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ORIGINAL ARTICLE

Prolonged and site-specific over-expression of corticotropin-releasing factor reveals differential roles for extended amygdala nuclei in emotional regulation

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Corticotropin-releasing factor (CRF) has a key role in the central stress response, and altered levels of this neuropeptide are linked to stress-related psychopathologies such as anxiety and depression. These disorders are associated with the inability to properly regulate stress response, specifically following exposure to prolonged stressful stimuli. Therefore, the current study assessed the effects of prolonged and site-specific over-expression of CRF, which mimics the state of chronic production, in extended amygdala nuclei that are known to be involved in mediating anxiety-like states. We first constructed and generated lentiviruses that overexpress (OE) CRF in a robust and stable manner, and then generated two male mouse models continuously over-expressing CRF, either at the central nucleus of the amygdala (CeA), or at the dorsolateral subdivision of the bed nucleus of the stria terminalis (BNSTdl). After 4 months, behavioral assessments were conducted for anxiety and depressive indices on these mice. Surprisingly, prolonged CRF OE at the CeA attenuated stress-induced anxiety-like behaviors, whereas prolonged CRF OE in the BNSTdl increased depressive-like behaviors, without affecting anxiety levels. These results show possible differential roles for CRF expressed by distinct loci of the extended amygdala, in mediating stress-induced emotional behaviors.
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Keywords: corticotropin-releasing factor (CRF); amygdala; stress; bed nucleus of the stria terminalis (BNST); lentiviruses

Introduction

The neuropeptide corticotropin-releasing factor (CRF) is an essential regulator of the neuroendocrine and behavioral response to stress, and was implicated in the control and maintenance of an organism's dynamic homeostatic equilibrium.[1–7] A significant body of evidence has accumulated suggesting that inappropriate regulation, disproportional intensity, or chronic and/or irreversible activation of the stress response mechanisms are linked to the etiology and pathophysiology of anxiety disorders and depression.[8–15]

Chronic hyperactivation of the CRF system was linked to stress-related mental disorders such as anxiety and depression.[16–20] Evidence from studies using competitive CRF peptide or small molecule CRF type 1 receptor (CRFR1) antagonists, provided strong support for the hypothesis that the brain CRF system has a key function in mediating behavioral responses to stressors.[8,16,18,21–23] Animal studies showed the anxiogenic-like behavioral effects of CRF administration and anxiolytic-like activity of CRFR1-selective antagonists, supporting the suggestion that CRF may be involved in anxiety-related disorders.[8,16]

A role for CRFR1 in modulating anxiety-like behavior was further suggested based on the behavioral phenotypes of the CRFR1-deficient mice models. Mice deficient in CRFR1, both centrally and peripherally, display decreased anxiety-like behavior and impaired hypothalamic-pituitary-adrenal (HPA)-axis response to stress.[24,25] In addition, mice lacking CRFR1 exclusively within the limbic system exhibited a similar anxiolytic phenotype.[26] Clinical and pre-clinical studies also suggested a central role for the CRF/CRFR1 system in major depression.[8–12] Using different animal models, potential antidepressant-like effects of selective CRFR1 antagonists have been shown.[10,22,23]

Animal models of fear and anxiety showed the involvement of specific, highly connected brain regions, including the hippocampus, central nucleus of the amygdala (CeA), basolateral amygdala (BLA), bed nucleus of the stria terminalis (BNST) and lateral septum, as key players in anxiety-like states and stress responses.[27–30] In extra-hypothalamic regions, CRF is mainly expressed by the CeA and the BNST nuclei, thus suggesting these extended amygdala sites

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as the source for CRF effects on fear, anxiety and the subsequent development of depression. Direct administration of CRF into the amygdala resulted in an anxiogenic-like behavioral phenotype, further supporting the role of amygdalar CRF in mediating the behavioral responses to stressors.[30]

Delineating the contributions of CRF expression in specific brain nuclei to the strictly regulated stress response may improve our understanding of the pathways by which the brain translates stressful stimuli into integrated biological responses. As chronic exposure to stressors has been implicated in the etiology of stress-related psychopathologies, this study assessed the behavioral and neuroendocrine effects of prolonged CRF over-expression (OE) at endogenously expressing nuclei of the extended amygdala, by establishing a lentiviral-based system for site-specific expression of CRF. Using this system, we generated mice over-expressing CRF at either the CeA or the dorsolateral subdivision of the BNST (BNSTdl) nuclei of the extended amygdala. After 4 months of continuous CRF OE, behavioral assessments were performed for anxiety indices, both under basal conditions and following exposure to a stressor, and for depressive-like indices.

Materials and methods

Lentiviral vectors design and production

Lentiviral vectors were constructed to produce lentiviruses expressing rat CRF, as described earlier.[31,32] Rat and mouse CRF mature peptides (GeneBank Accession number NM_031019 and NM_205769, respectively) are identical in their amino acid sequence. The following oligonucleotide primers were used for the polymerase chain reaction (PCR) isolation of rat CRF (GeneBank accession No. NM_031019): sense primer: 5′-ATGCGGCTGCGGCTGC TGGTGTCC-3′ and the antisense primers: 5′-TCATTTC CCGATAATCTCCATCAG-3′, carrying restriction sites for *AgeI* and *SfiI*, respectively. The purified PCR product was cloned into the lentiviral expression vector, pCSC-SP-PW-IRES/GFP, which was kindly provided by Dr Inder Verma (Salk Institute for Biological Studies, La Jolla, CA, USA).

Production of lentiviral vectors

Recombinant lentiviruses were produced by transient transfection in HEK293T cells, as described earlier,[31] using pCSC-SP-PW-rCRF-IRES/GFP (CRF OE), or pCSC-SP-PW-IRES/GFP (control). Briefly, infectious lentiviruses were harvested at 48 and 72 h post-transfection, filtered through 0.45-μm-pore cellulose acetate filters and concentrated by ultracentrifugation. Aliquots were kept at −70 °C. Viral titers were determined by infection of 293T cells and green fluorescent protein (GFP) visualization.

In vitro *validation of lentiviral vectors*

The ability of the CRF OE vectors to express CRF was assessed by western blot analysis and immunocytochemistry. HEK293T cells were infected with the CRF OE lentiviruses or with non-related-gene-expressing viruses. For western blot analysis, the cells were harvested 48 h post infection in a lysis buffer (25 mM Tris–Hcl, pH 7.4/150 mM KCl/1.5 mM $MgCl_2$/1% (wt/vol) glycerol/1% (wt/vol) NP40 containing protease inhibitors). The cell lysates were subjected to gel electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel; separated proteins were transferred onto nitrocellulose membranes and probed with rabbit antiserum against CRF (final dilution of 1:2000, kindly provided by Dr Wylie Vale, The Salk Institute). Subsequently, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody. Thereafter, the blots were stripped and re-probed with anti-β-actin (Sigma-Aldrich, St Louis, MO, USA), using horseradish peroxidase-conjugated anti-mouse as secondary antibody (Amersham Biosciences, Piscataway, NJ, USA). The immunoreactive proteins were visualized using EZ-ECL Chemiluminescence detection kit (Biological Industries, Kibbutz Beit Haemek, Israel).

