Site-Specific Genetic Manipulation of Amygdala Corticotropin-Releasing Factor Reveals Its Imperative Role in Mediating Behavioral Response to Challenge

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Background: Faulty regulation of the central extrahypothalamic corticotropin-releasing factor (CRF) expression is associated with stress-related psychopathologies including anxiety disorders and depression. Extensive pharmacological literature describes the effects of CRF agonists or antagonists' administration on anxiety-like behavior. However, the relevance of the endogenous agonist, presumed to be CRF, has never been explicitly demonstrated. Several genetic models have been used to study the role of CRF in the physiological response to stress and in stress-related disorders. Nevertheless, developmental compensatory mechanisms and lack of spatial and temporal specificity limited the interpretations of these studies.

Methods: Two lentiviral-based systems were designed, generated, and used to knockdown (KD) or conditionally overexpress (OE) CRF in the central amygdala (CeA) of adult mice. Behavioral responses associated with the CeA, such as anxiety, depression and fear memory, and the plasma corticosterone levels were evaluated under both basal and stressful conditions.

Results: Changing the CeA-CRF levels mildly affected anxiety-like behaviors under basal conditions. However, following exposure to an acute stressor, CeA-CRF-KD strongly attenuated stress-induced anxiety-like behaviors, whereas a short-term CeA-CRF-overexpression enhanced the stress-induced effects on these behaviors. Interestingly, a significant increase in basal corticosterone levels in the CeA-CRF-KD mice was observed, demonstrating the importance of endogenous CeA-CRF levels for basal, but not stress-induced, corticosterone levels.

Conclusions: These results highlight the pivotal role of CeA CRF expression regulation in mediating adequate behavioral responses to stress and introduce these novel viral tools as a useful approach for dissecting the role of central CRF in mediating behavioral and neuroendocrine responses to stress.

Key Words: Amygdala, anxiety, corticotropin-releasing factor, lentiviruses, site-specific genetic manipulation, stress

Corticotropin releasing factor (CRF) is an established and essential regulator of the neuroendocrine and behavioral stress response and was implicated in the control and maintenance of the organism's dynamic homeostatic equilibrium (for review, see 1–3). Maladaptive stress responses were suggested to underlie anxiety disorders and depression (2,4–10) and were repeatedly linked to dysregulation of the CRF system (11–15). Studies using CRF type 1 receptor (CRFR1) antagonists indicated the brain CRF system as pivotal in mediating behavioral responses to stressors (7,12,14,16,17). Other studies demonstrated anxiogenic-like behavioral effects of CRF administration and anxiolytic-like effects of CRFR1-selective antagonists, thus suggesting that CRF might be involved in anxiety-related disorders (7,14). The role of the CRF/CRFR1 system in modulating anxiety-like behaviors was further supported by the behavioral phenotypes of CRFR1-deficient mice models. CRFR1 knockout (KO) mice, which are depleted of *CRFR1* both centrally and peripherally, display decreased levels of anxiety-like behaviors and an attenuated hypothalamic-pituitary-adrenal (HPA) axis response to stress (18,19). Similarly, mice lacking *CRFR1* specifically within the limbic system exhibit an anxiolytic phenotype (20). In contrast, mice deficient of *CRF* exhibited altered HPA axis regulation yet did not differ behaviorally, potentially because of compensatory mechanisms (21,22).

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Studies that utilized models of fear and anxiety in rodents demonstrated that specific, highly connected brain regions, including the hippocampus, central nucleus of the amygdala (CeA), basolateral amygdala, bed nucleus of the stria terminalis (BNST), and lateral septum, are key players in anxiety-like states and stress responses (23–26). Within these regions, CRF is highly expressed in the CeA, suggesting it as one of the key loci of extrahypothalamic CRF-induced effects on fear and anxiety. Indeed, CRF administration to the amygdala induced anxiogenic-like behaviors (13,27). Although extensive pharmacologic literature describes the effects of CRF agonists or antagonists' administration on initiating or blocking anxiety-like behavior, respectively, the relevance of the endogenous ligand, presumed to be CRF, has never been explicitly demonstrated. In addition, to date, transgenic animal models have not reached sufficient spatial and temporal specificity to affect distinct brain nuclei and frequently exhibit developmental compensatory changes that make interpreting the observed phenotype problematic.

This study describes the preparation and use of two lentiviral-based systems for site-specific genetic manipulation in adult mice, allowing knockdown or inducible and temporally controlled overexpression of CRF levels in selected brain nuclei. Knockdown or short-term overexpression of CRF specifically in the CeA of adult male mice was followed by behavioral assessments of anxiety-like behaviors and corticosterone measurements under basal and stress-induced conditions.

Methods and Materials

Lentiviral Vectors Design and Construction

All constructs were assembled using standard cloning methods and confirmed by deoxyribonucleic acid (DNA) sequencing. For a detailed description of the design and cloning process, see Methods in the Supplement 1.

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Production of Lentiviruses

Recombinant lentiviruses were produced as described previously (28). See a brief description in Methods in Supplement 1.

In Vitro Validation of Lentiviral Vectors

The ability of the short hairpin (sh)CRF vectors to knockdown CRF expression was assessed by Western blot analysis. The inducible CRF overexpression viral system was assessed using fluorescence microscopy of infected HEK293T cells, with or without doxycycline (Dox) treatment. For detailed information, see Methods in Supplement 1.

Animals and Housing

Adult C57BL/6J male mice (8 weeks old) (Harlan, Jerusalem, Israel) were housed in a temperature-controlled room (22 ± 1°C) on a reverse 12 hour light/dark cycle (lights on at 7:00 PM). Food and water were given ad libitum. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Weizmann Institute of Science.

In Vivo Validation of Lentiviral Vectors

The ability of the shCRF vectors to knockdown CRF expression was assessed in vivo using a CRF-specific in situ hybridization protocol, reported previously (29), alone or combined with green fluorescent protein (GFP) immunohistochemistry. For detailed information see Methods in Supplement 1.

Surgical Procedure

Mice were stereotaxically injected as previously described (30). For a detailed description of the procedure see Methods in Supplement 1.

Behavioral Assessments

The assessment of anxiety-like behaviors utilized the dark-light transfer (DLT) and the elevated plus maze (EPM) tests, as previously described (31). Repeating the DLT test immediately following 30 minutes of restraint stress assessed stress-induced anxiety-like behaviors. General locomotor activity in the home cages was assessed using an automated system (InfraMot; TSE Systems, Bad Homburg, Germany) as described in Neufeld-Cohen *et al.* (29). Depressive-like behaviors were assessed using the forced-swim (FST) and the tail suspension (TS) tests. Fear-conditioning (adapted from 32) was performed using a computer-controlled fear conditioning system (TSE Systems). Memory tests were performed 24 hours following the conditioning. For detailed protocols, see Methods in Supplement 1.

