Optogenetic silencing of a corticotropin-releasing factor pathway from the central amygdala to the bed nucleus of the stria terminalis disrupts sustained fear

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Abstract

The lateral central nucleus of the amygdala (CeA_L) and the dorsolateral bed nucleus of the stria terminalis (BNST_DL) coordinate the expression of shorter and longer-lasting fears, respectively. Less is known about how these structures communicate with each other during fear acquisition. One pathway, from the CeA_L to the BNST_DL, is thought to communicate via corticotropin-releasing factor (CRF), but studies have yet to examine its function in fear learning and memory. Thus, we developed an adeno associated viral-based strategy to selectively target CRF neurons with the optogenetic silencer archaerhodopsin tp009 (CRF-ArchT) to examine the role of CeA_L CRF neurons and projections to the BNST_DL during the acquisition of contextual fear. Expression of our CRF-ArchT vector injected into the amygdala was restricted to CeA_L CRF neurons. Furthermore, CRF axonal projections from the CeA_L clustered around BNST_DL CRF cells. Optogenetic silencing of CeA_L CRF neurons during contextual fear acquisition disrupted retention test freezing 24 hours later, but only at later time-points (> 6 minutes) during testing. Silencing CeA_L CRF projections in the BNST_DL during contextual fear acquisition produced a similar effect. Baseline contextual freezing, the rate of fear acquisition, freezing in an alternate context after conditioning and responsivity to foot-shock were unaffected by optogenetic silencing. Our results highlight how CeA_L CRF neurons and projections to the BNST_DL consolidate longer-lasting components of a fear memory. Our findings have important implications for understanding how discrete amygdalar CRF pathways modulate longer-lasting fear in anxiety- and trauma-related disorders.
Keywords
central nucleus of the amygdala; bed nucleus of the stria terminalis; corticotropin releasing factor; contextual fear; optogenetics

Introduction

The neural mechanisms encoding aversive experiences into both short-term and longer-lasting fear and anxiety behaviors are unclear. Two structures that have received considerable attention in recent years for their role in fear and anxiety are the amygdala and bed nucleus of the stria terminalis (BNST), respectively. Individuals diagnosed with post-traumatic stress disorder, phobias, and anxiety-related disorders often exhibit heightened amygdala and BNST activity to various types of threat. Consistent with these findings, recent work has proposed that BNST dysfunction may lie at the heart of a number of psychiatric disorders. Despite substantial progress in identifying how fear and anxiety-like behaviors are expressed, less is known about how specific amygdala and BNST subdivisions and neurotransmitter systems contribute to initially acquiring fear.

Pre-clinical animal models of fear- and anxiety-like behaviors have been valuable for two key reasons. First, they have identified the functional importance of specific amygdala and BNST subdivisions in fear learning and memory. Second, they have provided insight into some of the core mechanisms that may regulate anxiety- and trauma-related dysfunction in humans. One mechanism that has received considerable attention for its role in fear and anxiety is corticotropin-releasing factor (CRF), a 41 amino-acid neuropeptide expressed in the lateral central nucleus of the amygdala (CeA_L) and BNST. Over the last few decades, a number of pharmacological studies have unraveled how CRF within the CeA_L and BNST modulates fear and anxiety-like behaviors, but recent studies have yet to assess CRF’s function with novel approaches (e.g., optogenetics with cell-type specific targeting). Antisense and viral knockdown studies have revealed that CeA_L CRF is necessary for contextual fear memory consolidation and stress-enhanced anxiety-like behaviors, but the functional importance of CeA_L CRF neurons themselves during the formation of a fear memory is just beginning to receive attention. Indeed, CeA_L-CRF neurons are known to send axonal projections to the BNST_DL and these long-range projections have long been suspected to serve a critical function in fear- and anxiety-like behaviors.

