the injection, the microinjector was left in place for additional 5 min for adequate diffusion. The doses of OT and OTA were chosen based on previous studies on fear and anxiety in rats (Bale et al., 2001; Lahoud and Maroun, 2013; Neumann and Slattery, 2016; Neumann et al., 2000; Toth et al., 2012).

2.5 Acoustic startle apparatus

All experiments were conducted in eight, identical SR-LAB startle chambers with cylindrical animal enclosures (San Diego Instruments, San Diego, CA). A high-frequency loudspeaker, mounted 24 cm above the enclosures, provides background noise as well as the startle eliciting white-noise bursts (WNB). During the FPS, a single LED bulb positioned on the ceiling inside the startle chamber was used as the visual conditioned stimulus (CS). In addition, a grid floor made of stainless steel bars placed inside the enclosures was used to deliver foot shocks as the unconditioned stimulus (US). The presentation and sequence of all stimuli as well as recording of the responses were automatically controlled by the SR-LAB software (San Diego Instruments).

2.6 Acoustic startle response (ASR)

On day 1 (habituation), rats were placed in the cylindrical enclosures inside the chambers for 20 min. On day 2 (pre-test), rats were placed in the same enclosures, where after 5 min acclimation they were presented with 30 startle eliciting WNB (95 dB, 50 ms, inter-trial-interval 30 s). A background white-noise (70 dB) was continuously played throughout the whole session. On day 3 (test), rats received 30-startle eliciting WNB (same as above) 10 min after bilateral administration of OT (n=7), OTA (n=7) or ACSF (n=7) into the BNST_{dl} (Fig. 1).

In all experiments, animals were assigned to the experimental groups based on their baseline ASR (pre-test) in order to generate experimental groups with balanced average ASR and to reduce variability in stress-reactivity. All chambers were cleaned with a 70% ethanol solution between sessions.

2.7 Fear potentiated startle (FPS)

The FPS procedures were modified based on previous studies (Ayers et al., 2011; Missig et al., 2010; Sink et al., 2013; Walker et al., 2009a). On days 1 and 2, separate cohort of rats underwent habituation and pre-test sessions, respectively (as above). On the following day (fear conditioning), animals were placed in the cylindrical enclosures containing a grid floor conveying foot shocks. After 5 min acclimation, animals received 10 presentations of a 3.7 s cue light (CS), each co-terminating with a 0.5 s foot shock (US; 0.5 mA, inter-trial-interval 60–180 s). A background noise was absent during the conditioning session. Twenty-four hours later, rats were tested for the FPS expression (test), where after 5 min acclimation they were exposed to 30 startle eliciting WNB (as above) and their levels of cued and non-cued fear, were measured. A background white-noise of 70 dB was continuously played throughout the whole session. The session consisted of 10 baseline startle trials (not included in the analysis) followed by additional 20 trials, with half presented in the presence of the cue light (CS) and the other half without the CS (noise-alone), mixed in a pseudorandom order (inter-trial-interval 30 s). During the FPS testing, grid floor was removed from the enclosures.

To examine the role of OT in the acquisition of fear conditioning, animals were divided based on pre-test ASR into three treatment groups: OT (n=13), OTA (n=20), or ACSF

(n=21). Fear conditioning session was performed 10 min after the intra-BNST_{dl} injections (Fig. 1).

Another cohort of rats was used for the FPS consolidation experiment. Here, rats received OT (n=7), OTA (n=7), or ACSF (n=8), 30 min after completing the fear conditioning session (Fig. 1).

2.8 Histology

Following the experiments, rats were bilaterally injected with 1% Chicago Sky Blue dye (Alfa Aesar, MA) into the BNST_{dl} (0.5 μ l). The brains were removed and coronal sections (50 μ m) were cut on a Leica freezing-stage sliding microtome (SM200R, Leica Biosystems Inc., IL) and photographed to assess proper cannula placement (Fig. 2). Data from rats with guide cannula placement outside the BNST_{dl}, including posterior, anterior, or sub-commissural BNST were excluded from the analyses. Rats with both unilateral and bilateral hits were included in the analysis.