For fluorescent microscope visualization of CRF (co-expressed with GFP), HEK293T cells were grown on glass cover slips. The cells were fixed with 4% paraformaldehyde in PBS for 10 min, 48 h post infection, permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 5 min and blocked in PBS containing 0.1% Triton and 3% bovine serum albumin for 1 h at room temperature (RT). For immunostaining, the cells were incubated for 2 h at RT with the same rabbit antiserum against CRF as in the immunocytochemistry, then incubated for 1 h with CY3 conjugated second antibodies (Chemicon-Millipore, Schwalbach, Germany) at a final dilution of 1:500, followed by 5 min of Hoechst 33342 (Invitrogen Corporation, Carlsbad, CA, USA) staining at a final dilution of 1 μg ml^{-1}. The cover slips were mounted with Immu-mount (Thermo scientific, Pittsburg, PA, USA) mounting media, and the cells were viewed and images captured under a fluorescence microscope.

In-situ *hybridization*

Antisense and sense (control) RNA probes were generated using rat CRF complementary DNA and labeled with DIG-11-UTP using a labeling kit from Roche Applied Science (Mannheim, Germany). *In situ* hybridization was carried out with the free-floating section method, as reported earlier.[33]

Animals and housing

Adult C57BL/6J male mice (7 weeks old) (Harlan, Jerusalem, Israel) were housed in a temperature-controlled room (22 ± 1 °C) on a reverse 12 h light/dark cycle (lights on at 19:00). Food and water were available *ad libitum*. All experimental protocols were approved by the Institutional Animal Care and Use Committee of The Weizmann Institute of Science.

Surgical procedure
Mice were anesthetized with a mix of 10% ketamine/10% xylazine in saline solution at 100 μl per 10 g body weight, and placed on a computer-guided stereotaxic instrument (Angle Two Stereotaxic Instrument, myNeurolab, Richmond, IL, USA), which is fully integrated with the Franklin and Paxinos mouse brain atlas coordinates through a control panel. The lentiviral vectors were delivered bilaterally using a 2 μl Hamilton syringe connected to a motorized nanoinjector, at a rate of 0.3 μl min^{-1}. To allow the solution to diffuse into the brain tissue, the needle was left in the brains for an additional 2 min after injection. The coordinates relative to bregma were as follows: CeA ML ±2.52, AP −1.34, DV −4.7; BNSTdl ML ±0.9, AP +0.14, DV −4.35. The animals experienced 4 months of continuous CRF and/or GFP expression and were then assessed for anxiety-like behavior and depression-like behavior. Fifteen mice were injected in each experimental group.

Behavioral assessments
All behavioral assessments were performed during the dark phase following habituation to the test room for 2 h before any test.

For the assessment of anxiety-like behaviors, the open field (OF), the dark–light transfer (DLT), the elevated plus maze and the acoustic startle response (ASR) tests were used. The OF consisted of a Plexiglas box (50 × 50 × 22 cm). The arena was illuminated with 120 lux. Each mouse was placed in the corner of the apparatus to initiate a 10-min test session. The time spent in the center of the arena, the latency to cross the center, and the number of entries into the arena center, were measured. The DLT test consisted of two compartments, a dark one (14 × 27 × 26 cm) and a 1050 lux illuminated light compartment (30 × 27 × 26 cm), connected by a small passage. The mice were placed in the dark compartment to initiate a 5-min test session. The time spent in the light compartment, the number of entries to the light compartment and the latency of entering the light zone were measured. The elevated plus maze apparatus comprised a central part (5 × 5 cm) with two opposing open arms (30.5 × 5 cm) and two opposing closed arms (30.5 × 5 × 15 cm). The apparatus was elevated at a height of 53.5 cm and the open arms were illuminated with 6 lux. The mice were placed in the center, facing an open arm to initiate a 5-min session test. The time spent in the open arms and the number of entries to the open arms was measured. The indices collected in these tests were quantified using an automated video tracking system (VideoMot2; TSE Systems, GmbH, Bad Homburg, Germany).

The ASR apparatus (StartleResponse, TSE Systems) consisted of a sound-attenuated, well-ventilated cabinet. In this cabinet, the mice were placed in a small Plexiglas and wire mesh box, which does not discomfort the mice, mounted on a vibration-sensitive platform. Movement in this box was detected by a high-precision sensor that was integrated in the measuring platform. Two high-frequency loudspeakers inside the cabinet produced all audio stimuli. Protocol (Adapted from Bilkei-Gorzo *et al*., 2008[34] and Groenink *et al*., 2008[35]): as illustrated in Figure 5a, the session started with a 5-min acclimation period, with a background white noise (70 dB(A)), which was maintained throughout the session; during the last 2 minutes of this period an individual activity baseline was recorded. Overall, 32 startle stimuli (120 dB(A), 40 ms; inter-trials interval: randomly varying, 12–30 s) were presented; the stimuli presentation was divided into three 'Blocks': Blocks 1 and 3 consisted of six startle stimuli each, whereas Block 2 consisted of 10 startle stimuli and 10 'no stimuli' (70 dB(A), 40 ms.; i.e. equivalent to the background white noise) that were presented in a quasirandom manner. The entire session was completed in less than 20 min. Two indices were recorded for each of the blocks: (1) Max ASR (g) = mean maximal force (g) produced in response to the startling stimuli, and (2) RT ASR (ms) = mean reaction time to respond to the startle stimuli (latency to exceed the individual activity baseline). Stress-induced anxiety-like behaviors were assessed immediately after 30 min of restraint stress.

Depressive-like behaviors were assessed using the forced swim (FST) and the tail suspension tests. In the FST, mice were placed individually in a translucent round container, filled with water (22 ± 2 °C) to a depth of 30 cm. The test consisted of a 15 min conditioning trial on day 1, followed by a 5 min test performed 24 h later (day 2). The tests were video recorded for later scoring. An observer blinded to the experimental conditions assessed the time the animals spent being immobile, swimming, and climbing. As the differences observed in the FST can be attributed to altered recall faculties, learning and memory were evaluated using the fear conditioning paradigm. The percentage of time spent freezing (% freezing), indexing fear, was assessed among CRF-OE and control mice throughout the habituation session, the conditioning session, the contextual memory test and the cue memory test.

