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Fear extinction learning can be impaired or enhanced by modulation of the CRF system in the basolateral nucleus of the amygdala

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Abstract

The neuropeptide corticotropin-releasing factor (CRF) is released during periods of anxiety and modulates learning and memory formation. One region with particularly dense concentrations of CRF receptors is the basolateral nucleus of the amygdala (BLA), a critical structure for both Pavlovian fear conditioning and fear extinction. While CRF has the potential to modify amygdala-dependent learning, its effect on fear extinction has not yet been assessed. In the present study, we examined the modulatory role of CRF on within-session extinction and fear extinction consolidation. Intra-BLA infusions of the CRF binding protein ligand inhibitor $CRF_{(6-33)}$ which increases endogenous levels of free CRF, or intra-BLA infusions of exogenous CRF made prior to fear extinction learning did not affect either fear expression or within-session extinction learning. However, when these animals were tested twenty-four hours later, drug free, they showed impairments in extinction memory. Conversely, intra-BLA infusions of the CRF receptor antagonist α-helical $CRF_{(9-41)}$ enhanced memory of fear extinction. These results suggest that increased CRF levels within the BLA at the time of fear extinction learning actively impair the consolidation of long-term fear extinction.

Keywords

Fear extinction; amygdala; corticotropin-releasing factor; anxiety; fear learning

Introduction

Human subjects with anxiety disorders exhibit abnormalities in how they acquire and/or extinguish conditioned fear responses [1, 2]. Understanding how anxiety and conditioned fear interact at the neuronal level may thus yield fundamental insights into the causes of anxiety disorders and provide a foundation for clinical investigation. Corticotropin-releasing factor (CRF) is a key neuropeptide for initiating behavioral, endocrine and autonomic

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responses to stress [3–5]. It is released by neurons in the paraventricular nucleus of the hypothalamus to regulate pituitary ACTH secretion, thus triggering a key component of the hypothalamic-pituitary-adrenal (HPA) axis [6]. Growing evidence suggests that the CRF system plays a significant role in the regulation of anxiety [7, 8]. For example, chronic hyperactivation of the CRF system has been linked to several anxiety disorders and depression [9, 10]. Levels of CRF are enhanced in patients with post-traumatic stress disorder (PTSD) [11], while single nucleotide polymorphisms in the CRF gene have been associated with childhood risk factors for panic disorders [12].

Traditionally, stress-related behavior was thought to be mediated solely by activation of the HPA axis. However, growing evidence suggests that the extrahypohalamic CRF system plays a significant role in the regulation of anxiety. Both the basolateral nucleus of the amygdala (BLA) and central nucleus of the amygdala (CE) are rich in CRF immunoreactive cell bodies, terminals and receptors [13–15]. Acute stress elevates extracellular CRF levels in the amygdala [16–17]. A large number of studies have implicated the amygdala as a key player in mediating anxiety responses [18–20]. Moreover, altered amygdala function has been implicated in several anxiety disorders such as generalized anxiety disorder as well as in PTSD [21–23]. While the amygdala is only one of a constellation of structures involved in mediating anxiety [24], it plays a crucial and clearly defined role is Pavlovian fear conditioning and extinction.

In classical fear conditioning, an initially neutral stimulus, such as a tone (conditioned stimulus; CS) is paired with a noxious stimulus such as a brief electrical footshock (unconditioned stimulus; US). Afterwards, when presented alone, the CS elicits responses in the animal characteristic of fear [25]. Extinction of conditioned fear is a form of new learning in that the CS is repeatedly presented alone so that it ceases to elicit a fear response [26–27]. The original fear memory is inhibited, but not erased, as extinction actively suppresses fear responses in a context-dependent fashion [27]. Evidence suggests that the BLA is a critical site of plasticity for fear conditioning and is also required for the acquisition and storage of extinction memory [28–30].