Data Analyses

The results are presented as means ± SEM. Behavioral indices were analyzed by independent Student's *t* test (two-tailed) or by a two-way analysis of variance. For a detailed description, see Methods in Supplement 1.

Immunohistochemistry

Specific immunohistochemistry of the brain slices for GFP was performed as previously described (33). Mice that did not show GFP at the aimed injection location were excluded from data analysis. For a detailed description, see Methods in Supplement 1.

Blood Collection and Corticosterone Analysis

Tail blood samples were collected before (basal), immediately after 30 minutes of restraint stress and 60 and 120 minutes from stress initiation. For a detailed description of corticosterone analysis, see Methods in Supplement 1.

Ribonucleic Acid (RNA) Preparation and Real-Time Polymerase Chain Reaction (PCR)

Immediately following decapitation, brains were removed and the area of interest was punched using a microdissecting needle of an appropriate size. RNA was extracted and reverse transcribed to generate complementary DNA that was later used as a template for quantitative real-time PCR analysis using specific primers. See detailed procedures in Methods in Supplement 1.

Results

Central Amygdala CRF Knockdown has a Limited Effect on Basal Anxiety While Attenuating Stress-Induced Anxiety

To knockdown CRF in a site-specific manner, we recently established a lentiviral-based system expressing short hairpin RNA against CRF, followed by a GFP reporter (34). For a description of the design, establishment and verification of this system, see Results and Figure S1 in Supplement 1.

To assess the effects of CeA CRF-knockdown (KD) on basal anxiety-like behavior and stress-induced anxiety levels, small interfering (si)RNA or control lentiviruses were stereotaxically injected bilaterally into the CeA of male C57BL/6 mice. In vivo verification of the CRF-KD at the CeA is demonstrated in Figure 1. Immunostaining for GFP (Figure 1C) and in situ hybridization with specific probe for CRF (Figure 1, B and D), performed on brain sections obtained from mice injected with siCRF into the CeA of adult mice, demonstrated the efficiency of these viruses to knockdown the endogenous levels of CRF messenger RNA (mRNA) in the CeA. Mice injected with the siCRF lentiviruses showed a robust reduction in CeA-CRF mRNA levels (Figure 1D) compared with control mice (Figure 1B).

Following 2 weeks' recovery, the effects of the CRF-KD on anxiety-like behavior were examined under two stress conditions: basal (no additional stressors other than the inherent stressful properties of the tests) and immediately following exposure to 30 minutes of restraint stress, using the EPM and DLT tests.

In the EPM test, CeA CRF-KD significantly increased the percent of time spent in the open arms [$t(18) = 2.37$; $p = .033$] (Figure 2A) and the percent of entries to those arms [$t(18) = 2.63$; $p = .020$] (Figure 2B). In the DLT test, under basal conditions, no differences were observed in the percent of time spent in the light compartment [$t(17) = .93$; $p = .365$] (Figure 2C) or in the number of entries into the light [$t(17) = .61$; $p = .553$] (Figure 2D). Following stress, a within-subject change index was calculated for the percent of time spent in the light compartment and the number of entries to the light in the DLT test (stress-induced change = 100*([stress-basal]/basal). Although control mice reacted to the stressor by an increase in anxiety-like behaviors, i.e. significant reduction in percent of time spent in the light compartment [$t(6) = 2.47$; $p = .050$] (Figure 2E, left bar), and the number of entries to the light [$t(6) = 2.63$; $p = .039$] (Figure 2F, left bar), CeA CRF-KD mice appeared unaffected by the stress exposure. CeA CRF-KD mice spent a similar percent of the time in the light compartment [$t(8) = 1.29$; $p = .233$] (Figure 2E, right bar), and entered the light compartment as often as under basal conditions [$t(9) = .03$; $p = .975$] (Figure 2F, right bar). Further analyses comparing the stress-induced changes in those anxiety indices between the groups indicate that exposure to the stressor affected the control mice significantly more than the CeA CRF-KD mice; stress-induced change in percent of the time in the light compartment [$t(14) = 2.81$; $p = .014$]; a similar trend appeared in stress-induced change in number of entries to the light compartment [$t(15) = 1.94$; $p = .071$].

No differences were observed between CeA CRF-KD mice and their controls in home-cage locomotion (Figure 2, G and H). For full