A computer-controlled fear conditioning (FC) system (TSE Systems) monitored the procedure while measuring the mice freezing behavior. The procedure includes: habituation (day 1): the mice were habituated for 5 min to the FC conditioning chamber, a clear Plexiglas cage (21 × 20 cm × height 36 cm) with a stainless steel grid floor within a constantly illuminated (250 lux) FC 'housing'.

Conditioning (day 2): during this 5 min training session, the mice initially explored the context for 2 min. Thereafter, two pairings of a co-terminating tone (conditioned stimulus (CS): 30 s, 3000 Hz, pulsed 10 HZ, 80 dbA) and shock (unconditioned stimulus: 0.7 mA, 2 s, constant current) with a fixed inter-trials intervals of 60 s. The unconditioned stimulus was delivered through the metal grid floor; mice were

taken out of this chamber 1 min after the last CS-unconditioned stimulus paring. The chamber was thoroughly cleaned with 10% ethanol before each session. The conditioning box 'housing' a ventilating fan provided a constant auditory background noise (white noise, 62 dB(A)).

Contextual memory test (day 3): context-dependent memory was tested 24 h after the conditioning by re-exposure to the conditioning box for 5 min without any stimuli.

Cued memory test (day 3): the tone-dependent memory test was performed 2 h after the contextual memory test in a novel context. The novel context was distinct from the conditioning context; the walls and floor of the box were opaque black Plexiglas (the dimensions were similar), the apparatus 'house-lights' and ventilating fan were turned off and the box was cleaned with 1% acetic acid before each session. Initially, the behavior was monitored for 2 min without any stimuli before the CS (tone) presentations; thereafter, two CSs were presented separated by a fixed 1 min inter-trials interval; the mice were taken out of this box 1 min after the last CS.

General locomotor activity during three consecutive days was assessed by an infrared-based automated system (InfraMot; TSE Systems).

Blood collection and hormone analysis

Basal dark phase blood samples were obtained at 1300 hours, 6 h after the beginning of the dark phase, from individually housed mice. For the evaluation of the endocrine response to stress, tail blood samples were collected before (basal), immediately after 30 min of restraint stress, and 60 and 120 min after stress initiation. Restraint stress was induced using a 50 ml ventilated conical tube. Plasma samples were immediately centrifuged and stored at −80 °C until assays for hormone measurement were conducted. Corticosterone concentrations were quantified using Corticosterone EIA kit (Cayman Chemical Company, Ann Harbor, MI, USA).

RNA preparation and real-time PCR

Immediately after decapitation, the brains were removed and placed into a 1 mm metal matrix (Stoelting Co, Wood Dale, IL, USA, catalog no. 51386). The brains were sliced using standard razor blades (GEM, Personna American Safety Razor Co., Cedar Knolls, NJ, USA, 62–0165) into 2 mm slices that were quickly frozen on dry ice. The area of interest was punched using a microdissecting needle of an appropriate size and stored at −80 °C. RNA was extracted using a 5 PRIME Manual PerfectPure RNA Cell & Tissue kit (5 Prime GmbH, Hamburg, Germany). The RNA samples were reverse transcribed to generate complementary DNA pools that were later used as templates for quantitative real-time PCR analysis using specific primers. The expression of hypoxanthine–guanine phosphoribosyltransferase mRNA served as an internal control. The real-time reaction was performed in an AB-7500 thermocycler (Applied biosystems inc, Foster City, CA, USA) using power SYBR Green PCR mix (Applied Biosystems, Warrington, UK). The following specific primers were designed using Primer Express software (PE Applied Biosystems, Perkin Elmer, Foster City, CA, USA): mCRFR1 primers: 5′-TGCCAGGAGATTCTCAACGAA-3′ and 5′-AAAGCCGAGATGAGGTTCCAG-3′ corresponding to nucleotides 495–515 and 656–676, respectively. Hypoxanthine–guanine phosphoribosyltransferase primers: 5′-GCAGTACAGCCCCAAAATGG-3′ and 5′-GGTC CTTTTCACCAGCAAGCT-3′ corresponding to nucleotides 540–559 and 571–591, respectively. Melting curve analysis was used to check the specificity of the amplification products.

Immunohistochemistry

Specific immunohistochemistry of brain slices for GFP was performed as described earlier.[36] In brief, the animals were anesthetized with chloral hydrate (1.4 g per kg body weight, intraperitoneal) and perfused transcardially with 10 ml of PBS followed by 100 ml of 4% paraformaldehyde in a borate buffer, pH 9.5. The brains were removed and post-fixed in 30% sucrose using the same fixative at 4 °C, frozen and sectioned coronally at 25 μM using a sliding microtome (Leica Microsystems GmbH, Wetzlar, Germany) and stored in PBS at 4 °C until used.

Brain slices were blocked for 1 h with PBS containing 0.3% Triton and 20% normal horse serum to prevent non-specific binding and incubated overnight at RT with rabbit anti-GFP antibody (1:100) as the primary antibody (MBL, Naka-ku, Japan), followed by Cy2 conjugated secondary antibody (Chemicon-Millipore, Schwalbach, Germany). The slices were then washed and mounted on gelatin-coated slides, and screened using a fluorescent microscope for GFP expression at the injection sites. Representative images were captured. The mice that did not show GFP at the aimed injection location were excluded from data.

Data analysis

The results are presented as means ± s.e.m. Behavioral parameters were analyzed by independent Student's *t*-test (two-tailed). Corticosterone dynamics were assessed using two-way analysis of variance (ANOVA) for 'time' (after exposure to restraint stress), as a with-in factor (repeated measures), 'virus' (as between factor) and the interaction 'time' × 'virus' of circulating corticosterone levels during the response to the stressor. Fear conditioning results were assessed using two-way ANOVA for 'time intervals' (with-in factor with repeated measures), 'virus' (between factor) and the interaction 'time intervals' × 'virus' of the percentage of freezing during all phases of the FC procedure (habituation, conditioning, context test and cue test).

Results

Establishment of site-specific CRF OE mouse models
To generate continuous and site-specific CRF over-expressing mouse models, we designed and generated lentiviruses with the ability to express CRF under the control of the ubiquitous promoter, cytomegalovirus. The CRF complementary DNA was isolated and subcloned into a lentiviral expression vector that also contains the complementary DNA sequence for the reporter protein GFP (Figure 1a). The ability of the CRF-OE lentiviruses to infect cells and induce the expression of CRF was confirmed by infecting HEK293T cells. The cell lysates were tested by western blot analysis, revealing a band of approximately 20 kDa, which correlates with the expected size of CRF precursor (Figure 1b). Such a band did not appear in cells infected with a non-related virus (Figure 1b; upper panel). The blotted membrane was re-probed with anti-β-actin antibodies and no significant difference was detected in the amount of the total protein loaded and transferred (Figure 1b; lower panel). To visualize the expression of CRF, HEK293T cells were plated on glass cover slips, infected with the CRF-OE lentiviruses and 48 h later were fixed and immunostained using specific CRF antiserum. CRF immunoreactivity was clearly detected in all the cells that were infected and showed expression of the GFP reporter (Figure 1c).