2.9 Data analysis

Startle amplitude was defined as the maximum peak voltage within the first 200 ms after the onset of WNB. Shock reactivity amplitude was recorded during the fear conditioning session and was defined as the maximum peak voltage that occurred during the 0.5 s foot shock delivery. Cued and non-cued fear were calculated as percent change scores of startle amplitude based on (Ayers et al., 2011; Missig et al., 2010; Walker et al., 2009a). Cued fear = [(light-noise trials – noise-alone trials)/noise-alone trials] $\times$ 100. Non-cued fear = [(noise-alone trials – pre-shock trials)/pre-shock trials] $\times$ 100 (Fig. 2).

2.10 Statistical analysis

All data are presented as mean $\pm$ standard error of mean (SEM). For the ASR experiment, the mean startle amplitude was analyzed by two-way repeated measures analysis of variance (ANOVA) with the factors TIME (pre- and post-treatment) and TREATMENT (OT, OTA or ACSF). For the FPS experiment, the mean startle amplitude was analyzed by two-way repeated measures ANOVA with the factors TRIAL TYPE (pre-shock, noise-alone, and light-noise) and TREATMENT (OT, OTA or ACSF). Where the F-ratio was significant, all pairwise post hoc comparisons were made using Bonferroni's tests. The percent change scores (cued fear, and non-cued fear) were analyzed separately for each measure using one-way ANOVA. The effects of drug treatments on shock reactivity during fear conditioning were analyzed by one-way ANOVA. The percent change scores (cued and non-cued fear) and shock reactivity between low and high responders infused with ACSF were calculated with unpaired *t*-test. The statistical analyses were completed using GraphPad Prism version 6 (GraphPad Software Inc., San Diego, CA). *P* $\leq$ 0.05 was considered significant.

3. Results

3.1 Effects of OT or OTA administration into the BNST_{dl} on the ASR

Quantitative analysis revealed that neither OT nor OTA changed the ASR amplitude when administered intra-BNST_{dl} 10 min before the test. There was no main effect of TIME (*F* (1,

18) = 2.92, $P = 0.10$) or TREATMENT ($F(2, 18) = 0.12$, $P = 0.89$), and no interaction between TIME and TREATMENT ($F(2, 18) = 0.46$, $P = 0.64$, two-way repeated measures ANOVA) (Fig. 3).

3.2 Effects of OT or OTA administration into the BNST_{dl} on the acquisition of FPS

3.2.1 Acquisition of cued fear conditioning—All animals exhibited a significantly potentiated startle response in light-noise trials compared to noise-alone trials. There was a significant main effect of TRIAL TYPE (noise-alone, light-noise) ($F(1, 51) = 91.33$, $P < 0.0001$), but no main effect of TREATMENT ($F(2, 51) = 0.52$, $P = 0.60$). However, there was a significant interaction between TRIAL TYPE and TREATMENT ($F(2, 51) = 5.63$, $P = 0.0062$ two-way repeated measures ANOVA). Post hoc comparisons revealed significant differences in startle amplitude in light-noise trials compared to noise-alone trials in ACSF-treated group ($t(20) = 7.78$, $P < 0.0001$), OT-treated group ($t(12) = 5.70$, $P < 0.0001$), as well as in OTA-treated rats ($t(19) = 3.19$, $P < 0.01$), Bonferroni's post hoc tests (Fig. 4A, B). The percent change analysis revealed significant effect of TREATMENT on cued fear ($F(2, 51) = 4.36$, $P = 0.017$), and Bonferroni's post hoc comparisons test revealed a significant reduction in cued fear in OTA-treated group compared to ACSF-treated animals ($P < 0.05$, Fig. 4C).

3.2.2 Acquisition of non-cued fear conditioning—Quantitative analysis of the non-cued fear showed a significant enhancement of startle amplitude in noise-alone trials compared to pre-shock trials across all groups. There was a main effect of TRIAL TYPE ($F(1, 51) = 20.79$, $P < 0.0001$) but no main effect of TREATMENT ($F(2, 51) = 1.39$, $P = 0.26$) and no interaction between TRIAL TYPE and TREATMENT ($F(2, 51) = 1.15$, $P = 0.32$) (Fig. 4A). Similarly, the mean percent change in non-cued fear was not different between treatment groups ($F(2, 51) = 0.66$, $P = 0.52$) (Fig. 4D).

3.2.3 Shock reactivity—The mean shock reactivity during the fear conditioning session was not different between ACSF, OT and OTA-treatment groups ($F(2, 51) = 0.57$, $P = 0.57$) (Fig. 4E).