A number of studies suggest that CRF may modulate both amygdala-dependent and amygdala-independent learning. CRF antagonists infused into the BLA disrupt contextual fear conditioning [31] and impair memory formation of an inhibitory avoidance task [32], suggesting that in general CRF may enhance learning. Moderate increases in CRF also enhance performance in a spatial learning task, visual discrimination paradigm and an inhibitory avoidance task [32–33]. However, a recent report suggests that infusions of CRF into the BLA might impair fear conditioning [34]. The effects of CRF or CRF antagonists on fear extinction learning have not been assessed. However, levels of CRF are enhanced in PTSD patients [35] and PTSD patients exhibit deficits in their ability to extinguish learned fear [36–38], suggesting that CRF in the amygdala might impair fear extinction learning as well.

Here we manipulated the CRF system within the BLA in several different ways prior to fear extinction learning to test the involvement of this neuropeptide on the extinction of fear memories. We took advantage of the fact that endogenous CRF binds to the high affinity

binding protein (CRF-BP), a membrane-associated protein which sequesters and inhibits CRF [39]. Administration of CRF-BP ligand inhibitors displace CRF which is then free to act at available CRF receptors [40]. This increase in endogenous CRF is thought to be comparable to administration of a low concentration of exogenous CRF and has been shown to enhance performance in several learning tasks [32–33, 40]. In this study, we evaluated the effects of intra-BLA infusions of 1) a CRF-BP ligand inhibitor, 2) CRF itself and 3) a CRF receptor antagonist each administered prior to fear extinction learning. Our results suggest that increased levels of CRF within the BLA inhibit the formation of long-term memory of fear extinction.

Methods

Subjects

Adult male Sprague Dawley rats (Charles River Laboratories; 250–325g) were housed individually with *ad libitum* access to food and water and maintained on a 12 hour light/dark cycle. All procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by Columbia University's Animal Care and Use Committee.

Surgery

Rats were anesthetized with a mixture of isoflurane and oxygen and mounted in a stereotaxic apparatus. Betadine was applied to the scalp and a local anesthetic (bupivacaine, s.c.) was injected under the scalp. The scalp was incised and small burr holes were made in the skull above the BLA (−2.8 AP, +/− 5.3 ML) [41]. 26-gauge guide cannula (Plastics One) were inserted just dorsal to the BLA (−6.0 DV) and cemented, with skull screws, to the skull. Dummy cannulas were inserted to prevent clogging. Rats received an analgesic (carprofen, 5 mg/kg, i.p.) and 5 ml of lactated ringer (s.c.). Animals recovered for one week before behavioral testing.

Behavior

24 hours after habituation to the training context, rats were placed in a rodent conditioning chamber with a metal grid floor (Coulbourn Instruments). Rats received 3 tone-shock pairings: CS = 5 kHz tone, 80dB, 30 sec; US = 0.5mA shock, 1 sec, tone coterminating with the shock. 24 hours later rats were placed in a different context (B) with a black plexiglass floor washed with peppermint soap. They received 10 CS tones (30 sec duration; 60–120 sec inter-tone intervals). To assess extinction recall, rats were then placed 24 hours later in context B and received 1 CS tone. Behavior was recorded by video camera and analyzed off-line. Time spent freezing to each CS (immobility with the exception of breathing) was manually scored for each animal by an observer blind to group assignment. At the end of behavioral experiments, animals were sacrificed by carbon dioxide inhalation, their brains removed and stored in 4% paraformaldehyde in phosphate buffer. Brains were sectioned at a thickness of 100 μm. Nissl staining and light microscopy were used to verify cannula placements within the amygdala (Figure 1).

Infusions

15 min prior to fear extinction learning, animals received bilateral intra-BLA infusions of vehicle (saline, 0.5μL), one of two doses of the CRF-BP inhibitor rat/human $CRF_{(6–33)}$ dissolved in vehicle (0.1μg/side or 1.0μg/side in 0.5μL; Tocris Bioscience) or CRF dissolved in vehicle (15 ng/side in 0.5μL; Tocris Bioscience) or the non-selective CRF receptor antagonist α-helical $CRF_{(9-41)}$ (1μg/side in 0.5μL vehicle; Tocris Bioscience). Animals were infused at a rate of 1 μL/min and the infusion cannulas (extending 2 mmfrom the guide cannulas) were left in place for 1–2 min to allow drug diffusion away from the tip.