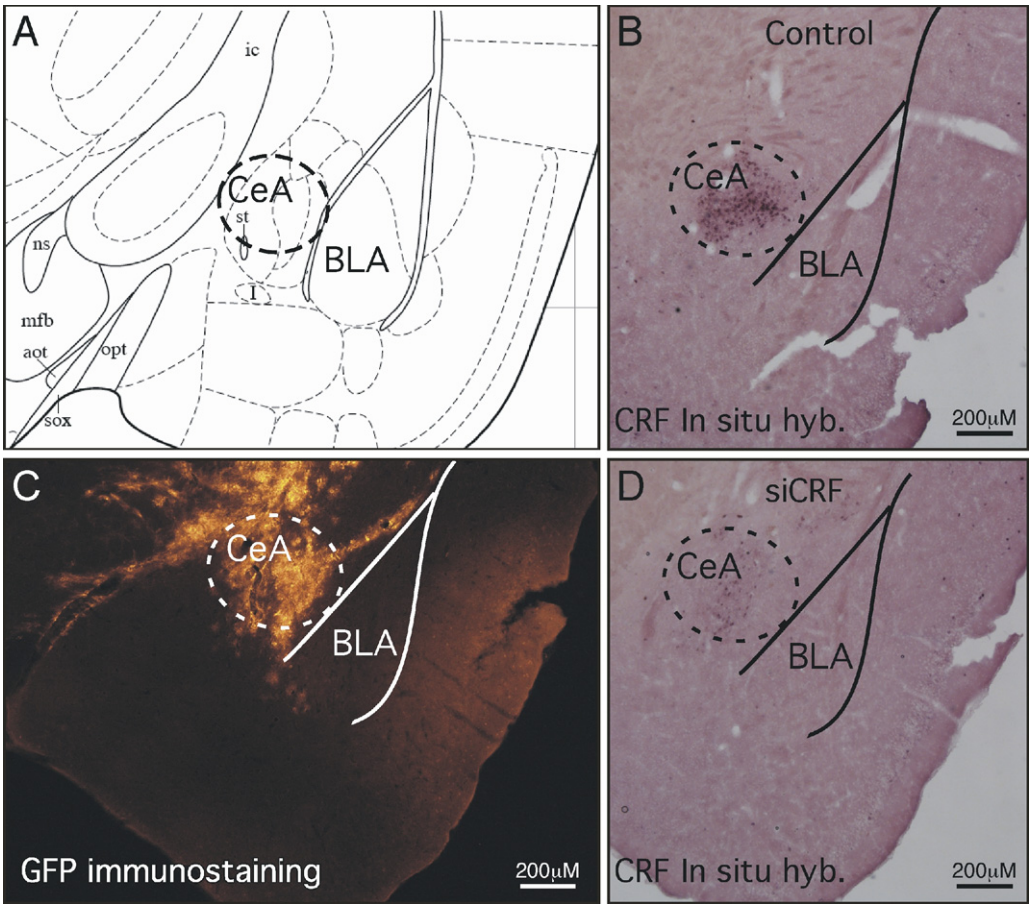

Figure 1. Knockdown of endogenous central amygdala (CeA) corticotropin-releasing factor (CRF) by small interfering (si)CRF lentiviruses. **(A)** Brain section map showing the site of CeA injection, adapted from the Paxinos and Franklin mouse brain atlas (50). **(B, D)** Endogenous CRF messenger RNA levels detected using specific CRF in situ hybridization at the CeA of control **(B)** and siCRF injected mice **(D)**. **(C)** Immunohistochemistry image showing green fluorescent protein (GFP) immunostaining at site of injection. Scale bar: 200 μM. aot, accessory olfactory tract; BLA, basolateral amygdaloid nucleus; ic, internal capsule; mfb, medial forebrain bundle; ns, nigrostriatal bundle; opt, optic tract; sox, supraoptic decussation.

statistical analysis, see Results in Supplement 1. Thus, the home-cage activity data suggest that the observed differences between the groups' behaviors in the DLT and EPM tests derive from stress-induced changes in exploratory behaviors that are related to anxiety in mice (35).

Establishment of Lentiviral-Based System for Inducible and Site-Specific Overexpression of CRF

To study the effects of an opposite manipulation, we established a second lentiviral-based, site-specific system, for inducible overexpression of CRF. The system is composed of two complementary lentiviral vectors. The Effector construct consists of a reverse tetracycline transactivator (rtTA) gene and the reporter GFP (Figure 3A, upper panel). The CRF-Target construct includes the tetracycline-responsive element (TRE) DNA sequence, upstream to the nucleotide coding sequence of the CRF, followed by the reporter red fluorescent protein (RFP) (Figure 3A, lower panel). Transcription of the CRF and the RFP is initiated only in the presence of the inducer, Dox (Figure 3A).

To verify the functionality of the established system in vitro, HEK293T cells were infected with a mixture of the two lentiviruses and incubated with or without the presence of Dox in the growing media. A fluorescence microscope analysis of the cells showed, as expected, a robust GFP expression with or without the presence of Dox and a strong Dox-dependent RFP expression (Figure 3B). Stereotaxic injection of a viral cocktail, containing both lentiviruses, to the CeA generates a mouse model in which CRF is overexpressed specifically at the CeA and only during Dox administration (Figure 3C). To also determine in vivo the functionality of the conditional CRF overexpression lentiviral system in the brain region studied, we microdissected the CeA of mice injected with the mixture of the two viruses treated with or without Dox-containing water for 3 days. The level of exogenous CRF (originated from the lentiviral infection) was determined by performing a real-time PCR using primers that are specific to the CRF-RFP mRNA, which exist only in lentiviral construct. As demonstrated in Figure 3D, Dox administration increases the levels of CRF-RFP levels by approximately 8-fold.

Central Amygdala CRF Inducible Overexpression Enhances Stress-Induced Anxiety Without Affecting Basal Anxiety Levels

C57BL/6 male mice were injected as described above, with a mixture of the two lentiviruses bilaterally into the CeA. Following recovery, half of the group was subjected to Dox administration (CRF-OE), and the other half was kept on normal drinking water (control). After 3 days of Dox induction, basal anxiety-like behavior of mice from both groups was evaluated using the DLT test.

Under basal anxiety conditions, mice induced to overexpress CRF in the CeA (+Dox) did not differ from control mice (–Dox) in the time spent in the light compartment [$t(27) = .58$; $p = .564$] (Figure