3.3 FPS in rats with low and high levels of pre-shock ASR

Peripherally administered OT was shown to reduce background anxiety measured in FPS only in rats with low levels of ASR (Ayers et al., 2016). To assess whether OT or OTA might differently affect rats with low and high levels of ASR, animals were divided into low and high responders based on their pre-shock ASR values. We used a median split within each experimental group to divide animals into low (ACSF=11, OT=7, OTA=10) and high responders (ACSF=10, OT=6, OTA=10).

In ACSF-treated controls, low and high responders showed equivalent percentage of cued fear and an unpaired t -test comparing the mean percent change in cued fear revealed no significant difference between low and high responders ($P = 0.79$, Fig. 5A). However, there was a significant difference in percentage of non-cued fear between low and high responders ($P = 0.03$, unpaired t -test, Fig. 5B). Finally, shock reactivity during fear conditioning session

was not significantly different between low and high responders ($P = 0.37$, unpaired t -test, Fig. 5C).

3.3.1 Effects of OT or OTA administration into the BNST_{dl} on the acquisition of FPS in rats with low levels of pre-shock ASR

—In low responders, quantitative analysis showed a significant enhancement of startle amplitude in light-noise trials compared to noise-alone trials such that there was a significant main effect of TRIAL TYPE (noise-alone, light-noise) ($F(1, 25) = 68.45$, $P < 0.0001$), but no main effect of TREATMENT ($F(2, 25) = 2.27$, $P = 0.12$). However, there was a significant interaction between TRIAL TYPE and TREATMENT ($F(2, 25) = 6.87$, $P = 0.004$, two-way repeated measures ANOVA). Post hoc comparisons revealed significant differences in startle amplitude in light-noise trials compared to noise-alone trials in ACSF-treated group ($t(10) = 6.01$, $P < 0.0001$), OT-treated group ($t(6) = 6.20$, $P < 0.0001$), but not in OTA-treated rats ($t(9) = 1.97$, $P > 0.05$), Bonferroni's test. In addition, the startle amplitude in light-noise trials was significantly different between OT and OTA-treated rats ($P < 0.01$, Bonferroni's post hoc tests) (Fig. 6A). Notably, the comparison of mean percent changes in cued fear revealed significant effect of TREATMENT ($F(2, 25) = 4.10$, $P = 0.03$) and Bonferroni's multiple comparisons test showed a significant reduction in cued fear in OTA-treated group in comparison to ACSF-treated group ($P < 0.05$, Fig. 6B).

Quantitative group analysis revealed a significant potentiation of startle amplitude during noise-alone trials in comparison to pre-shock startle trials (non-cued fear) in all low responders, but this was not affected by intra-BNST_{dl} injections. There was a significant main effect of TRIAL TYPE (pre-shock ASR, noise-alone) ($F(1, 25) = 26.95$, $P < 0.0001$) but no effect of TREATMENT ($F(2, 25) = 1.70$, $P = 0.20$), and no interaction between TRIAL TYPE and TREATMENT ($F(2, 25) = 0.57$, $P = 0.57$) (Fig. 6A). Comparison of percent changes in background anxiety did not reveal any differences between treatment groups ($F(2, 25) = 0.29$, $P = 0.75$, Fig. 6C).

Finally, the shock reactivity during fear conditioning was not affected by intra-BNST_{dl} injections in rats with low levels of ASR ($F(2, 25) = 2.56$, $P = 0.097$, one-way ANOVA, Fig. 6D).

3.3.2 Effects of OT or OTA administration into the BNST_{dl} on the acquisition of FPS in rats with high levels of pre-shock ASR

—In high responders, quantitative analysis revealed a significant cued fear across all groups. There was a significant main effect of TRIAL TYPE (noise-alone, light-noise) ($F(1, 23) = 37.14$, $P < 0.0001$) but no main effect of TREATMENT ($F(2, 23) = 0.27$, $P = 0.76$), and no interaction between TRIAL TYPE and TREATMENT ($F(2, 23) = 2.21$, $P = 0.13$, Fig. 6E). Accordingly, the mean percentage of cued fear was not significantly different among experimental groups ($F(2, 23) = 1.38$, $P = 0.27$, one-way ANOVA, Fig. 6F). However, variability observed in a percentage change of cued fear in the OT-treated group of high responders (Fig. 6F) might have resulted in the lack of significant ANOVA. We have therefore recalculated the results after excluding two outliers from the OT-treated group (cued fear: -5.15% and 737.01% , respectively). Here, the comparison of mean percent changes in cued fear revealed significant effect of TREATMENT ($F(2, 21) = 5.24$, $P = 0.01$) and Bonferroni's multiple comparisons test

showed a significant reduction in cued fear in OTA-treated group in comparison to ACSF-treated group ($P < 0.05$). Based on the ANOVA results, we cannot exclude the possibility that the effect of OTA on cued fear is observed in both low and high responders.