The BNST, like the CeA_L, expresses CRF and lesions of the BNST disrupt the retention of contextual fear memories. The majority of preclinical work examining BNST and CRF function has focused on the expression of fear by examining enhanced startle behavior to light and long-duration cues (for reviews see), with limited work evaluating its function in fear conditioning (for review see). Because both the BNST and CeA_L have CRF expressing neurons, and the dorsolateral BNST (BNST_DL) and CeA_L project to each other, the functional contributions of CeA_L CRF neurons and projections to contextual fear learning and memory have been difficult to sort out. Understanding the function of CRF systems outside the HPA-axis is essential given their importance in anxiety- and trauma-
Fear of phasic threats (i.e., short-lasting cues) is in part regulated by the CeA, whereas fear of sustained threats (i.e., long-lasting cues, lights, and contexts) is in part regulated by the BNST. However, the neuronal and molecular mechanisms that process these different types of threat are poorly understood. This is especially true with regard to how CeAL neurons and their projections to the BNSTDL might modulate fear learning and memory. More so, this focus is translationally relevant given that dysfunction in amygdala circuits may be a core feature in pathological fear and anxiety.

Therefore, in the present study, we focus on how CRF neurons in the CeAL and specific CeAL CRF projections to the BNSTDL modulate contextual fear learning and memory by selectively disrupting activity during fear acquisition. We developed an adeno-associated viral construct to selectively target CRF neurons with the optogenetic neural silencer archaerhodopsin tp009 (ArchT). We used immunohistochemical, \textit{in situ} hybridization, and in vitro electrophysiological techniques to validate the selectivity and physiological characteristics of CeAL CRF-ArchT infected neurons. Finally, we used optogenetics to examine how silencing CeAL neurons and projections to the BNSTDL at the time of fear-acquisition affected the retention of contextual fear memory.

\textbf{Materials and Methods}

\textbf{Subjects}

Adult Male Sprague-Dawley rats (10–18 weeks of age) obtained from Envigo (Indianapolis, IN) 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. Animals were randomly assigned to experimental conditions. Animals were pair-housed prior to implantation of fibers, after which they were single-housed. All behavioral experiments occurred between ~12:00 P.M. – 5:00 P.M. Given the nature of the optogenetic studies, blinding of the experimenter was not possible. All procedures were approved by the University of Delaware, or the NIDA IRP Institutional Animal Care and Use Committees (IACUC), in accordance with guidelines specified by the US National Institutes of Health Guide for the Care and Use of Experimental Animals.

\textbf{Viral Vectors}

Two viral plasmids were constructed: pAAV-CRF-ArchT-EGFP-WPRE-SV40 (abbreviated CRF-ArchT) and a control construct pAAV-CRF-EGFP-WPRE-HGH (abbreviated CRF-EGFP; Figure 1A). Both constructs contained a woodchuck hepatitis posttranscriptional regulatory element (WPRE) and a polyadenylation signal (SV40 or HGH) and were packaged into an AAV2/2 by the Penn Vector Core (Philadelphia, PA). More details about the viral constructs can be found in the Supplementary Methods.
Surgery

Rats received two surgeries spaced 4 weeks apart: one for viral infusions and another for implantation of the fiber optic ferrule assemblies. Animals were sacrificed following behavioral procedures to confirm viral expression and correct placement of cannula (see Supplementary Methods).

Contextual and Auditory Fear Conditioning

Contextual fear conditioning was conducted by providing five 0.6mA shocks spaced three minutes apart. An 18-min retention test was conducted 24 h after conditioning. Auditory fear conditioning used five 30-s tones co-terminating with foot-shock. A five tone retention test was provided in an alternate context 24-h after conditioning (see Supplementary Methods).

Shock responsivity testing

Shock responsivity testing was conducted similar to previous reports as detailed in the Supplementary Methods.

Immunohistochemistry and In Situ Hybridization

For confirming targeted expression of our construct, we used immunohistochemical and situ hybridization techniques (described in Supplementary Methods).

Whole-Cell Patch Clamp

Slice preparation and recordings were conducted using procedures previously described and are presented in detail within the Supplementary Methods.