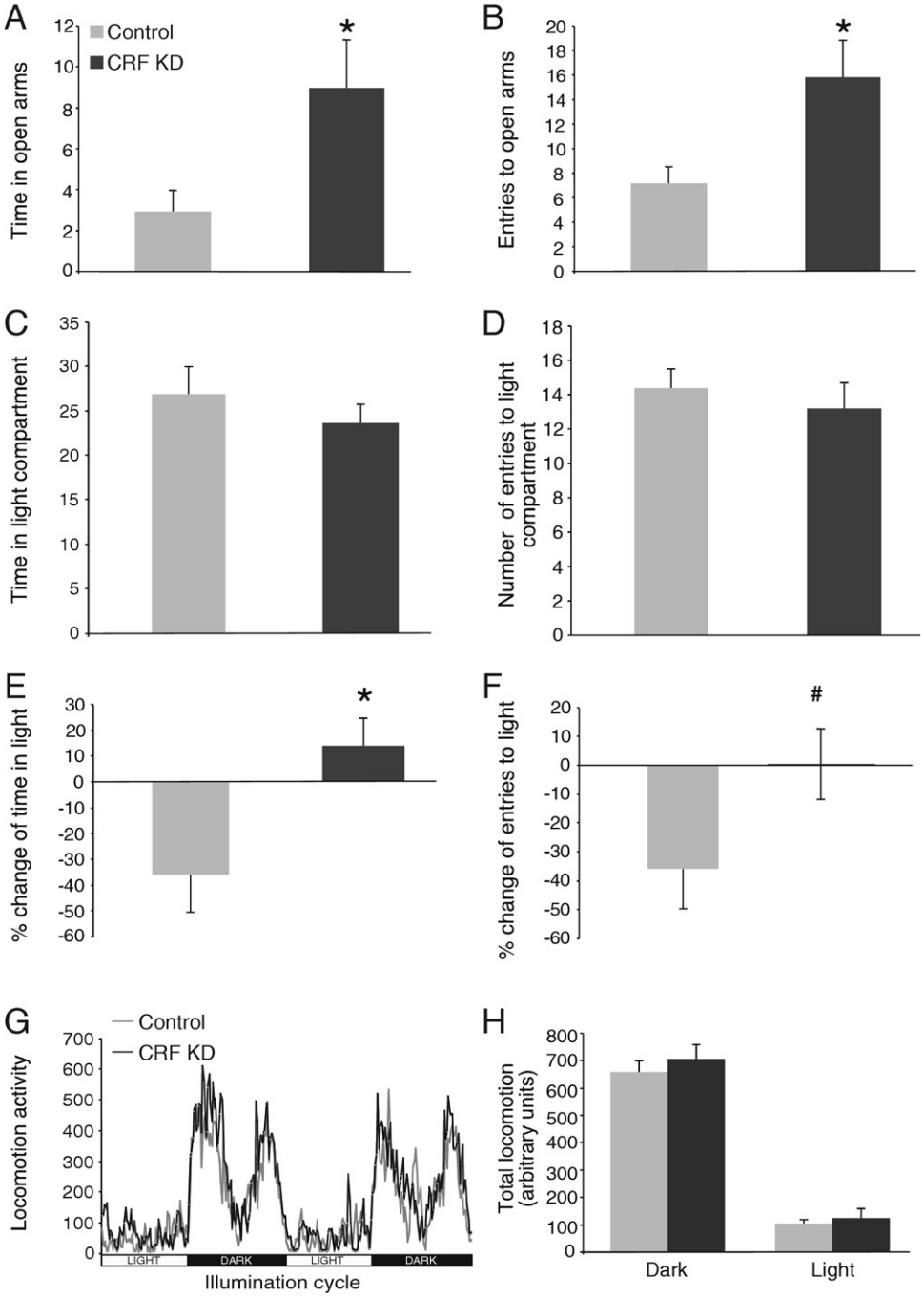

Figure 2. Basal and stress-induced anxiety-like behavior in mice subjected to corticotropin-releasing factor (CRF) knockdown at the central amygdala (CeA). **(A, B)** Under basal conditions, mice subjected to CRF- knockdown (KD) at the CeA spent more time in the open arms of the elevated plus maze **(A)** and entered the open arms more frequently **(B)** than control mice. **(C, D)** No differences in the time spent in the light compartment **(C)** or in the number of entries to the light compartment **(D)** in CeA CRF-KD mice tested using the dark-light transfer test. **(E, F)** Following exposure to 30 minutes of restraint stress, control mice reacted to the stressor by an increase in anxiety-like behaviors, i.e. significant reduction in percent of time spent in the light compartment (**E,** left bar), and the number of entries to the light (**F,** left bar), whereas CeA CRF KD mice appeared unaffected by the stress exposure. CeA CRF KD mice spent a similar percent of the time in the light compartment (**E,** right bar) and entered the light compartment as often as under basal conditions (**F,** right bar). **(G, H)** Monitoring of home-cage locomotion indicated no differences between control and CeA CRF-KD mice. Values are expressed as the mean ± SEM ($n = 8–11$ mice per group; *$p < .05$; #$p < .06$).

4A) or in the frequency of entering this compartment [t(27) = .98; $p = .335$] (Figure 4B). However, immediately following exposure to an acute stressor, +Dox mice displayed an enhanced anxiogenic effect. Stress-induced change in time spent in the light compartment of the DLT appeared more pronounced in +Dox mice than that of –Dox mice, although the difference was not significant [t(25) = .94; $p = .357$] (Figure 4C), yet +Dox mice did exhibit a significantly greater reduction in the number of entries to this compartment than –Dox mice [t(25) = 3.31; $p = .003$] (Figure 4D). It is worth noting that we recently confirmed that Dox administration to wild-