CRF is endogenously expressed at the mouse-extended amygdala by neurons of the CeA and the BNST. The anatomical location of CRF expressing neurons at the CeA (Figure 2a; upper panels) and BNSTdl (Figure 2b; upper panels) nuclei is shown by *in situ* hybridization, using a specific cRNA probe of CRF (Figures 2a and b; lower left panels). The CRF-OE lentiviruses were stereotaxically injected into the CeA or BNSTdl of male C57BL/6 mice. The harvested brains were fixed, sliced and immunostained for GFP. Viral infected cells showed strong immunoreactivity to GFP at the specific loci of injection (Figures 2a and b; lower right panels).

Attenuated anxiety-like behavior in response to acute stress among mice subjected to prolonged CRF OE in the CeA
The effects of prolonged CRF OE in the CeA or the BNSTdl, on anxiety-like behavior, were examined by injecting the CRF-OE lentviruses to the CeA or BNSTdl of male C57BL/6 mice. The mice were kept under standard housing conditions for 4 months and then tested for basal anxiety-like behavior and stress-induced anxiety, compared with the mice injected with control (GFP-expressing) lentiviruses. The OF, DLT and elevated plus maze tests that were used in this study, take advantage of the conflict between the mouse exploratory drive and its aversion to open or brightly lit spaces. To estimate anxiety levels, the time spent in the center of the OF or in the light compartment of the DLT, the number of entries into the center/light and their latency were measured. Mice over-expressing CRF in the CeA showed basal anxiety-like behavior, which is similar to their control group as estimated by the time spent in center (Figure 3a; left bars) and their latency to enter the center (Figure 3b; left bars) of the OF test. Yet, their number of entries to the center of the arena was

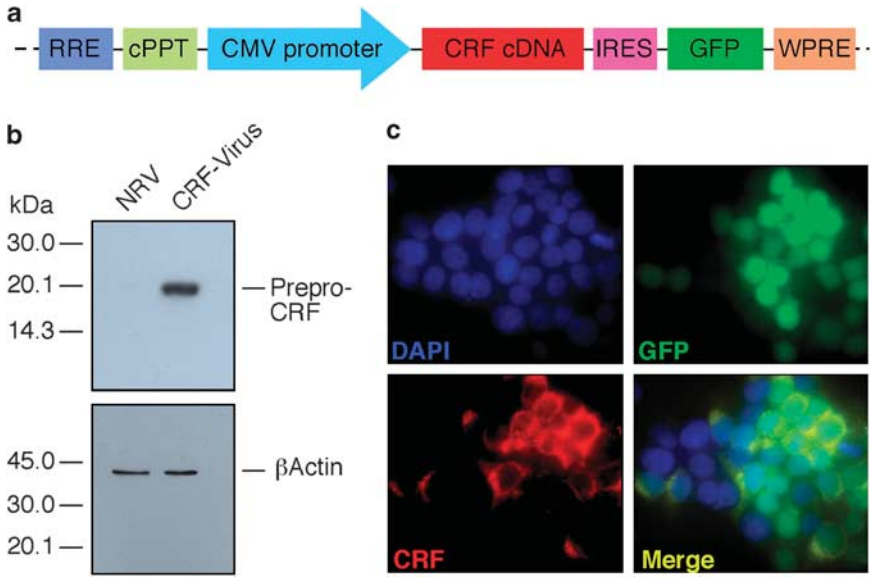


Figure 1 Design and *in vitro* validation of lentiviruses over-expressing CRF. (**a**) Schematic representation of the lentiviral construct designed to over-express rat CRF (rCRF) together with a GFP protein. (**b** and **c**) HEK293T cells were infected with rCRF over-expressing lentiviruses and confirmation of CRF OE was performed using western blot analysis (**b**) and immunocytochemistry (**c**). Cells infected with non-related virus (NRV) were used as control. Abbreviations: cDNA, complementary DNA; CMV, cytomegalovirus.