Statistical Analyses of fear conditioning and shock responsivity

Violations in homogeneity of variance were tested prior to statistical analyses. The number of animals in each group was selected based off pilot experiments (data not shown). Freezing during fear acquisition and retention of contextual fear conditioning was analyzed separately for test phase using a two-group (CRF-EGFP vs CRF-ArchT) between factor by 6 time bin within factor-repeated measure analysis of variance. A Holm-Bonferroni sequential correction test for non-independent samples was used to compare freezing of the two groups at select time bins. One animal (CRF-ArchT) in the CeA_L → BNST_DL CRF pathway experiment was removed for improperly placed fibers (see placement highlighted in blue in Supplementary Fig. 8).

Some animals were lost due to damaged head-stages and improper patch cord coupling (final group numbers shown in the Results section). Freezing at baseline during exposure to all contexts was assessed with an independent samples t-test to evaluate (1) if optogenetic stimulation itself could induce freezing before and after conditioning or (2) if stimulation in an alternate context could act as a retrieval cue. For auditory delay fear conditioning, we conducted analyses excluding outliers > 2 S.D. and computing a difference score for each CS. Mann-Whitney U tests were used to examine ordinal shock responsivity data. Electrophysiological data (pre vs. post laser effects) were analyzed using a one-way repeated measures ANOVA, followed by Dunnett’s post-hoc comparison.
Results

CRF-ArchT-EGFP Selectively Targets CeA\textsubscript{L} CRF+ Neurons

In order to selectively target CRF neurons with an inhibitory opsin, we reconstructed an AAV2/2 archaerhodopsin tp009 (ArchT) enhanced green fluorescent protein (EGFP) vector using a ~2.2kb rat CRF promoter (CRF-ArchT; Fig. 1a; Supplementary Fig. 1–2). In parallel, we created a control construct that did not express ArchT (CRF-EGFP; Fig. 1a; Supplementary Fig. 1–2). Immunohistochemical labeling for EGFP confirmed that ArchT expression was restricted to the CeA\textsubscript{L} following injections into this structure with visible processes in the basolateral amygdala and coursing upwards to the stria terminalis (Fig. 1b–c; Supplementary Fig. 8a–b; n=4). Co-labeling of CRF with EGFP further showed that the CRF-ArchT-EGFP protein was produced in CeA\textsubscript{L} CRF+ neurons (Supplementary Fig. 3d).

Given differences in basal vs. physiological driven levels of CeA\textsubscript{L} CRF expression, poor antibody specificity, and the fact that ArchT is also expressed in axonal projections, we wanted to confirm that CRF-ArchT-EGFP protein expression was in fact restricted to CeA\textsubscript{L} CRF synthesizing cells. To further validate the CRF-ArchT-EGFP construct, we compared the expression pattern of CeA\textsubscript{L} CRF-ArchT-EGFP protein to that of CRF mRNA using radiolabeled in situ hybridization. CRF-ArchT-EGFP protein and CRF mRNA CeA\textsubscript{L} expression patterns were highly similar (Supplementary Fig. 3b–c; n=4). Additionally, using RNAscope in situ hybridization, we were able to better confirm that only cells that synthesized CRF also synthesized EGFP mRNA and, critically, expressed the CRF-ArchT-EGFP protein (Fig. 1d–e; n=2).

CRF-ArchT-EGFP is Selective for Other Types of CRF+ Neurons

Although the focus of the present paper was on optogenetic manipulation of CRF cells in the CeA, we also examined whether our viral construct could be expressed in other CRF populations. CRF cells in the CeA and BNST are GABAergic whereas CRF cells in the paraventricular nucleus (PVN) of the hypothalamus are glutamatergic. Injection of CRF-ArchT into the PVN demonstrated co-localization and selectivity of cellular expression in PVN CRF neurons (Supplementary Fig. 4). These data suggest that CRF-ArchT can be targeted to CRF+ neurons across the brain, irrespective of regional phenotypic and co-localized neurotransmitter differences.