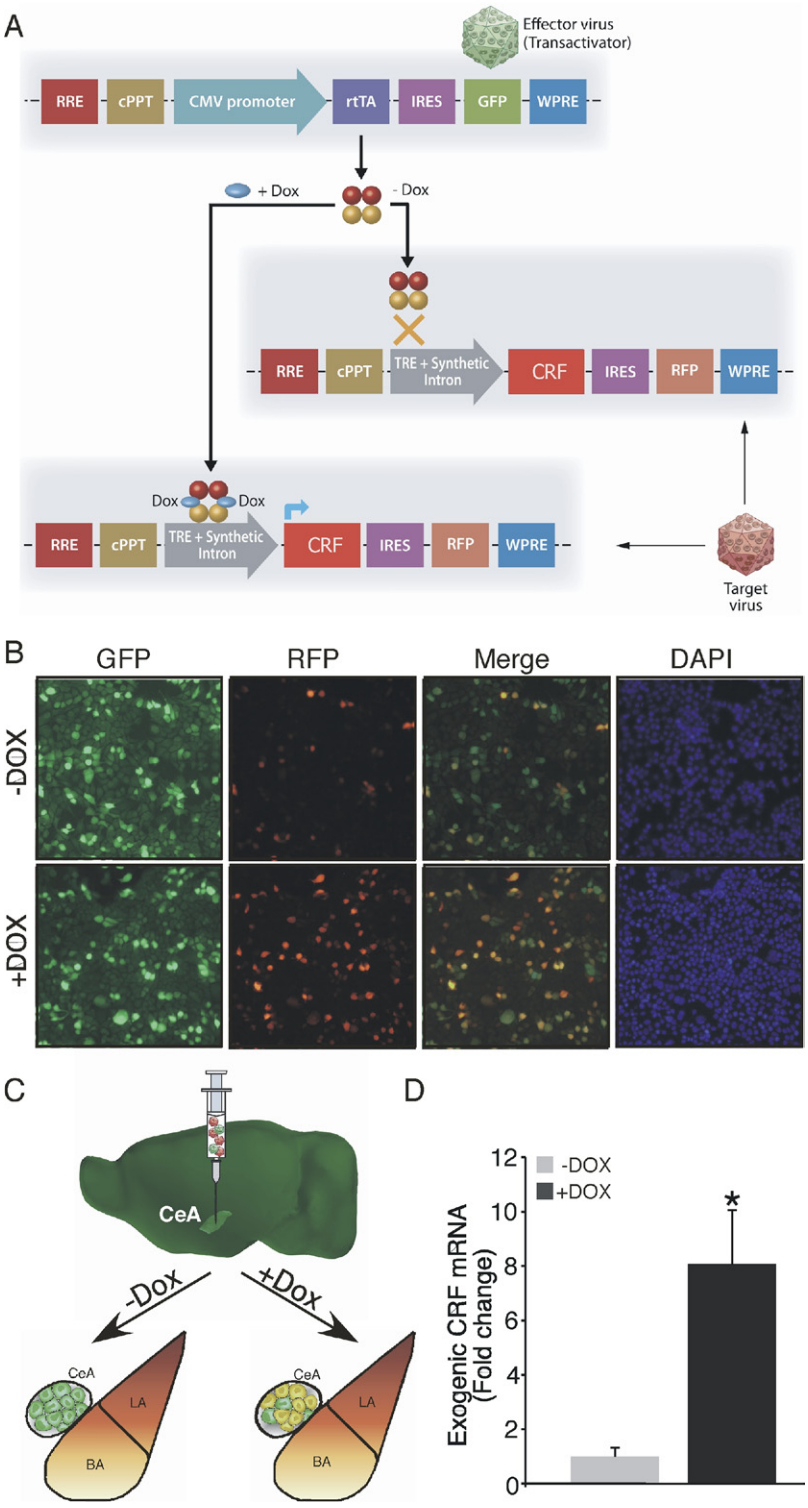

Figure 3. Design and in vitro validation of inducible corticotropin-releasing factor (CRF) overexpression lentiviral-system. **(A)** Schematic representation of the lentiviral constructs and system components. rtTA, delivered by the Effector virus and constitutively expressed at the infected cells, binds TRE in the presence of doxycycline to induce the expression of CRF delivered by the Target virus. **(B)** In vitro validation-HEK293T cells infected by both viruses express GFP in the presence or absence of doxycycline, whereas the RFP is expressed only sporadically in the absence of doxycycline and robustly in its presence. **(C)** A mixture of the two lentiviruses is injected directly into the CeA and the delivered genes are incorporated into the DNA of the infected cells. Initiation of transcription, limited to the infected cells, is induced by administrating doxycycline-containing drinking water and results in overexpression of CRF. **(D)** Real-time polymerase chain reaction analysis shows a robust increase of CRF-RFP messenger RNA transcript in CeA of injected mice following doxycycline administration (+Dox), compared with noninduced (–Dox) injected mice. CeA, central amygdala; CMV, cytomegaloviruse; cPPT, central polypurine tract; DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescent protein; IRES, internal ribosome entry site; RFP, red fluorescent protein; RRE, Rev-responsive element; rtTA, reverse tetracycline transactivator; TRE, tetracycline-responsive element; WPRE, Woodchuck hepatitis posttranscriptional regulatory element.

type mice did not affect the behavioral parameters tested in the current study (30).

As in the previous experiment, monitoring home-cage locomotion of –Dox and +Dox mice indicated that CeA CRF-overexpression (OE) did not induce any differences between these groups (Figure 4, E and F). For full statistical analysis, see Results in Supplement 1.

Knockdown or Conditional Overexpression of CRF in the Central Amygdala Does Not Affect Depression-Like Behavior or Fear Learning

Because anxiety disorders and depression are often cooccurring and altering CRF levels were associated with alterations in indices of depressive-like behaviors, two paradigms of behavioral despair assessment were utilized to evaluate the effects of

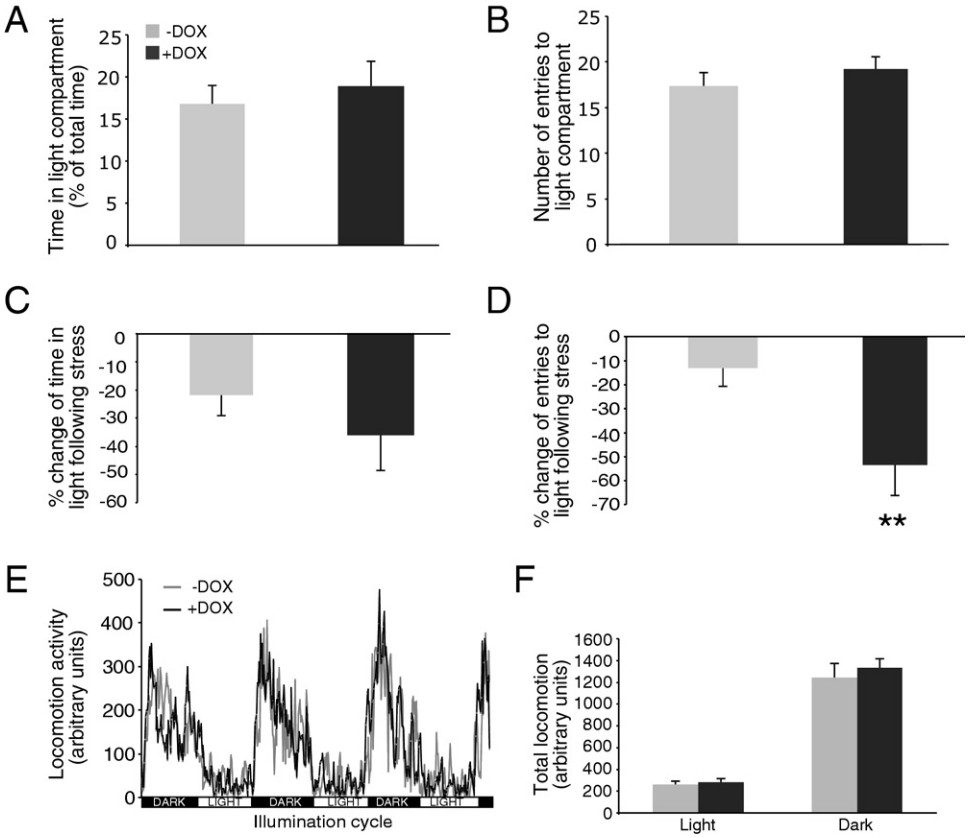

Figure 4. Basal and stress-induced anxiety-like behavior in mice overexpressing corticotropin-releasing factor (CRF) at the central amygdala (CeA). **(A, B)** Under basal conditions, mice subjected to CRF-overexpress at the CeA did not differ significantly from control mice in time spent in the open arms **(A)** and in number of entries to the open arms **(B)** of the elevated plus maze. **(C, D)** Following exposure to an acute stressor, doxycycline-administered (+Dox) mice displayed an enhanced anxiogenic effect. Stress-induced change in time spent in the light compartment of the dark-light transfer by +Dox mice appeared more pronounced than that of noninduced (–Dox) mice, although the difference was not significant **(C)**, yet +Dox mice did exhibit a significantly greater reduction in the number of entries to this compartment than –Dox mice **(D)**. **(E, F)** Monitoring of home-cage locomotion showed no differences in locomotion between the two experimental groups. Values are expressed as the mean ± SEM ($n = 14–15$ mice per group; $^{**}p < .01$).

CeA CRF knockdown and CeA CRF conditional overexpression: the TS test and the FST. As depicted in Figure 5, A–C, CeA CRF-KD did not affect depressive-like behaviors indicated as immobility in both the TS and FST assessments. Likewise, CeA CRF-OE did not affect indices of behavioral despair in both the TS and FST assessments (Figure 5, D–F). See Results in Supplement 1 for complete statistical description.