Quantitative analysis revealed no significant non-cued fear in any group of high responders. There was no main effect of TRIAL TYPE (pre-shock baseline, noise-alone) ($F(1, 23) = 2.88$, $P = 0.10$), no main effect of TREATMENT ($F(2, 23) = 0.55$, $P = 0.58$), and no interaction between TRIAL TYPE and TREATMENT ($F(2, 23) = 1.75$, $P = 0.20$), Fig. 6E). In addition, the comparison of mean percent change in non-cued fear revealed no significant difference between treatment groups ($F(2, 23) = 2.26$, $P = 0.30$, one-way ANOVA, Fig. 6G).

Similarly to the low responders, the shock reactivity measured during fear conditioning was not different between treatment groups in high responders ($F(2, 23) = 0.15$, $P = 0.86$, one-way ANOVA, Fig. 6H).

3.4 Effects of OT or OTA administration into the BNST_{dl} on the consolidation of FPS

3.4.1 Consolidation of cued fear conditioning—To determine the role of OT in cued fear consolidation, the rats were injected with ACSF, OT or OTA into the BNST_{dl} after fear conditioning session. All animals showed increased startle amplitude in light-noise trials compared to noise-alone trials, indicative of robust cued fear. There was a main effect of TRIAL TYPE (noise-alone, light-noise) ($F(1, 19) = 24.94$, $P < 0.0001$) but no main effect of TREATMENT ($F(2, 19) = 0.17$, $P = 0.85$), and no interaction between TRIAL TYPE and TREATMENT ($F(2, 19) = 0.50$, $P = 0.61$, two-way repeated measures ANOVA) (Fig. 7A). The comparison of mean percent change in cued fear revealed no significant difference between treatment groups ($F(2, 19) = 1.58$, $P = 0.23$, one-way ANOVA) (Fig. 7B).

3.4.2 Consolidation of non-cued fear conditioning—The animals did not show a significant enhancement of startle amplitude in noise-alone trials compared to pre-shock trials. There was no main significant main effect of TRIAL TYPE (pre-shock baseline, noise-alone) ($F(1, 19) = 0.30$, $P = 0.59$), and no main effect of TREATMENT ($F(2, 19) = 0.39$, $P = 0.68$) or interaction between TRIAL TYPE and TREATMENT ($F(2, 19) = 0.006$, $P = 0.99$) (Fig. 7A). The percent change analysis revealed that neither OT nor OTA affected consolidation of non-cued fear ($F(2, 19) = 0.42$, $P = 0.66$, one-way ANOVA, Fig. 7C).

4. Discussion

Here, we demonstrate for the first time that intra-BNST_{dl} administration of OTA, but not OT, prior to the fear conditioning session reduces the acquisition of cued fear in the FPS paradigm. Our results suggest that OTR neurotransmission in the BNST_{dl} facilitates the acquisition of conditioned fear to a short, discrete cue, without affecting the formation of non-cued fear. The effect of OTA on the acquisition of cued fear was specific to the process of associative learning, because OTA did not affect ability to startle or shock reactivity measured during the fear conditioning session. Furthermore, OTR neurotransmission appears specifically involved in the process of fear acquisition because neither OTA nor OT affected consolidation of the cued fear.