Results

We first asked whether increasing endogenous levels of CRF in the BLA by inhibiting CRF-BP affects fear extinction. Rats were habituated to the training context on day 1 and conditioned with 3 tone-shock pairings on day 2 (Figure 2A; each CS was a 5kHz 30 sec tone coterminating with a footshock US = 0.5mA, 1 sec). Freezing to each tone was quantified (Figure 2B). A repeated measures ANOVA across all 3 tones found no significant difference between groups ($F_{(2,16)} = 0.86$; p=0.44), ensuring that there were no *a priori* differences between groups.

24 hours later, rats received intra-BLA infusions of vehicle (n=8), a low concentration of the CRF-BP inhibitor $CRF_{(6–33)}$ (0.1 μg/side; n=4) or a high concentration of $CRF_{(6–33)}$ (1.0 μg/side; n=7). These concentrations were chosen based on the literature [33, 42]. 15 minutes later, rats were placed in context B and presented with 10 CS tones (Figure 2C). A one-way ANOVA showed no difference between groups in freezing to the pre-tone period (defined as the 30 sec before the first CS tone) ($F_{(2,16)} = 1.18$; p=0.33). To determine if $CRF_{(6–33)}$ affects extinction learning, a repeated measures ANOVA was performed across all 10 tones. A repeated measures ANOVA showed a significant effect of tone ($F_{(9,153)} = 2.4$; $p<0.05$) no effect of drug ($F_{(2,16)} = 0.235$; p=0.79) and no tone*drug interaction ($F_{(18,153)} = 0.53$; p=0.94) These data suggest that animals successfully extinguished to the CS, but that $CRF_{(6–33)}$ does not affect fear expression or within-session fear extinction learning.

24 hours later, animals were again placed in context B to assess extinction recall (Figure 2D). To measure extinction recall in its purest form with no opportunity for further extinction, animals were tested with a single 30 second CS tone, and freezing during the pre-CS period and CS period was analyzed. A one-way ANOVA showed no difference between groups in freezing to the pre-tone period (defined as the 30 sec before the first CS tone) ($F_{(2,16)} = 0.21$; p=0.82). However, when freezing to the tone CS was analyzed, a one-way ANOVA showed a significant main effect of group ($F_{(2,16)} = 4.35$; $p<0.05$). Tukey's *post hoc t* tests revealed that a significant difference existed between vehicle controls and animals receiving the high concentration (1.0 μg) of $CRF_{(6–33)}$ ($p < 0.05$). These data suggest that if endogenous levels of CRF within the BLA are increased at the time of extinction learning, long-term extinction memory is impaired.

We next asked whether intra-BLA infusions of exogenous CRF also impair fear extinction and, conversely, whether intra-BLA infusions of a CRF receptor antagonist can enhance fear extinction. A pilot study revealed that high concentrations (30ng) of CRF infused into the

BLA impaired post-shock freezing and fear expression, while a lower dose (15ng) did not impair footshock sensitivity or fear expression (Supplementary Figure 1). We therefore tested the effects of the lower concentration of CRF on fear extinction learning. This concentration is also consistent with the literature [34, 43–44]. Rats were habituated to the training context on day 1 and conditioned with 3 tone-shock pairings on day 2 (Figure 3A). Freezing to each tone was quantified (Figure 3B). A repeated measures ANOVA across all 3 tones found no significant difference between groups ($F_{(2,25)} = 0.47$; p=0.63), ensuring that there were no *a priori* differences between groups.