Green Light Silences CRF-ArchT-EGFP Neurons

To validate that CRF-ArchT-EGFP was a viable neuronal silencer, we conducted in vitro whole-cell patch clamp on CeA\textsubscript{L} CRF-ArchT infected rats. Light-activated silencing of CeA\textsubscript{L} neurons by ArchT was confirmed in amygdala brain slices (Fig. 2). Action potentials elicited during 1-s photostimulation periods were compared to those preceding and following stimulation. Neuronal firing was significantly inhibited during laser illumination (one way repeated measures-ANOVA, F(2,6) = 17.03, p = 0.0034). Post hoc analysis found firing was decreased during laser illumination compared to both pre and post illumination.
CeA<sub>L</sub> CRF+ Neurons project to the BNST<sub>DL</sub> cluster around BNST<sub>DL</sub> CRF+ cells

To confirm that CeA<sub>L</sub> CRF neurons project to the BNST<sub>DL</sub> as previously reported<sup>19</sup>,<sup>20</sup>,<sup>40</sup>, we examined immunoreactivity in the BNST<sub>DL</sub>. Following CRF-ArchT injection into the CeA<sub>L</sub>, immunohistochemical labeling confirmed the presence of EGFP in the BNST<sub>DL</sub> (Fig. 5b–c, Supplementary Fig. 5b and 8e, n=4). Given that the BNST<sub>DL</sub> is known to contain a number of CRF+ neurons<sup>24</sup> and the known role of BNST<sub>DL</sub> CRF type one receptors<sup>41</sup> in fear and anxiety-like behaviors<sup>21</sup>,<sup>42</sup> from our recent work, we examined if CeA<sub>L</sub> CRF fibers were present near BNST<sub>DL</sub> CRF+ neurons (Supplementary Fig. 5; n=3). Co-labeling of BNST<sub>DL</sub> sections for EGFP and CRF, following injections of CRF-ArchT into the CeA<sub>L</sub>, revealed that CeA<sub>L</sub>→BNST<sub>DL</sub> long range CRF projections were in fact clustered around BNST<sub>DL</sub> CRF producing cells.

Silencing CeA<sub>L</sub> CRF+ Neurons During Fear Acquisition Only Disrupts Later Time-Points of Fear Memory Retention

To explore the role of CeA<sub>L</sub> CRF+ neurons during the formation of a fear memory to a specific environment, we trained CRF-ArchT (n=13) and CRF-EGFP controls (n=11) in contextual fear conditioning (Fig. 3a–b). Optogenetic silencing of CeA<sub>L</sub> CRF+ neurons did not affect freezing between groups (Fig. 3c; F(1,22)=0.0005, ns) or the rate of acquisition (interaction; F(5,18)=0.44, ns). Both groups increased freezing with each subsequent shock, reaching ~80% freezing after 3 shocks, F(5,18)=59.08, p<0.0001 (Fig. 3c). Thus, silencing CeA<sub>L</sub> CRF+ neurons did not affect acquisition.

However, at retention testing 24-h later, CRF-ArchT animals showed reduced freezing relative to CRF-EGFP controls (Fig. 3d; F(1,22)=7.30, p<0.013). The interaction effect was marginally significant, F(5,18)=2.72, p<0.091, with freezing during the third, fourth and fifth time bins (minutes 6–15) lower in the CRF-ArchT group (Holm’s sequential Bonferroni, bin 3: p<0.046, bin 4: p<0.008, bin 5: p<0.046).

Laser silencing of CeA<sub>L</sub> CRF+ neurons in an alternate context after conditioning did not induce freezing in CRF-ArchT (n=10) relative to CRF-EGFP controls (n=9; (t(17)=.607, ns; Supplementary Fig. 6c) or alter shock responsivity (Supplementary Fig. 6d; p’s > .05). Additionally, laser silencing had a marginally disrupting effect on retention of auditory fear to a discrete 30-s tone CS in CRF-ArchT (n=11) relative to CRF-EGFP controls (n=10; Fig. 4). A repeated measures ANOVA on difference scores at each CS presentation (i.e., CS1 testing – CS1 training, etc.) with outliers > 2 S.D (n=3/group) removed revealed a significant interaction (F(4,52)=3.402, p <.016) with freezing during CS3 (p <.05), CS4 (p<.05), and CS5 (p<.05) in the CRF-ArchT group lower than controls. Holms-Bonferroni sequential correction confirmed effects at CS4: p< .05, and CS5: p< .05.