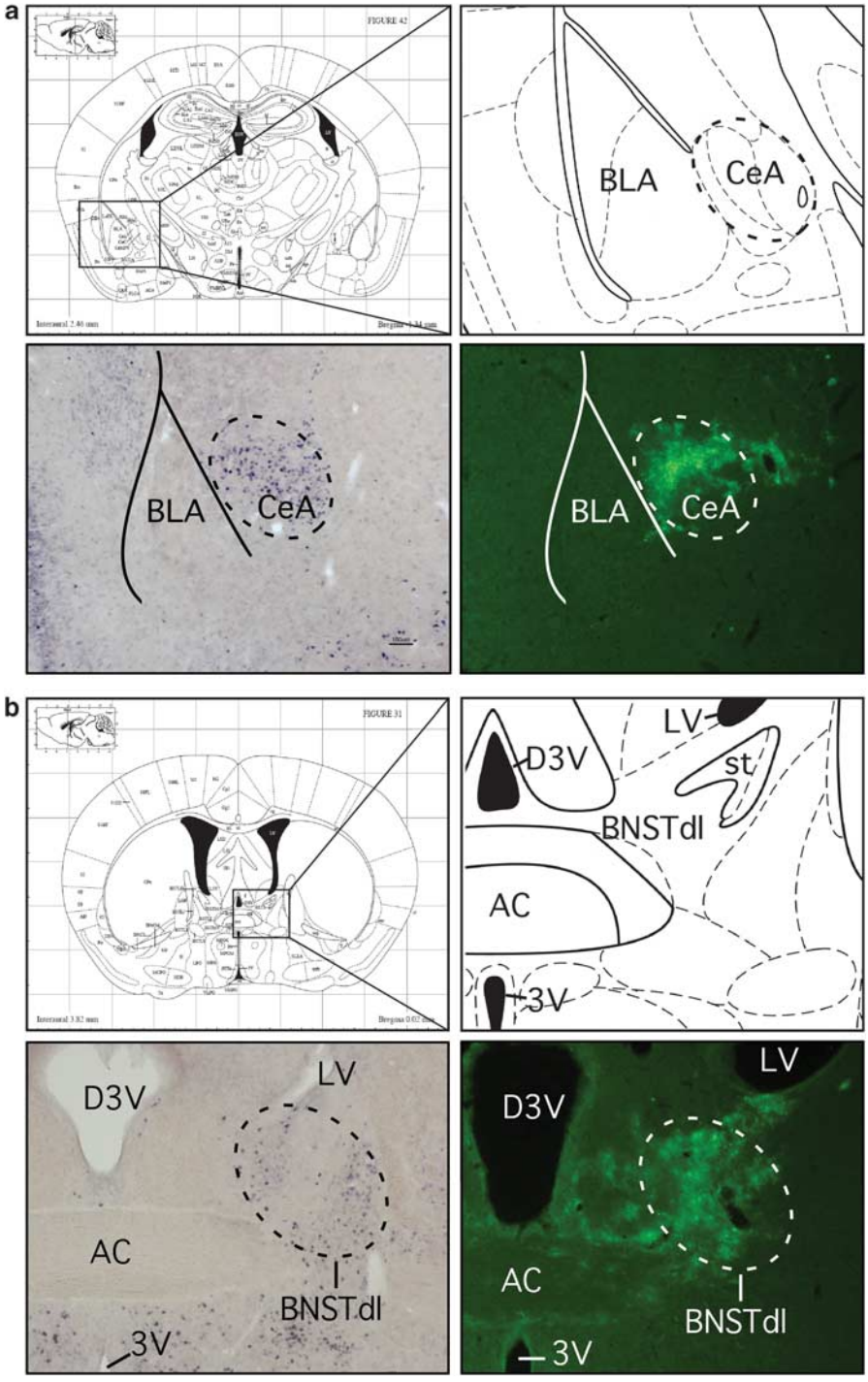


Figure 2 *In vivo* validation of CRF over-expressing lentiviruses injected into the CeA and the BNSTdl brain nuclei. Brain sections adapted from the Paxinos and Franklin mouse brain atlas, showing the sites of CeA (**a**, upper panels) and BNST (**b**, upper panels). Endogenous mRNA of CRF was detected using *in-situ* hybridization at the CeA (**a**, lower left panel) and at the BNSTdl (**b**, lower left panel). Mice injected with CRF over expressing lentiviruses to the CeA (**a**, lower right panel) and at the BNSTdl (**b**, lower right panel) show immunoreactivity to GFP at site of injection. Scale bar = 200 μM. AC, anterior commissure; D3V, dorsal third ventricle; LV, lateral ventricle; 3V, third ventricle.

significantly higher (Figure 3c; left bars; $P \leqslant 0.05$), suggesting lower anxiety-like behavior. After exposure to an acute stressor (30 min of restraint stress), an attenuated stress-induced anxiety level was observed among mice over-expressing CRF in the CeA, as indicated by significantly lower anxiety indices

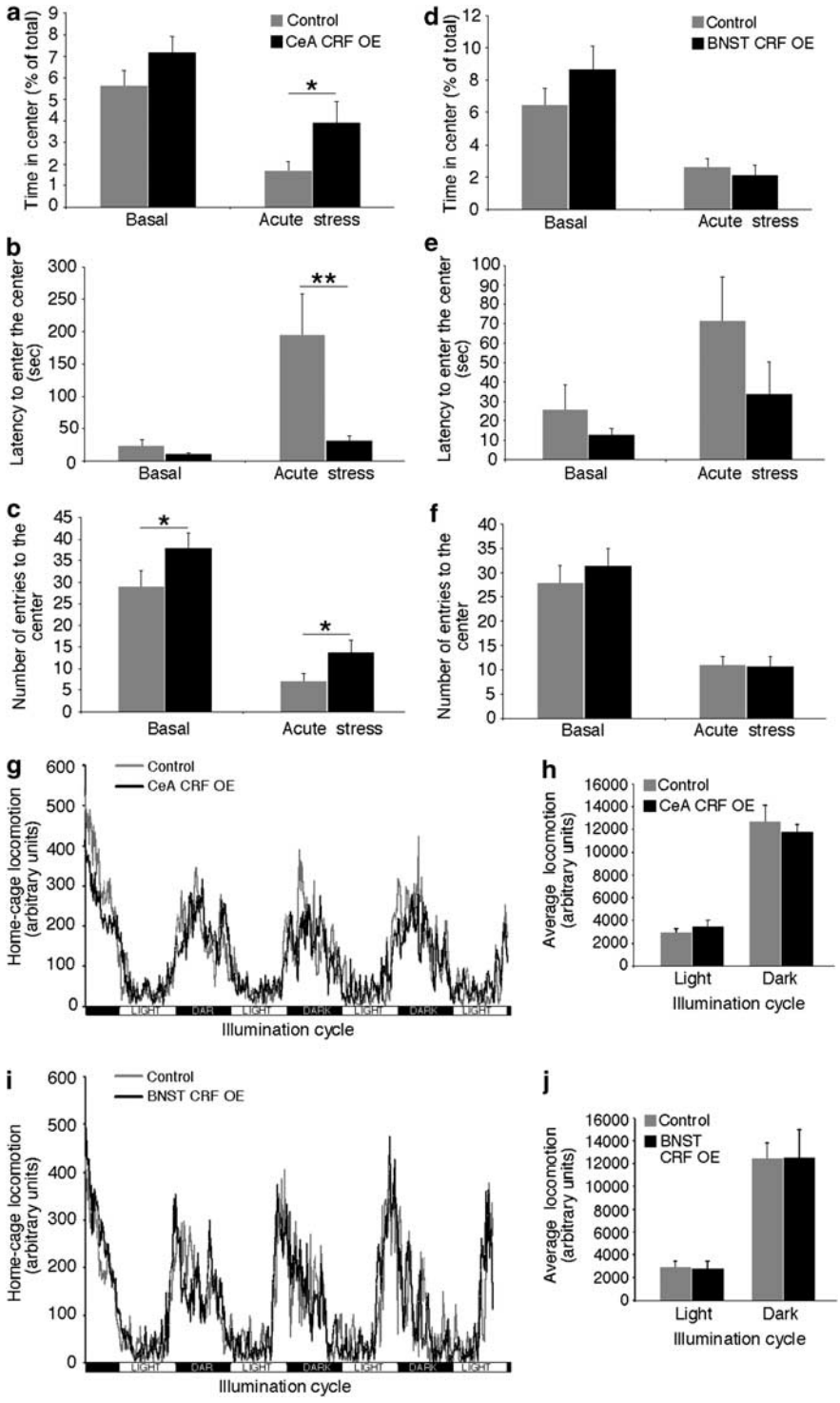


Figure 3 Basal and stress-induced anxiety-like behavior in mice chronically over expressing CRF at CeA or at BNSTdl, as measured by the OF test. (**a–c**) Mice chronically over-expressing CRF at the CeA showed a tendency for spending more time in center of arena (**a**), showed a lower latency for crossing the center (**b**) and entered the center more often (**c**) compared with their control group, when tested under basal conditions. These differences were strongly enhanced following exposure to acute stress (**d–f**). Mice chronically over-expressing CRF at the BNSTdl did not show significant differences as compared with the control group in the above parameters. (**g–j**). Home-cage monitoring of locomotion showed no difference in locomotion of mice expressing CRF at CeA (**g** and **h**) and at BNSTdl (**i** and **j**). Values are expressed as the mean ± s.e.m. ($n = 12$–15 mice per group; $^{**}P < 0.01$, $^{*}P < 0.05$, $^{\#}P < 0.06$).