24 hours later, rats received intra-BLA infusions of vehicle (n=11), CRF (15 ng/side; n=8) or the CRF receptor antagonist α-helical $CRF_{(9-41)}$ (1.0 μg/side; n=8). The concentration of antagonist was chosen based on the literature [42, 45]. 15 minutes later, rats were placed in context B and presented with 10 CS tones (Figure 3C). A one-way ANOVA showed no difference between groups in freezing to the pre-tone period ($F_{(2,25)} = 1.22$; p=0.31). To determine if CRF or a CRF receptor antagonist affected extinction learning, a repeated measures ANOVA was performed across all 10 tones. A repeated measures ANOVA showed a significant effect of tone ($F_{(9,216)} = 2.16$; $p<0.05$) no effect of drug ($F_{(2,25)} = 0.21$; p=0.81) and no tone*drug interaction ($F_{(18,216)} = 1.62$; p=0.11), suggesting no effect of these drugs on within-session fear extinction learning.

24 hours later, animals were again placed in context B and received one CS tone to assess extinction recall (Figure 3D). A one-way ANOVA showed no difference between groups in freezing to the pre-tone period ($F_{(2,25)} = 0.98$; p=0.39). However, when freezing to the tone CS was analyzed, a one-way ANOVA showed a significant main effect of group ($F_{(2,25)} = 12.61$; $p < 0.001$). Tukey's *post hoc t* tests revealed that significant differences existed between vehicle controls and animals receiving CRF ($p < 0.05$), between vehicle controls and animals receiving α-helical $CRF_{(9-41)}$ ($p<0.05$) and between animals receiving CRF and animals receiving α-helical $CRF_{(9-41)}$ ($p<0.001$). Together, these data suggest that increased levels of CRF at the time of extinction learning impair the consolidation of long-term memory of fear extinction. Conversely, blocking CRF receptors with an antagonist enhances the formation of fear extinction memory.

Discussion

In this study we evaluated whether modulating the CRF system within the BLA affects fear extinction. Our data suggest that increasing the concentration of CRF within in the BLA impairs the consolidation of long-term fear extinction memories.

While this is the first study to examine the effects of CRF on fear extinction, it has been previously demonstrated that CRF enhances fear learning in the BLA. CRF antagonists infused into the BLA disrupt contextual fear conditioning [31] and impair memory formation of an inhibitory avoidance task [42]. Furthermore, inhibitors of CRF-BP indirectly increase endogenous levels of CRF and enhance performance on a spatial learning task, visual discrimination paradigm and inhibitory avoidance task [32–33]. Using specific CRF-1 receptor deletions in glutamatergic, GABAergic, dopaminergic or serotonergic cells, it has been shown that the selective deletion of forebrain CRF-1 receptors in forebrain

glutamatergic neurons reduces anxiety [46]. Interestingly, neurotransmitter-specific deletion of CRF-1 receptors did not influence auditory fear conditioning, although fear extinction was not tested [46].

CRF administration has profound effects on the excitability of neurons within the BLA. When the CRF agonist urocortin is infused *in vivo* into the BLA for 5 days, rats develop anxiety. Brain slices collected from these animals reveal a decrease in spontaneous and stimulus-evoked IPSPs in the BLA leading to hyperexcitability of the principal excitatory neurons of the BLA [47]. Similarly, acute CRF *in vitro* increases the excitability of BLA neurons by reducing the slow afterhyperpolarization [48]. Mice in which CRF-1 receptors are deleted specifically on glutamatergic cells show decreased excitatory neurotransmission within the basolateral amygdala [43].

While long-term changes to the CRF system can have profound changes on amygdalar circuits, transient elevations in CRF are induced by acute stress and can modulate behavior. Immobilization stress elevates extracellular CRF levels in the amygdala [16–17] as does neonatal stress [49] and predator stress [50]. An aversive footshock can increase CRF levels in the CE [45]. Interestingly, the effects of stress on learning can be counteracted by manipulating the CRF system. Although immobilization stress impairs contextual fear memory, systemic injections of a CRF-1 receptor antagonist reverse the stress-induced memory impairment [51]. The source of endogenous CRF within the BLA is unclear: while the BLA contains large concentrations of CRF receptors, the CE is rich in CRF-expressing neurons [14–15, 52]. Yet neurons in the CE do not project to the BLA [53]. It is possible that increases in extracellular CRF in the CE are volume-conducted to the BLA [45]. However, this also raises the possibility that our pharmacological manipulations in this study affected not only neurons in the BLA, but neurons in the CE. Because fear learning is expressed, in part, by activity in the CE [54], future experiments should examine the role of CRF in the CE in modulating fear extinction.