In summary, laser silencing during fear acquisition only disrupts freezing at later time-bins of contextual, and to a lesser extent auditory, fear retention 24-h later. However, short-term memory during acquisition, early time-points of long-term fear, and perception of environmental cues or pain responsivity are unaffected.

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Silencing CeA\textsubscript{L} → BNST\textsubscript{DL} CRF+ Neuronal Projections Also Disrupts Later Time-Points of Fear Memory Retention

Next, we examined the role of the CeA\textsubscript{L} → BNST\textsubscript{DL} CRF pathway in contextual fear conditioning (Fig. 5a–c)\textsuperscript{19}. Similar to silencing CeA\textsubscript{L} CRF+ neurons, optogenetic silencing of CeA\textsubscript{L} → BNST\textsubscript{DL} CRF projections did not affect freezing at baseline (p > .05) and the rate of fear acquisition was normal (F(5,12) = 105.05, P<0.0001), with no between group effect (F(1,16) = 0.61, NS) nor interaction (F(5,12) = 0.97, NS; Figure 5d).

Also similar to silencing CeA\textsubscript{L} CRF+ neurons, silencing CeA\textsubscript{L} → BNST\textsubscript{DL} CRF projections produced decreased freezing at retention testing (Fig. 5e). There was a highly significant repeated measure effect of time bins, suggesting changes in freezing as the testing session progressed (F(5,12)=105.05, p<0.0001), but no between group difference (F(1.16=0.61, ns) nor interaction effects (F(5,12)=0.97, ns). However, the decrease in freezing at retention was due to reduced freezing in CRF-ArchT animals at time bins 4 and 6 (Fig. 5e; Holm’s sequential Bonferroni procedure time bin 4: p<0.018, time bin 6: p<0.046).

Similar to CeA\textsubscript{L} experiments, freezing in an alternate context after conditioning (CRF-ArchT n=5 and CRF-EGFP controls n=5, levene’s test, p=.032, t\textsubscript{corrected}(4.763)=1.108, ns) and shock responsivity (CRF-ArchT n=8 and CRF-EGFP controls n=8, ns; Supplementary Fig. 7c–d) were unaffected.

Additionally, restricting silencing to 1-minute periods surrounding each shock (30s before, 1s during shock, and 29s after) produced the same effect as prolonged stimulation. CRF-ArchT animals (CRF-ArchT\textsubscript{short}, n=5) were compared to animals that received laser silencing during the entire conditioning session (CRF-ArchT\textsubscript{long}, n=8, from Fig. 3). Shorter periods of laser silencing did not differentially affect fear acquisition (between groups (F(1,12)=.344, ns), interaction (F(5,60)=.785, ns), but main effect of time bin (F(5,60)=37.996, p < .001) or fear retention (between groups (F(1,12)=1.020, ns), interaction (F(5,60)=.471, ns), but main effect of time bin (F(5,60)=2.931, p=.020).

Exploratory post-hoc analyses confirmed the lack of a difference. Thus, long or short periods of CeA\textsubscript{L} → BNST\textsubscript{DL} CRF+ silencing during acquisition similarly disrupt later stages of retention.