Whereas the amygdala is strongly associated with fear and anxiety, it is also imperative for emotional learning and memory (for review, see LeDoux [36]). Therefore, we evaluated the effects of CRF-KD and short-term CRF-OE in the CeA on fear learning. Using the fear-conditioning paradigm, mice were conditioned and subsequently tested for contextual memory and for cue memory.

Neither knockdown of CRF (Figure 5, G and H) nor overexpressing it (Figure 5, I and J) in the CeA had any effect on contextual learning (Figure 5, G and I) or cue learning (Figure 5, H and J). For full statistical analysis, see Results in Supplement 1.

Knockdown But Not Overexpression of CRF in the Central Amygdala Affects Basal Corticosterone Levels

CRF expressed in the paraventricular nucleus of the hypothalamus (PVN) is known to activate the HPA axis. Although CRF in the CeA does not directly activate this axis, efferents from this nucleus might affect the PVN directly or indirectly and modulate the neuroendocrine response to stress. To evaluate whether changes in the levels of CRF in the amygdala modified the HPA axis activity, we used separate groups of mice to determine the corticosterone levels under basal conditions and following acute stress. CRF-KD or CRF-OE in the CeA did not alter plasma corticosterone concentrations following restraint stress (Figure 6, A and C). See complete statistical analysis in Results in Supplement 1. Nevertheless, the basal (nonstress) corticosterone plasma levels of the CRF-KD group was significantly higher compared with the control-injected group (Figure 6B), suggesting a possible role for CeA-CRF– expressing neurons in regulating basal HPA axis activity. No significant differences in basal corticosterone levels were observed between the CRF-OE mice and their controls (Figure 6D).

Modified levels of the CeA-CRF may result in compensatory changes in the levels of CRF, CRFR1 and glucocorticoid receptor (GR) expressed by connected brain regions such as the PVN and the BNST. To this end, both the PVN and the BNST were microdissected from the CRF-KD group of mice and the respective controls and the CRF, CRFR1, and GR mRNA levels were determined using real-time PCR analysis. Interestingly, the BNST-CRFR1 mRNA levels were significantly elevated in the CRF-KD group (Figure 6E). CRF and GR mRNA levels in the BNST and the CRF, CRFR1, and GR mRNA levels in the PVN were unchanged (Figure 6, E and F).

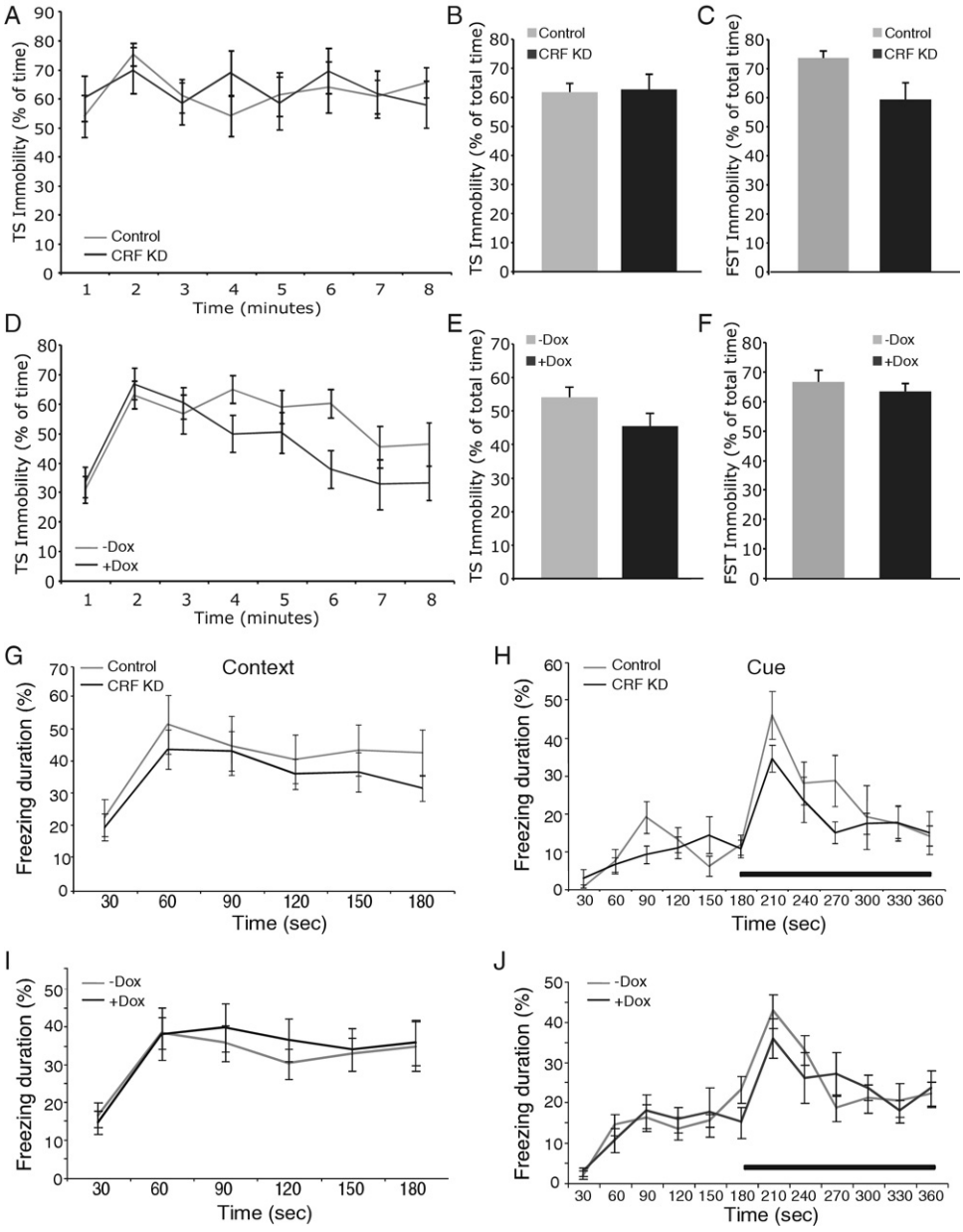

Figure 5. Knockdown or conditional overexpression of *CRF* in the central amygdala does not affect depression-like behavior or fear learning. **(A–F)** Reducing or enhancing CeA *CRF* levels did not affect immobility in both the tail-suspension (TS) and forced swim test (FST). No significant differences were observed between CRF-KD ($n = 11$) and control ($n = 10$) mice immobility (%time) throughout **(A)** and overall **(B)** the TS test or overall the FST **(C)**. Similarly, no significant differences were observed between mice overexpressing CRF (+DOX; $n = 14$) and their controls (–DOX; $n = 15$) immobility (%time) throughout **(D)** and overall **(E)** the TS test or overall the FST **(F)**. **(G–J)** Knocking down **(G, H)** or overexpressing CRF **(I, J)** in the CeA had no effect on contextual learning **(G, I)** or cue learning **(H, J)**. Values are expressed as the mean ± SEM ($n = 14$–15 mice per group).