The studies described above suggest that CRF enhances learning, possibly by increasing glutamatergic neurotransmission. Since fear extinction is a form of new learning dependent on the BLA, [26–27, 29], one might expect that CRF would enhance fear extinction, rather than impair it. Interestingly, a number of anxiety disorders as well as depression have been associated with increased levels of CRF in the CSF [55–56]. Crucially, these enhanced levels of CRF are also seen in patients with PTSD [35, 57]. It is well known that PTSD patients exhibit deficits in their ability to extinguish learned fear [36–38]. Moreover, PTSD patients display an overgeneralization of fear responses and/or deficits in learning to discriminate threat vs. safety cues [36, 58]. Thus it is possible that the deficits in fear extinction learning seen in PTSD may be directly related to increased CRF levels. Interestingly, increasing CRF within the amygdala and BNST, via deletion of *GABA(A)α1* receptors specifically on CRF-containing neurons, had no effect on fear conditioning but impaired fear extinction [59]. The current study is in agreement with these results, suggesting that the CRF system might differentially modulate fear conditioning and fear extinction learning.

It should be noted that animals treated with CRF or $CRF_{(6–33)}$ showed enhanced freezing to the tone during extinction recall compared to the first CS tone during extinction training day.

One possible explanation for this result relies on the fact that extinction involves not only the formation of a new memory, but weakening of the original CS-US association [29]. Moreover, fear extinction may involve activation and/or plasticity of inhibitory circuits within the amygdala [60]. If CRF administration leads to hyperexcitability of BLA principal excitatory neurons [47], one consequence would be a reduction in the influence of GABAergic inhibition. This might be manifested by increased freezing during extinction recall, although this possibility remains to be tested. A second possibility is that CRF infusions produced an aversive state, and that higher levels of freezing during the extinction recall test reflect new learning of the association between the context and CRF-induced aversion. However, this would also manifest itself as higher freezing levels during the pre-CS period, which was not seen in any drug group. Relatedly, the disruptive effects of CRF infusions on extinction recall might be explained by state-dependent effects. Although we did not explicitly test this possibility, infusions of the CRF receptor antagonist produced enhanced fear learning.

CRF-mediated hyperexcitability within the amygdala may underlie several anxiety disorders [61]. Here, we found that both endogenous and exogenous increases of CRF within the BLA impaired the consolidation of fear extinction memory, while treatment with CRF receptor antagonists increased fear extinction learning. These data add to our understanding of how CRF receptors contribute to anxiety-related psychological disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Fear extinction memory is impaired by increases in endogenous CRF in the BLA
- Fear extinction memory is impaired by intra-BLA infusions of CRF
- Fear extinction memory is enhanced by intra-BLA infusions of a CRF receptor antagonist

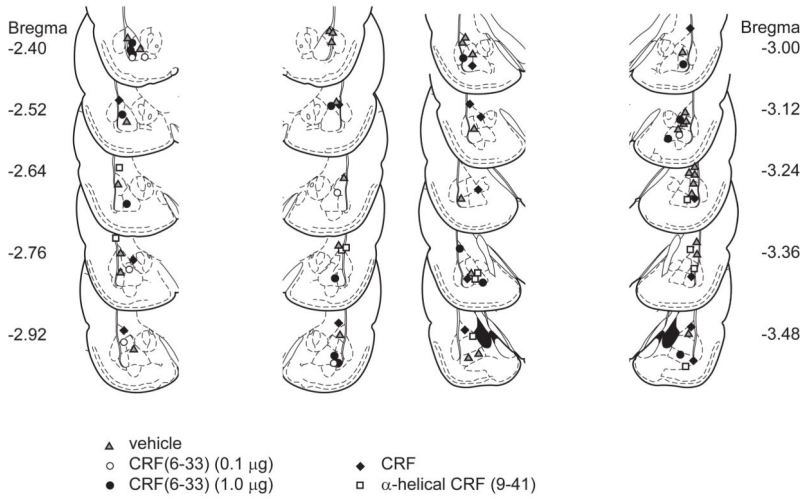

Figure 1.
Histological verification of cannula placements.