Discussion

In this study, we demonstrate the fidelity of an AAV2/2 CRF-specific optogenetic neural silencer in selectively targeting CRF cells. Our CRF-ArchT construct should be a valuable addition to the optogenetic toolbox for fear, stress, and anxiety researchers to ask specific questions about the function of CRF neurons and projections. Our data provide strong support for the selectivity of CRF-ArchT within CRF+ cells. In particular, we show that: (1) the expression of CRF-ArchT parallels that of CRF mRNA in both the CeA\textsubscript{L} and PVN\textsuperscript{33}, (2) CRF-ArchT mRNA is only transcribed in CeA\textsubscript{L} cells that transcribe CRF, and (3) the induction of firing in CeA\textsubscript{L} CRF-ArchT cells is abolished with green light stimulation. Our use of a high-titer AAV2/2 explains, in part, why our construct successfully targeted phenotypically distinct CRF populations across the CeA\textsubscript{L} and PVN (i.e., GABAergic CeA\textsubscript{L} and glutamatergic PVN)\textsuperscript{43, 44}. Furthermore, we used a long promoter fragment\textsuperscript{45}, that has
previously been used and validated, to selectively target CRF cells – an approach which has been successfully applied to targeting other peptidergic cells of the CeA\textsubscript{L} and PVN. There have been mixed results in the literature with using transgenic approaches to target CRF+ cells, across mice and rats, but our data provide compelling evidence for the selectivity of our construct in CRF+ CeA\textsubscript{L} and PVN cells. Future studies will fully quantitate overlap of our CRF-ArchT construct with CRF neurons across the anterior/posterior axis, with other CeA\textsubscript{L} cellular markers (e.g., GABA, somatostatin, PKC-δ, TAC-2), and see, and other CeA\textsubscript{L} CRF projections.

**CeA\textsubscript{L} CRF Neurons Are Involved in Consolidating Sustained Fear**

CeA\textsubscript{L} neurons are critical during the earliest stages of fear learning and memory. Our study shows that silencing CeA\textsubscript{L} CRF neurons during acquisition disrupts consolidation of longer-lasting components of a contextual fear memory, given that baseline activity, fear acquisition, freezing in a novel context after conditioning, or responding to varying foot-shock intensities were unaffected. Silencing CeA\textsubscript{L} CRF neurons did not affect fear acquisition to asymptote, suggesting CeA\textsubscript{L} CRF neurons are not critical for short-term memory. Given that freezing was disrupted 24 h later, but only beginning 6 minutes after the start of the retention test, CeA\textsubscript{L} CRF neurons appear to preferentially modulate longer-lasting components of long-term fear memories. Alternatively, it is possible that silencing CeA\textsubscript{L} CRF neurons during fear acquisition accelerates extinction learning. However, given that the rate of fear acquisition was unaffected, the most parsimonious conclusion is that the consolidation of longer-lasting components of fear were disrupted. Our optogenetic findings add to previous reports showing that neurotoxic lesions, functional inactivation, and CRF knockdown in the CeA prior to or during the acquisition phase of contextual fear disrupts fear retention.

**CeA\textsubscript{L}→BNST\textsubscript{DL} CRF Projections Represent a Critical Pathway in Consolidating Sustained Fear**

Given that CeA\textsubscript{L} CRF projections target a number of other brain regions, it is unclear which specific CeA\textsubscript{L} CRF cells and/or pathways regulate this effect. We found that silencing CeA\textsubscript{L}→BNST\textsubscript{DL} CRF axonal projections within the BNST\textsubscript{DL}, similar to silencing CeA\textsubscript{L} CRF neurons within the CeA\textsubscript{L}, disrupted fear memory retention across a similar time course, indicating these projections are critical to modulating longer-lasting fragments of the fear memory. Because silencing is spatially selective to presynaptic boutons in the illuminated area and has no effects on action potentials, fibers of passage, or back propagation to the cell bodies in the CeA\textsubscript{L}, it can be concluded that only the CeA\textsubscript{L}→BNST\textsubscript{DL} CRF ArchT expressing projections mimicked the silencing of CeA\textsubscript{L} CRF neurons. Whether CeA\textsubscript{L} projections to other regions have similar effects are for future studies.

Our findings agree with previous work indicating that the BNST is involved in consolidating long-term contextual fear memories. Pharmacological work from our lab has shown that pre-training antagonism of CRF type 1 receptors in the BNST\textsubscript{DL} blocks the retention, but not short-term acquisition, of contextual fear. Similarly, antisense knockdown of CRF in the CeA\textsubscript{L} before or immediately after contextual fear conditioning produces a similar effect. However, these studies do not differentiate between the shorter and longer-
lasting aspects of contextual fear memories – a key component of the present work. Our data support the hypothesis that CeA_L \rightarrow BNST_{DL} CRF projections regulate learning and memory of longer lasting fragments of contextually conditioned fear memories \(^1\).