The role of the BNST in mediating conditioned fear responses elicited by long-duration cues (Waddell et al., 2006; Walker et al., 2009b) or context (Duvarci et al., 2009; Sullivan et al., 2004) is well documented. Although initial lesion studies did not associate the BNST with modulation of conditioned fear to short-duration cues (Gewirtz et al., 1998; Hitchcock and Davis, 1991; LeDoux et al., 1988), accumulating evidence suggests otherwise, for review see (Gungor and Pare, 2016). During the recall of classically conditioned fear responses, 25% of BNST neurons show alterations in firing rates in response to a discrete CS (Hauffer et al., 2013). Deactivation of the BNST enhances discrimination of CS⁺ and CS⁻ during conditioned fear responses (Duvarci et al., 2009). Recent studies have shown the critical role of the BNST in serotonin-induced enhancement of cued fear (Marcinkiewicz et al., 2016; Pelrine et al., 2016). These studies suggest that the BNST is also involved in the processing of cued fear conditioning. Notably, the BNST is a heterogeneous structure that contains a variety of neuropeptides (Hazra et al., 2011; Walter et al., 1991), which are critical modulators of behavior, but often exert opposing behavioral effects (Kash et al., 2015). Therefore, specific roles of various inputs and peptidergic neuromodulators in the BNST might not have been revealed by the lesion studies. Our results not only support the role of the BNST_{dl} in the regulation of fear associated with short-duration cues but also indicate that the OTR transmission in the BNST_{dl} facilitates the acquisition of cued fear. In the BNST_{dl}, OTR mRNA is expressed in Type II, electrophysiologically defined neurons (Dabrowska et al., 2011), which are putative inhibitory interneurons (Daniel and Rainnie, 2016). In the CeA, OT was shown to reduce fear expression by activating inhibitory interneurons and enhancing GABA transmission, which results in reduced activity of CeA output neurons (Viviani et al., 2011). It is likely that OTRs in the BNST also regulate fear learning by enhancing GABA transmission, as intra-BNST infusion of GABA-A agonist was shown to potentiate FPS expression (Meloni et al., 2006). Nonetheless, our results suggest that OTR might have opposing roles in modulating fear in the CeA and the BNST, such that it has been shown to reduce fear expression in the CeA (Knobloch et al., 2012) and it appears to facilitate fear acquisition in the BNST. However, OTR transmission might also differentially contribute to distinct phases of fear learning even within the same brain region, like it has been demonstrated in the CeA (Knobloch et al., 2012; Lahoud and Maroun, 2013). As OTRs are also found in Type III, putative CRF neurons in the BNST_{dl} (Dabrowska et al., 2011), which might be involved in the modulation of fear and anxiety (Dabrowska et al., 2013; Marcinkiewicz et al., 2016); it is possible that OTR on the CRF neurons modulate their neuronal activity and as such regulate the acquisition of cued fear.

In addition to the cued fear, during FPS testing, we have also measured potentiation of the startle amplitude in noise-alone trials that occurs after the first presentation of CS (Walker and Davis, 2002). We referred to this FPS component as non-cued fear but in some FPS studies it has been described as background anxiety (Ayers et al., 2016; Ayers et al., 2011; Missig et al., 2010). This FPS component might represent a slow decay of cued fear from the preceding light-noise trial, which is sustained beyond the immediate threat (Ayers et al., 2011; Missig et al., 2010). More likely, it reflects potentiation of the startle amplitude in response to unpredictable (not signaled by a cue) vs. predictable stimuli (cued-fear) (Grillon et al., 2009). Our results show that OTA selectively impaired potentiation of the startle amplitude during CS⁺ presentation (cued fear), without affecting startle potentiation during

noise-alone trials (CS^- , non-cued fear or background anxiety). These results suggest that OTR neurotransmission in the $BNST_{dl}$ enables learning of the discrimination between light-noise (CS^+) and noise-alone (CS^-) trials during fear conditioning, as we have shown that blocking OTR during learning process disrupts the ability to differently respond to CS^+ and CS^- trials during FPS testing. The role of the BNST in mediating such discrimination has been shown before (Duvarci et al., 2009).

More broadly, our results imply that OTRs in the $BNST_{dl}$ facilitate the formation of adaptive fear response, which allows discrimination between the threatening and non-threatening stimuli. In contrast, OTR blockade does not have an effect on baseline startle response or background anxiety. The latter suggests that OTR in the $BNST_{dl}$ does not modulate response to an unpredictable threat (non-cued fear), which reflects inability to discriminate between threat and safety (Grillon and Morgan, 1999; Grillon et al., 2009; Rosen and Schulkin, 1998). Relevant to the human FPS studies, the enhanced startle in response to situations of unpredictable threat (aversive stimuli administered unpredictably) is potentiated in patients suffering from post-traumatic stress disorder (PTSD), whereas startle increase in response to predictable threat (signaled by a cue) is not further enhanced in these patients (Grillon et al., 2009).