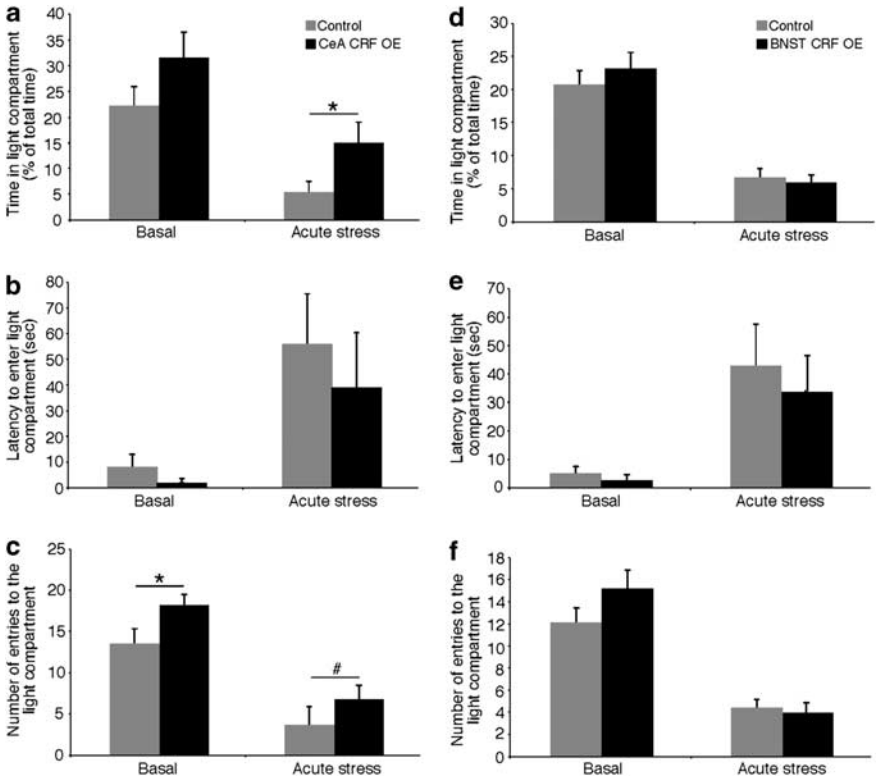


Figure 4 Basal and stress-induced anxiety-like behavior in mice chronically over expressing CRF at CeA or at BNSTdl, as measured by the Dark-Light transfer test. (**a–c**). Mice chronically over expressing CRF at CeA spent more time in light compartment (**a**), showed a lower latency to enter light compartment (**b**) and entered the light compartment more often (**c**), compared with their control group, when tested under basal conditions and following acute stress. (**d–f**). Mice chronically expressing CRF at BNSTdl did not show significant differences as compared with control group in the above parameters. Values are expressed as the mean ± s.e.m. ($n = 12$–15 mice per group; $^{*}P < 0.05$, $^{\#}P < 0.06$).

in both tests (Figures 3a–c; right bars. $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$). At the BNSTdl, prolonged CRF OE did not lead to significant differences in any of the OF anxiety indices, both under basal conditions, nor following exposure to an acute stressor (Figures 3d–f). No differences were observed between CeA CRF-OE or BNSTdl CRF-OE mice and their controls in the home-cage locomotion (Figures 3g–j). In the DLT test, a similar pattern was observed under basal conditions among CeA CRF OE mice. These mice exhibited a tendency to spend more time in the light compartment (Figure 4a; left bars), had a shorter latency for the first visit to the light compartment (Figure 4b left bars) and visited the light compartment significantly more times (Figure 4c; left bars, $P \leqslant 0.05$). After exposure to an acute stressor, these mice spent a significantly longer time in the light compartment (Figure 4a; right bars, $P \leqslant 0.05$) and showed a tendency for shorter latency to the first visit to the light compartment (Figure 4b; right bars) and more visits to the light compartment (Figure 4c; right bars). No differences were observed between CeA CRF-OE or BNSTdl CRF-OE mice and their controls in the elevated plus maze test (Supplementary Figure S1). Collectively, these observations indicate attenuated anxiety-like behavior, in response to acute stress, after prolonged CRF OE in the CeA. Prolonged CRF OE in the BNSTdl did not affect anxiety levels (Figures 4d–f). An additional group of mice was used to assess the effects of prolonged CRF OE in the CeA and in the BNSTdl on ASR, under basal conditions and after acute stress. *t*-test comparisons of the Max ASR index in the different blocks indicated no differences between the mice injected with the control virus and those injected with the CRF OE in either the CeA or the BNSTdl (Supplementary Figure S2). However, *t*-tests comparisons of the RT ASR indicated that prolonged CRF OE in the CeA significantly retarded the response to the startling stimuli in Block 3 under basal conditions ($t_{(15)} = 2.56$; $P = 0.022$) (Figure 5b). Furthermore, prolonged CRF OE in the CeA induced an habituation-like process to the startling stimuli following exposure to stress; Block 3 RT ASR was significantly slower than Block 1 ($t_{(7)} = 3.43$; $P = 0.011$) only among CRF OE mice (Figure 5c). No such differences were observed between BNSTdl injected mice (Figures 5d and e). Thus, the RT ASR data may support the observations in the OF and DLT tests indicating attenuated anxiety among stress-exposed CeA CRF OE mice.

