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