In the previous FPS studies, systemic, but not ICV, administration of OT reduced background anxiety in rats (Ayers et al., 2011; Missig et al., 2010) without affecting cued or contextual fear. Given that OT does not readily cross the blood brain barrier and OTR are highly expressed in the periphery (Gimpl and Fahrenholz, 2001), systemic OT might trigger peripheral mechanisms that are independent of central OT release (Evans et al., 2014; Moaddab et al., 2015). Therefore, it is difficult to directly compare the effects observed in the current study after intra- $BNST_{dl}$ infusion to the effects of peripheral OT administration. Although more recent study by the same group did not reproduce the overall effect of OT on background anxiety, it demonstrated differential OT effects on background anxiety in rats with low and high levels of baseline startle (Ayers et al., 2016). Our results show that although background anxiety was not affected by intra- $BNST_{dl}$ administration of OT or OTA in high or low responders, there was a significant difference in non cued-fear (background anxiety) between low- and high- responders infused with ACSF, such as high responders did not develop a significant potentiation of the startle amplitude in noise-alone trials. Furthermore, although non-cued fear was not affected by any treatment, blockade of OTRs in the $BNST_{dl}$ impaired the acquisition of cued fear in rats with low, and to a lesser extent, high levels of baseline startle. Importantly, both low and high responders demonstrate equivalent levels of cued fear after ACSF administration. The mechanisms of the potentially different effects of OTA in high and low ASR responders are yet to be determined. However, CRF is one of the major neuromodulators of ASR in the BNST (Lee and Davis, 1997; Walker et al., 2009b), and it might interact with OTR in the BNST (Dabrowska et al., 2011). Hence, it is possible that differences in the baseline CRF transmission between low and high responders might contribute to distinct effects of OTA in the $BNST_{dl}$. However, it needs to be noted that high variability of cued fear (percentage change) observed in OT-treated high-responders had inevitably contributed to the lack of significant treatment effect, when analyzed with ANOVA. Based on these results, we cannot unequivocally state that the effect of OTA on cued fear was only observed in low-responding rats.

Previous studies in rodents have shown that background anxiety measure is mainly independent from the contextual fear (Missig et al., 2010). In our design this has been also assured by altering context between FPS training and testing sessions (presence of background noise, removal of shock-conveying grid) to prevent formation of a significant contextual fear. However, future studies are warranted to test the effects of OT and OTA in the BNST_{dl} on the contextual fear (startle amplitude to noise only measured in the training context without CS presentation). Currently we cannot entirely exclude a possibility that the observed non-cued fear might have been contaminated with contextual fear. In addition, although all rats displayed robust cued fear, non-cued fear (background anxiety) was not significant in the consolidation experiment; hence future studies are warranted to test the potential contribution of OTR neurotransmission to the consolidation of non-cued fear.

OT is currently being tested as a potential pharmacotherapeutic agent for stress-related anxiety disorders, yet the effects of OT on fear learning are often contrasting and dependent on a brain region (Cohen et al., 2010; Guzman et al., 2013; Knobloch et al., 2012; Lahoud and Maroun, 2013) or different phase of fear learning (Toth et al., 2012). Although studies on intranasal OT application for fear-based psychiatric disorders in humans are promising (Acheson et al., 2013; Koch et al., 2016), other studies have shown enhancing effect of OT on fear learning in humans, when given prior to the fear conditioning (acquisition) (Eckstein et al., 2016). Other studies have shown that OT can be anxiogenic in humans as it can potentiate startle reactivity in response to an unpredictable threat (Grillon et al., 2013). Importantly, most of the animal and human studies on the role of OT in the regulation of fear have relied on application of exogenous OT, whereas exposure to various stressors and aversive stimuli triggers central OT release (Ebner et al., 2005; Landgraf and Neumann, 2004). Therefore, in the BNST_{dl}, foot shock-induced OT release during fear conditioning might have prevented any additional effect of exogenous OT application on fear acquisition in our experiments. Furthermore, our results suggest that rather than in response to a non-specific stressor, like foot shock, OT in the BNST_{dl} might be released specifically during associative learning during CS-US exposure. In fact, the involvement of OTR neurotransmission specifically during learning process has been vastly reported outside the fear circuitry, especially as it pertains to a social behavior. This includes social recognition mediated by OTR in the posterior BNST (Dumais et al., 2016), pup calls' recognition mediated by OTR in maternal left auditory cortex (Marlin et al., 2015), and partner preference formation in prairie voles mediated by OTR in the nucleus accumbens (Ross and Young, 2009). Our results indicate that endogenous OT neurotransmission in the BNST_{dl} is specifically engaged during the formation of cued fear. Here we postulate that as the function of the OT release during fear learning processes remains poorly understood, OTR antagonist studies are needed to better understand the role of endogenous OT system in the regulation of fear vs. anxiety.