The present study does have some limitations. CeA_L CRF cells are primarily GABAergic \(^61\). Thus, the extent to which GABA and CRF within the CRF CeA_L \rightarrow BNST pathway contribute to fear learning and memory is unknown (for review see \(^26\)). However, previous work has shown that GABA-A(\(\alpha_1\)) receptor deletion from CRF neurons, which abolishes the effects of GABA and enhances CRF across the CeA and BNST, impairs auditory fear extinction \(^61\). Our study complements this work by showing that silencing CeA_L CRF neurons during fear acquisition produces the opposite effect by disrupting retention of longer-lasting contextual and auditory fear memories. Given that we did not measure if CeA_L \rightarrow BNST CRF release decreased with silencing, an important future direction would be to assess CRF and GABA release in the CeA_L \rightarrow BNST pathway and within local BNST CRF neurons with optogenetic silencing to better understand how different sources of CRF and GABA influence longer-lasting fear behavior.

Recent evidence has also suggested that prolonged stimulation of ArchT in glutamatergic thalamocortical cells can stimulate presynaptic Ca\(^{2+}\) transmitter release \(^62\), however it is unknown if prolonged stimulation of CeA_L CRF+ neurons, which are GABAergic \(^63\), produces a similar effect. Nonetheless, we found that curtailing ArchT silencing to 1-min periods (paralleling the low end of the Ca\(^{2+}\) ramp in glutamatergic cells observed by \(^62\)) produced a similar reduction in freezing relative to prolonged inhibition. Although we demonstrate successful laser silencing of CeA_L CRF neurons in vitro, studies are needed to examine how silencing CeA_L CRF neurons affect in vivo population activity. Finally, we did not test if silencing CeA_L CRF projections affected corticosterone release or if other CeA_L pathways could differentially modulate components of the fear memory \(^19, 64\). These are important future questions for understanding how CeA_L CRF projections (e.g., those mediating arousal and endocrine activity) may regulate freezing at earlier time-points during fear retention \(^19, 64, 65\).

Conclusions

The mechanisms for transitioning from normal fear to pathological anxiety are still unclear \(^66\), but the amygdala and BNST play a critical role (for reviews see \(^67, 68\)). Recent work in humans has shown that the amygdala is responsive to the onset of threat-predicting cues whereas the BNST is important for maintaining fear responses \(^2\). This work complements decades of pre-clinical animal work suggesting that the CeA is important for phasic short-lasting fear whereas the BNST, in part, is critical for sustained long-lasting fear \(^1, 69\). Our results shed light on how a select CRF network modulates sustained, long-lasting fear.