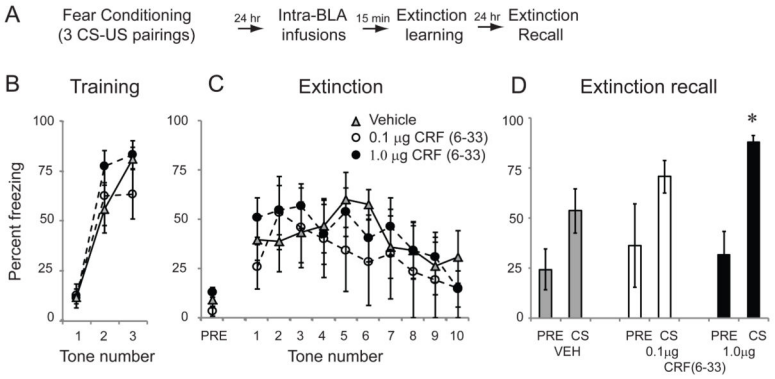

Figure 2.

Intra-BLA infusions of the CRF binding protein ligand inhibitor CRF(6-33) impair fear extinction. A: Schematic of behavioral protocol. B: Mean ± SE % freezing to three CS tones during fear conditioning. C: Mean ± SE % freezing to 10 CS tones 24 hr after training in rats given intra-BLA infusions of saline vehicle (n=8), 0.1 μg CRF(6-33) (n=4) or 1.0 μg CRF (6-33) (n=8) 15 min prior to training. D: Mean ± SE % freezing to the 30 sec pre-CS period (PRE) and 1 CS tone (CS) 24 hr after extinction training, drug-free. Animals that previously received infusions of CRF(6-33) showed impaired extinction memory.

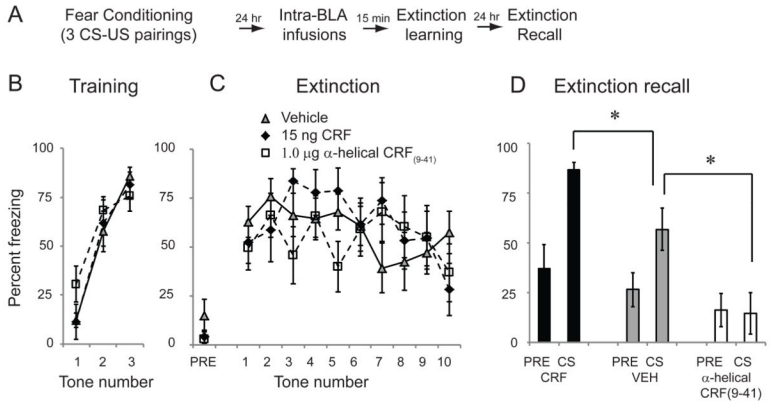

Figure 3.

Intra-BLA infusions of CRF impair fear extinction, while infusions of a CRF receptor antagonist enhance fear extinction. A: Schematic of behavioral protocol. B: Mean ± SE % freezing to three CS tones during fear conditioning. C: Mean ± SE % freezing to 10 CS tones 24 hr after training in rats given intra-BLA infusions of saline vehicle (n=11), 15ng CRF (n=8) or 1.0 µg α-helical CRF (9-41) (n=8) 15 min prior to training. D: Mean ± SE % freezing to the 30 sec pre-CS period (PRE) and 1 CS tone (CS) 24 hr after extinction training, drug-free. Animals that previously received infusions of CRF showed impaired extinction memory; animals that had received a CRF receptor antagonist showed enhanced extinction memory.