In summary, we demonstrate that blocking OTR in the BNST_{dl} reduces the acquisition, but not consolidation, of conditioned cued fear, yet leaves the baseline startle and non-cued fear (background anxiety) intact. Our study reveals the critical role of OTR neurotransmission in the BNST_{dl} in the acquisition of conditioned fear to a discrete cue. Future studies will focus on understanding the involvement of distinct neuronal populations in the BNST_{dl} and specific neurocircuitry mediating the OTR-induced facilitation of cued fear learning.

Acknowledgments

We thank Dr. David L. Walker, PhD, for helpful suggestions and discussions. We also thank Mrs. Daisy Martinon, MSc, for excellent technical assistance.

Funding and disclosures

This work was supported by Grant R00 MH-096746 and R01 MH113007 from the National Institute of Mental Health to JD and start-up funds from the Chicago Medical School, Rosalind Franklin University of Medicine Science to JD.

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Highlights

- Oxytocin receptor (OTR) transmission in the BNST facilitates acquisition of cued fear
- OTRs in the BNST do not contribute to consolidation of cued fear
- Manipulation of OTR in the BNST does not affect baseline startle or non-cued fear

Fig. 1.

Schematic representation of the experimental design. In the acoustic startle response (ASR) experiments, rats were given intra-BNST_{dl} ACSF (0.5 μ l in each side), OT (100 ng in 0.5 μ l) or OTA (200 ng in 0.5 μ l) 10 min prior to the test session. In the fear potentiated startle (FPS) experiments, rats were given intra-BNST_{dl} ACSF, OT or OTA (doses as above) either 10 min prior to the fear conditioning session (acquisition) or 30 min following the completion of the fear conditioning session (consolidation). FPS expression (measured as cued and non-cued fear, was tested 24 hours later. Cue light, conditioned stimulus (CS, 3.7 s); foot shock, unconditioned stimulus (US, 0.5 s, 0.5 mA); white-noise burst (WNB).

Fig. 2.

Proper cannula placement was verified with bilateral injection of 1% Chicago Sky Blue dye into the BNST_{dl} (dark arrows). Cued and non-cued fear were measured during FPS test session and calculated as percent change scores of startle amplitude.

Fig. 3.

Neither OT nor OTA administration into the BNST_{dl} affects the ASR. Bars show mean ($\pm$ SEM) startle amplitude before and after bilateral intra-BNST_{dl} administration of ACSF (0.5 μ l in each side, n=7, gray), OT (100 ng in 0.5 μ l, n=7, red) or OTA (200 ng in 0.5 μ l, n=7, blue).

Fig. 4.

Intra-BNST_{dl} administration of OTA, but not OT, reduces the acquisition of cued fear. **(A)** Group data for pre-shock, noise-alone, and light-noise startle amplitude from rats given bilateral intra-BNST_{dl} ACSF (0.5 μ l in each side, $n=21$, gray), OT (100 ng in 0.5 μ l, $n=13$, red) or OTA (200 ng in 0.5 μ l, $n=20$, blue), 10 min prior to the fear conditioning session.

Cued fear: All rats exhibited a significantly potentiated startle response in light-noise trials compared to noise-alone trials - ACSF-treated rats ($P < 0.0001$), OT-treated ($P < 0.0001$), and OTA-treated rats ($P < 0.01$), **** $P < 0.0001$, ** $P < 0.01$, Bonferroni's post hoc tests.

Non-cued fear: All rats exhibited a significant potentiation of startle amplitude in noise alone trials in comparison to pre-shock ASR ($P < 0.0001$), but this was not affected by intra-BNST_{dl} injections. **(B)** The mean ($\pm$ SEM) startle amplitude from ACSF-treated (gray), OT-treated (red), and OTA-treated (blue) rats is shown for grouped 10 noise-alone trials and 10 light-noise trials over the FPS test session. Group data for percent change scores: **(C)** cued fear shows main effect of TREATMENT ($P = 0.017$, one-way ANOVA) and a significant reduction of percentage of cued fear in OTA-treated rats in comparison to ACSF-treated rats (Bonferroni's post hoc tests, * $P < 0.05$); **(D)** non-cued fear background anxiety ($P = 0.52$) for rats given intra-BNST_{dl} ACSF, OT or OTA. **(E)** Group data for the shock reactivity amplitude ($P = 0.57$, one-way ANOVA).