Increase in depressive-like state among mice after prolonged CRF OE in the BNSTdl

Anxiety disorders and depression are often co-occurring, and the onset of depression is frequently observed after a long period of anxiety. To assess the effects of site-specific prolonged expression of CRF at the CeA or BNSTdl on depression-like behavior, we used the FST and the tail suspension tests. These tests consider the lack of motivation, manifested by immobility, as an indicator of a depressive state. Prolonged CRF OE at the CeA did not affect depressive-like indices in the FST (Figures 6a and b). However, prolonged CRF OE in the BNSTdl had a marginal effect, increasing climbing on day 1 of the FST (Figure 6c), whereas significantly reducing swimming time ($P \leqslant 0.01$) and increasing the immobile floating time ($P \leqslant 0.05$) on day 2 of the FST (Figure 6d). These results are suggestive of the increased depressive-like state among mice after prolonged CRF OE in the BNSTdl, but not among mice after prolonged CRF OE at the CeA. To eliminate the possibility that the increased immobility on day 2 observed after prolonged CRF OE in the BNSTdl are a result of altered memory compared with control mice, we used the FC test to assess learning and memory in these mice. Prolonged CRF OE in the BNSTdl did not affect learning or recall of context (Supplementary Figure S3a), nor of cue (Supplementary Figure S3b), in the auditory fear conditioning paradigm. Two-way ANOVA for 'time intervals' (with-in factor with repeated measures), 'virus' (between factor) and the interaction 'time intervals' × 'virus' of the percentage of freezing during all the phases of the FC procedure (habituation, conditioning, context test and cue test) indicated no differences between control mice and CRF-injected mice. Habituation: Two-way ANOVA indicated a significant main effect for 'time intervals' ($F_{(9,135)} = 12.10$; $P = 0.00$) but not for 'virus' ($F_{(1,15)} = 2.08$; $P = 0.17$); the interaction 'time intervals' × 'virus' was also not significant ($F_{(9,135)} = 1.00$; $P = 0.42$). Conditioning: Two-way ANOVA indicated a significant main effect for 'time intervals' ($F_{(9,144)} = 11.91$; $P = 0.00$) but not for 'virus' ($F_{(1,16)} = 0.00$; $P = 0.98$); the interaction 'time intervals' × 'virus' was also not significant ($F_{(9,144)} = 1.38$; $P = 0.24$). Context: Two-way ANOVA indicated a significant main effect for 'time intervals' ($F_{(9,144)} = 5.23$; $P = 0.00$) but not for 'virus' ($F_{(1,16)} = 0.14$; $P = 0.71$); the interaction 'time intervals' × 'virus' was also not significant ($F_{(9,144)} = 0.53$; $P = 0.77$). Cue: Two-way ANOVA indicated a significant main effect for 'time intervals' ($F_{(9,144)} = 43.83$; $P = 0.00$) but not for 'virus' ($F_{(1,16)} = 0.69$; $P = 0.42$); the interaction 'time intervals' × 'virus' was also not significant ($F_{(9,144)} = 0.71$; $P = 0.60$).

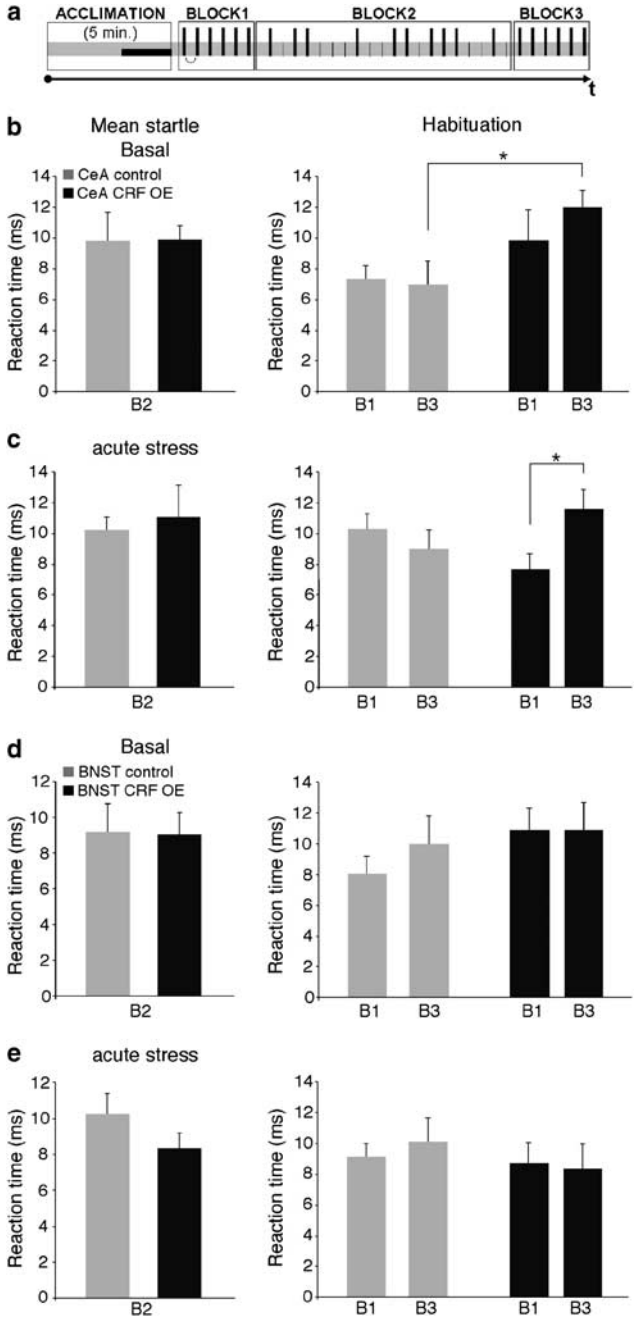


Figure 5 Basal and stress-induced anxiety-like behavior in mice chronically over expressing CRF at CeA or at BNSTdl, as measured by reaction time to acoustic startle. (**a**) A schematic representation of the ASR protocol. Gray vertical bar: background white noise; black horizontal bar: baseline measurement; black vertical lines: startle stimuli. (**b** and **c**) Reaction time to startle stimuli of mice continuously over-expressing CRF in the CeA in basal conditions (**b**), and following acute stress (**c**). Response at block 2 represents mean startle, while comparison between block 3 and block 1 indicates habituation. Extended reaction time of mice over expressing CRF in the CeA compared with controls at block 3 in basal conditions (**b**), and extended reaction time of these mice compared to block 1 following stress suggest lower anxiety than control mice. (**d** and **e**) Prolonged CRF OE in the BNSTdl did not affect reaction time in the ASR in basal conditions (**d**) and following acute stress (**e**). Values are expressed as the mean ± s.e.m. ($n = 8$–9 mice per group; $^*P < 0.05$). (B1: block 1; B2: block 2; B3: block 3).