Knockdown of CRF in the Central Amygdala Affects Urocortin 3 Levels in the BNST

Compensatory mechanisms involving different members of the CRF/Urocortin (Ucn) family had been previously suggested (21,22). To determine whether manipulating CRF levels at the CeA affected levels of other ligands of the family in connected brain areas, we evaluated the mRNA levels of Ucn2 and Ucn3 in the amygdala, BNST, PVN, and locus coeruleus, using real-time PCR. Significantly lower levels of Ucn3 mRNA were detected in the BNST of mice with CeA CRF-KD (Figure S2Ba, left panel, in Supplement 1) and a tendency for higher levels of this peptide mRNA in CeA CRF-OE (Figure S2Ba, right panel, in Supplement 1). No additional differences in Ucn3 mRNA levels were detected in other tested brain regions (Figure S2B, b and c, in Supplement 1). No significant differences in Ucn2 mRNA levels were found between any of the experimental groups (Figure S2A, a and b, in Supplement 1).

Discussion

The current study demonstrates the establishment and use of lentiviral-based systems to genetically manipulate CRF expression levels in a site-specific and temporally controllable manner in adult mice. Two separate lentiviral-based systems, designed to knockdown or conditionally overexpress CRF, were used to manipulate

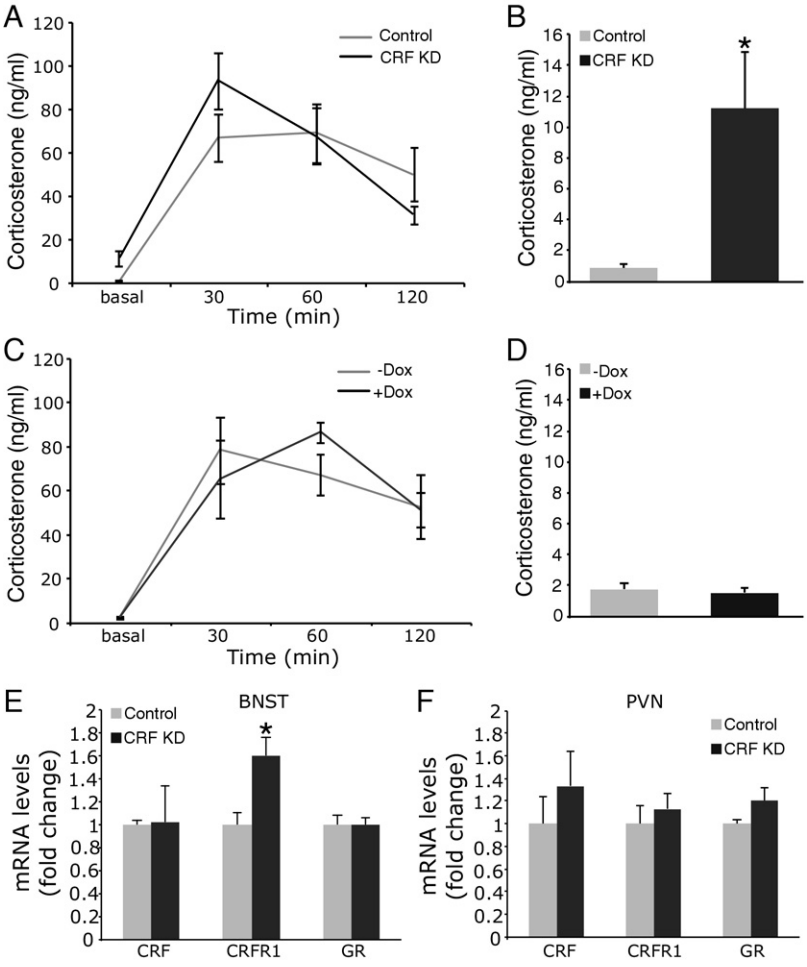

Figure 6. Knocking down corticotropin-releasing factor (CRF) in the central amygdala (CeA) affects basal corticosterone levels and CRFR1 messenger RNA (mRNA) levels expressed by the bed nucleus of the stria terminalis (BNST). **(A, C)** Stress-induced plasma corticosterone levels of CeA CRF-knockdown (KD) mice **(A)** and of CeA CRF-overexpress (OE) mice **(C)**, collected 30, 60, and 120 minutes from acute stress initiation did not differ from corticosterone levels of control mice in the same condition. **(B, D)** Basal corticosterone plasma levels of CeA CRF-KD mice **(B)** and not of CeA CRF-OE mice **(D)** are significantly elevated. Values are expressed as the mean ± SEM ($n = 11$-12 mice per group). **(E, F)** CRF, CRFR1, and glucocorticoid receptor (GR) mRNA levels, relative to hypoxanthine-guanine phosphoribosyltransferase (HPRT) in the BNST **(E)** and paraventricular nucleus of the hypothalamus (PVN) **(F)** of mice with CRF-KD in the CeA. CRF-KD in the CeA led to a significant increase in CRFR1 mRNA levels in the BNST **(E)** but not in the PVN **(F)**. CRF and GR mRNA levels were not affected in the BNST or PVN nuclei. Values are expressed as the mean ± SEM ($n = 6$ mice per group). *$p < .05$.

the expression levels of CRF specifically in the CeA of adult mice. The behavioral responses associated with the CeA, such as anxiety, depression, and fear memory, were evaluated under basal and immediately following acute stress conditions. Although changing the CeA-CRF levels only mildly affected the anxiety-like behaviors under basal stress conditions, it was evident that in more stressful conditions or following exposure to an acute stressor, CeA CRF-KD attenuated stress-induced anxiety and that short-term CeA CRF-OE enhanced the stress-induced effects on these behaviors.