Fig. 5. Group data for percent change scores of (A) cued fear ($P = 0.79$), (B) non-cued fear ($P = 0.03$), and (C) shock reactivity amplitude ($P = 0.37$, unpaired t -test) in rats with low and high levels of pre-shock startle after intra-BNST_{dl} ACSF injection.

Fig. 6.

Intra-BNST_{dl} administration of OTA impairs the acquisition of cued fear in rats with low levels of pre-shock ASR. **(A)** Group data for pre-shock, noise-alone, and light-noise startle amplitude from low responders given bilateral intra-BNST_{dl} ACSF (0.5 μ l in each side, $n=11$, gray), OT (100 ng in 0.5 μ l, $n=7$, red) or OTA (200 ng in 0.5 μ l, $n=10$, blue), 10 min prior to the fear conditioning session. **Cued fear:** In contrast to the ACSF and OT-treated animals ($P < 0.0001$), OTA-treated rats did not show a significant potentiation of ASR in response to light-noise trials in comparison to noise-alone trials ($P > 0.05$), **** $P < 0.0001$ and † $P < 0.01$, Bonferroni's post hoc tests. **Non-cued fear:** All rats show a significant potentiation of ASR in response to noise-alone trials in comparison to pre-shock ASR trials, but this was not affected by treatment. **(B)** Bars show mean ($\pm$ SEM) percentage of cued fear in rats given intra-BNST_{dl} ACSF (gray), OT (red) or OTA (blue). Percentage change of cued fear was significantly affected by TREATMENT ($P = 0.03$, one-way ANOVA), and Bonferroni's post hoc test revealed a significant reduction of cued fear in OTA-treated rats in comparison to ACSF-treated rats (* $P < 0.05$). **(C)** Bars show mean ($\pm$ SEM) percentage of non-cued fear in rats given intra-BNST_{dl} ACSF (gray), OT (red) or OTA (blue) ($P = 0.75$, ANOVA). **(D)** Bars show mean ($\pm$ SEM) shock reactivity during fear-conditioning after intra-BNST_{dl} ACSF (gray), OT (red) or OTA (blue) injections in low responders ($P = 0.097$). **(E)** Group data for pre-shock, noise-alone, and light-noise startle amplitude from high responders given intra-BNST_{dl} ACSF ($n=10$), OT ($n=6$) or OTA ($n=10$). **Cued fear:** All high responders demonstrated a significant cued fear, but this was not affected by intra-BNST_{dl} injections. **Non-cued fear:** High responders did not develop a significant non-cued fear, and this was not affected by intra-BNST_{dl} injections. Bars show mean ($\pm$ SEM) percentage change of cued (**F**, $P = 0.27$) and non-cued fear (**G**, $P = 0.30$) in rats with high levels of pre-shock startle. **(H)** Bars show mean ($\pm$ SEM) shock reactivity during fear-conditioning after

intra-BNST_{dl} ACSF (gray), OT (red) or OTA (blue) injections in high responders ($P = 0.86$, ANOVA).

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Fig. 7.

Neither OT nor OTA administration into the BNST_{dl} affects the consolidation of FPS. **(A)** Group data for pre-shock, noise-alone, and light-noise startle amplitude from rats given bilateral intra-BNST_{dl} ACSF (0.5 μ l in each side, n=8, gray), OT (100 ng in 0.5 μ l, n=7, red) or OTA (200 ng in 0.5 μ l, n=7, blue), 30 min after completing the fear conditioning session. There was a significant increase in startle amplitude in light-noise trials compared to noise-alone trials across all rats (cued fear) but this was not affected by intra-BNST_{dl} injections. There was no significant increase in startle amplitude in noise-alone trials compared to pre-shock ASR trials across all rats (non-cued fear) and this was not affected by intra-BNST_{dl} injections. Group data for percent change scores of cued fear **(B, $P = 0.23$)** and non-cued fear **(C, $P = 0.28$)** for rats given intra-BNST ACSF (gray), OT (red) or OTA (blue).