CeA_L CRF neurons and their receptors coordinate a number of fear memory processes. For example, CRF type 1 receptors (CRFr1s) within the basolateral amygdala modulate the consolidation of inhibitory avoidance memories \(^70\) and BNST_{DL} CRFr1s are important for the retention of contextual fear memories \(^26, 42\). Furthermore, a recent study demonstrated that CeA_L CRF neurons and receptors modulate weak, but not strong, fear conditioning \(^18\).
Our study adds an important piece to accumulating data over the last few decades on how CRF within the amygdala and BNST\textsuperscript{1,31,65} regulate a number of fear/anxiety behaviors including startle behavior to lights, long-lasting cues, and contextual fear memories\textsuperscript{16}. Future studies are needed to understand how suppression and activation of other CeA\textsubscript{L} CRF pathways modulate different fear- and anxiety-like behaviors (e.g., fear potentiated startle, elevated plus maze, etc.) and may serve as a therapeutic target in individuals suffering from a variety of fear and anxiety disorders.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
AAV2/2 CRF promoter driven ArchT vector selectively targets CRF cells. (a) CRF-ArchT and CRF-EGFP control vectors. (b) Schematic of CeA_L viral injection site. (c) Immunohistochemical labeling shows CRF-ArchT-EGFP+ protein is restricted to the CeA. Note the fluorescence extending into the basolateral amygdala (BLA; white arrow) and towards the stria terminalis (asterisk). (d) In situ hybridization, DAPI staining, and intrinsic EGFP+ fluorescence across three neurons in the CeA_L (two aqua arrows and one white arrow). Of the three cells with DAPI stained nuclei (blue stain), only one (white arrow) displays triple expression of cytoplasmic CRF-ArchT-EGFP+ protein (green) and mRNA (yellow-white) with CRF mRNA (red). (e) Magnification of panel d showing the CRF-ArchT-EGFP+ labeled CRF neuron (white arrow). Note the two adjacent cells (aqua arrows) which do not synthesize CRF mRNA also do not exhibit EGFP mRNA or protein. The green-labeled long process appears to be an EGFP+ axonal projection (asterisk).
Figure 2.
Confirmation of ArchT function in whole-cell recordings from CeA_L Neurons in an *in vitro* slice preparation. (a) Schematic showing site of CRF-ArchT injection into CeA_L. (b) Epifluorescent image demonstrating EGFP+ expression in a single CeA_L neuron. (c) Voltage clamp recording (holding potential −60mV) of a neuron demonstrating outward currents elicited by photostimulation (indicated by green bar); the size of the current increased with increasing laser output. (d) Current clamp recording of a neuron demonstrating ArchT-mediated inhibition of firing induced by depolarizing current injection (+100 pA, 3 s) through the patch pipette. The photostimulation period is indicated by the green box. (e) Summary of recordings from CeA_L neurons (n = 4, from 3 rats). Action potentials elicited during a 1-s photostimulation were compared to those preceding and following the stimulation. Neuronal firing was significantly inhibited by laser light. * denotes firing during laser-on was significantly different from pre and post illumination recording.
Figure 3.
Silencing of CeA_L CRF+ cells during acquisition disrupts later components of fear retention.
(a) Schematic of CeA_L viral injection site and fiber placement. (b) Parameters for contextual fear conditioning. (c) Laser silencing did not affect baseline activity/freezing or fear acquisition. (d) At fear retention, CRF-ArchT rats (n=13) exhibited reduced freezing at time bins 3, 4, and 5 relative to CRF-EGFP (n=11) controls. Each bin represents a three-minute period. *p<.05, error bars are ± S.E.M.
Figure 4.
Effects of silencing CRF+ CeA<sub>L</sub> cells on retention of auditory fear conditioning. (a) Schematic of CeA<sub>L</sub> viral injection site and fiber placement. (b) Schematic of experimental timeline. Animals were trained with five 30-s CSs co-terminating with a 1-s 0.6 mA foot-shock. (c) Silencing of CRF+ CeA<sub>L</sub> neurons did not statistically affect auditory fear acquisition, but freezing tended to increase over the last few CS-shock pairings (d) At fear retention, CRF-ArchT (n=11) rats tended to have reduced freezing during the last two presentations of the CS relative to CRF-EGFP (n=10) controls (e) A difference score removing outliers > 2 S.D. and examining the change from training to testing (i.e., CS1 testing – CS1 training, etc.) revealed CRF-ArchT (n=8) animals exhibited a greater reduction in freezing relative to CRF-EGFP (n=7) controls during the last few CSs. A lower difference score indicates a greater reduction in freezing. *p<.05, error bars are ± S.E.M.
Figure 5.
Silencing of the CeA\textsubscript{L} → BNST\textsubscript{DL} CRF pathway during acquisition only disrupts later components of fear retention. (a) Schematic of CeA\textsubscript{L} viral injection site and BNST\textsubscript{DL} fiber placement. (b) Rat atlas overlay showing EGFP expression in BNST\textsubscript{DL}. (c) Axonal projections in BNST\textsubscript{DL} (arrows). (d) Laser silencing with same parameters as shown in Fig. 2b did not affect baseline activity/freezing or fear acquisition. (e) At fear retention CRF-ArchT (n=8) animals exhibited reduced freezing at time bins 4 and 6 relative to CRF-EGFP (n=10) controls. Each bin represents a three-minute period. *p<.05, error bars are ± S.E.M.