Dysregulation of CRF expression was previously associated with stress-related psychopathologies including anxiety disorders and depression and was extensively studied using pharmacological tools or genetic manipulations of CRF or its principal receptor, CRFR1 (14,16–18,20–22). Several previous genetic studies aimed to define the role of CRF using different genetic mouse models, including establishment of constitutive *CRF* overexpression and *CRF* KO mice models (21,37–41) or *CRFR1* KO mice models (18–20). *CRFR1*-deficient mice had clearly shown an anxiolytic phenotype, yet the observed behavioral changes may result from a combination of different factors such as concomitant impairments in several CRFR1-containing brain nuclei and/or alterations in brain circuits involving Ucn1, a member of the CRF family with high affinity to the same receptor and thus may not be strictly CRF dependent. Mice with unrestricted CRF-OE described by Stenzel-Poore *et al.* (37,38) exhibited an anxiogenic phenotype. However, these mice also exhibited severe Cushing-like symptoms, resulting from a hyperactive HPA axis. In a central nervous system (CNS)–restricted model (39,40), HPA axis activity was still elevated, and although delayed, Cushing-like symptoms were still evident. More recently, Lu *et al.* (41) presented several CRF-overexpressing conditional transgenic mouse models. Using the Cre/LoxP system, they demonstrated stress-induced hypersecretion of stress hormones in mice overexpressing CRF in the entire CNS using the Nestin-Cre mouse line but not in mice with CRF-OE restricted to forebrain neurons using the Camk2a-Cre mouse line. Additionally, Lu *et al.* (41) showed that CNS-restricted CRF-OE promoted increased stress-coping behavior, whereas forebrain-restricted CRF-OE failed to affect behavior in these tests.

A deletion of the entire pre-proCRF coding region, resulting in CRF-deficient mice (21), indicated the critical role of CRF in mediating corticosterone secretion. These mice exhibited impaired production of corticosterone after different stressors, thus demonstrating a central role for CRF in the HPA axis stress response. However, it is of note that the effects of stress on anxiety-like behaviors that have been previously ascribed to CRF were not evident in these CRF-deficient mice, suggesting possible compensation mechanisms (21,22,42). All the above-mentioned CRF transgenic mice models, although differing in spatial specificity, are likely to be subject to compensational mechanisms because of the chronic elevation or developmental deletion of CRF in these mice. Therefore, developmental compensatory mechanisms and lack of spatial and temporal specificity limited these studies' interpretations regarding the role of CRF expression within discrete brain nuclei in these highly regulated processes.

The mice models used in the current study altered the expression levels of *CRF* in adulthood, thus avoiding developmental effects and minimizing long-term compensatory changes. Furthermore, the current genetic manipulations were site specific, restricted to the CeA, ensuring the effects to be mediated via CeA *CRF* expression alterations. In addition, the use of an inducible overexpression system allowed short-term overexpression of the *CRF* in the CeA, thus avoiding effects of chronic overexpression. The CeA expresses CRF endogenously; thus, CRF-KD in this nucleus or expressing CRF in addition to the endogenously expressed peptide altered CRF levels in this nucleus.

The results obtained from these two models may suggest that CRF expressed in the CeA is essential for responding to stressors but has a limited effect on basal anxiety levels. Although CRF overexpression in the CeA did not affect anxiety-like behaviors under basal conditions, CRF-KD in this region resulted in a decrease in anxiety-like behavior as measured using the EPM test. Intriguingly, following exposure to an acute stressor known to induce an endogenous increase in CeA CRF levels, knocking down CRF expression in the CeA blunted the effects of the stress exposure on anxiety-like behaviors in the DLT test, whereas the complementary manipulation of CRF-OE enhanced the stress-induced increase in anxiety-like behaviors in this test. These results may suggest a threshold for the anxiogenic effects of CRF, below which CRF in the CeA does not affect anxiety-like behaviors.

It is feasible to suggest that when CRF levels in the CeA exceed this threshold, anxiety-like behaviors are apparent. This suggestion is supported by findings demonstrating an amygdala CRF dose-dependent behavioral response to CRF, indicating a threshold below which no significant effects were evident on anxiety-like and panic-like behaviors (27). In the current study, CeA CRF-OE did not increase basal anxiety-like behavior; however, excess CRF levels augmented the behavioral response to the acute stress exposure, resulting in an enhanced anxiogenic effect. Exposing rodents to stressors is known to increase CRF expression in the CeA (43) and to result in elevated anxiety. The current study demonstrated a blunting of this effect by CRF-KD in the CeA; thus, it may be speculated that although stress affects several brain circuits and mechanisms, including CRF up-regulation in several brain loci, preventing such up-regulation in the CeA is sufficient for attenuating stress-induced anxiety.

A recent study by Keen-Rhinehart *et al.* (44) demonstrated that a chronic increase in CRF expression, specifically in CeA of female rats, using a lentiviral vector administration resulted, among others, in increase in the acoustic startle response and depressive-like behavior in the FST. Interestingly, although a short-term CeA-CRF-OE, conducted in the current study, enhanced the stress-induced effects on anxiety-like behavior, our recent study demonstrated that prolonged CRF-OE at the CeA attenuated stress-induced anxiety-like behavior (45).

Although CRF neurons in the CeA do not project to the primary plexus of the hypophysial portal vessels and therefore do not directly affect ACTH release from pituitary corticotropes, efferents from this nucleus might affect the PVN directly or indirectly and modulate the neuroendocrine response. CRF-KD or CRF-OE in the CeA did not alter plasma corticosterone concentrations following restraint stress; however, the basal (nonstress) corticosterone plasma level of the CRF-KD group was significantly higher compared with the control-injected group, suggesting a possible role for CeA-CRF– expressing neurons in regulating basal HPA axis activity.

The BNST receives innervation from many brain areas associated with initiation of the stress response, including CRF from the CeA. Its outputs include, among others, substantial innervations to the medial parvocellular neurons of the PVN, in which CRF is released to initiate the HPA axis (46,47). The CeA has only limited projections to the PVN (48), suggesting the BNST as a center for integration of a stress response and relay to the PVN to modulate the observed changes in the basal HPA activity.

The CeA has an established key role in emotional learning; it was reported essential for both acquisition and expression of fear in fear-conditioning studies (36,49). Fear conditioning is also affected by exposure to stress and by manipulating various members of the CRF family (32). In the current study, we demonstrated that CRF-KD in the CeA and that short-term CRF-OE in this nucleus do not affect cued or contextual fear learning, suggesting that our manipulations did not impair the functionality of the CeA. Yet because the effects of exposure to stressors on learning were not evaluated in this study, it is possible that manipulating CRF levels in the CeA may affect stress-induced learning impairments or enhancements.

To conclude, the current study introduced two novel CRF-related genetic tools and transgenic mice models, demonstrating the effects of CeA-specific, time-controlled manipulation of CRF expression. Results obtained with these models demonstrated the effects of CeA CRF on stress-induced anxiety, suggesting that whereas changes in CRF expression levels under basal conditions have a mild effect on anxiety, elevation of CRF in the CeA is essential for stress-induced anxiety and that levels of anxiety are associated with CRF levels. Further elucidation of these mechanisms may lead to a better understanding of the underlying processes of stress-responses and may aid in developing new therapeutic tools for stress-related psychopathologies.

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