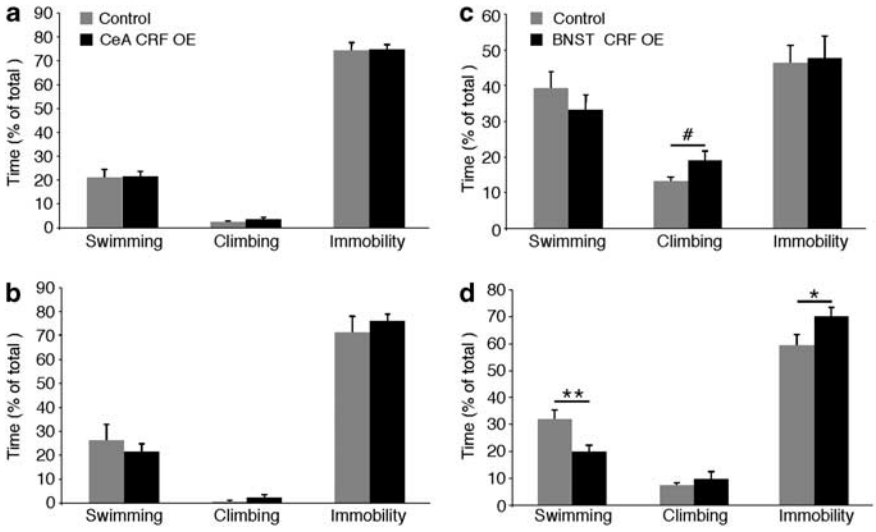


Figure 6 Depression-like behavior in mice chronically over expressing CRF at the CeA or at the BNSTdl brain nuclei. (**a** and **b**). Mice chronically over expressing CRF at the CeA did not show differences in the first day (**a**) or in the second day (**b**) of FST, as compared with their control group. (**c** and **d**). Mice chronically expressing CRF at the BNSTdl showed a tendency for more climbing on first day of the FST (**c**) and spent less time swimming and more time in immobile floating as compared with control mice on day two of the test (**d**). Values are expressed as the mean ± s.e.m. ($n = 12$–15 mice per group. $^{**}P < 0.01$, $^{*}P < 0.05$, $^{\#}P < 0.06$).

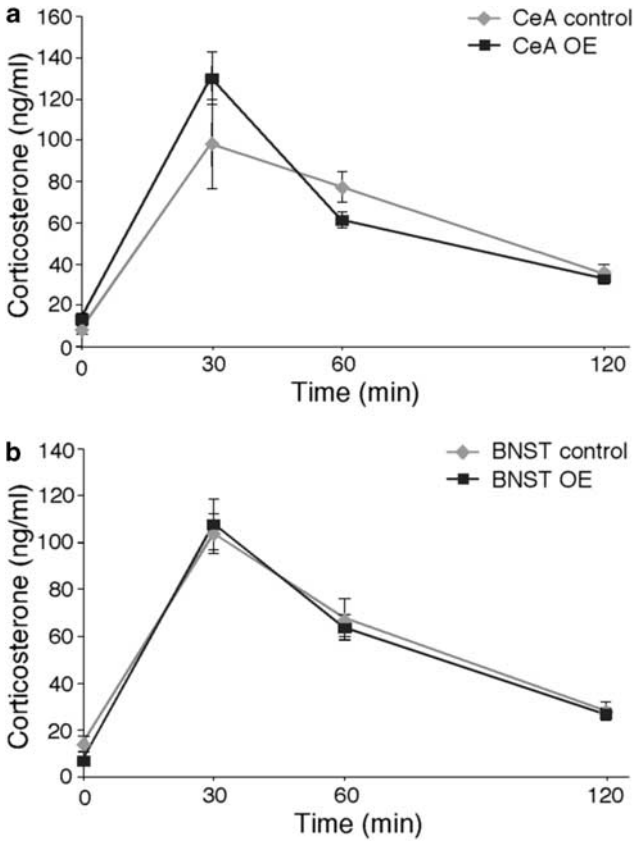


Figure 7 HPA axis is not modified by prolonged CRF OE in the CeA or in the BNSTdl. (**a** and **b**) Plasma corticosterone levels of CeA CRF OE mice (**a**) and of BNSTdl CRF OE (**b**) obtained before or after 30 min of acute stress did not differ from corticosterone levels of control mice in the same condition. Values are expressed as the mean ± s.e.m. ($n = 8$–9 mice per group).

No differences were observed between CeA CRF-OE or BNSTdl CRF-OE mice and their controls in the tail suspension test (Supplementary Figure S4).

Prolonged CRF OE in the CeA or the BNSTdl did not alter HPA axis activity

CRF expressed in the paraventricular nucleus (PVN) of the hypothalamus is known to activate the HPA axis. Although CRF in the CeA and in the BNST do not directly activate this axis, afferents from these nuclei might affect the PVN directly or indirectly, and modulate the neuroendocrine response. To evaluate whether changes in the HPA axis activity can be responsible for the observed behavioral differences between the experimental groups, we determined the corticosterone levels under basal conditions and after acute stress. Prolonged CRF OE in the CeA and in the BNSTdl did not alter the plasma corticosterone concentrations under basal (non-stressed) conditions, and did not have a significant effect on the corticosterone response to restraint stress (Figures 7a and b). Two-way ANOVA for 'time' (following exposure to restraint stress), as a with-in factor (repeated measures), 'virus' (as between factor) and the interaction 'time' × 'virus' of circulating corticosterone levels during the response to the stressor indicated no differences between the control virus and the CRF-injected mice in either the CeA or the BNSTdl. CeA: two-way ANOVA indicated a significant main effect for 'time' ($F_{(3,42)} = 46.56$; $P = 0.00$) but not for 'virus' ($F_{(1,14)} = 0.41$; $P = 0.53$); the interaction 'time'-'virus' was also not significant ($F_{(3,42)} = 2.37$; $P = 0.14$) (Figure 7a). BNSTdl: two-way ANOVA indicated a significant main effect for 'time' ($F_{(3,21)} = 85.83$; $P = 0.00$) but not for 'virus' ($F_{(1,7)} = 0.3.84$; $P = 0.09$);

the interaction 'time' × 'virus' was also not significant ($F_{(3,21)} = 0.16$; $P = 0.89$) (Figure 7b).

Effects of site-specific prolonged expression of CRF on CRFR1 expression

Prolonged exposure to CRF may result in modified levels of the CRF receptor type 1 (CRFR1), which could account for the behavioral changes observed. Several brain loci, known to endogenously express CRFR1 and to be involved in mediating stress-induced behaviors (CeA, BLA, BNST, septum, PVN, medial prefrontal cortex and the hippocampus) were micro-dissected and CRFR1 mRNA levels were determined using real-time PCR. Unexpectedly, we found that after prolonged CRF OE in the CeA, CRFR1 mRNA levels were significantly elevated at the CeA, compared with control mice (Figure 8a; left panel), and a tendency for higher levels of CRFR1 mRNA was detected at the septum (Figure 8c; left panel). CRFR1 mRNA levels in the BNST, PVN, BLA, medial prefrontal cortex and hippocampus were unchanged (Figures 8b and d–g; left panels). Following prolonged CRF OE in the BNSTdl a significant reduction in CRFR1 mRNA levels was observed in the BNST (Figure 8b, center panel), whereas no significant differences were observed in any of the examined other locations (Figures 8a and c–g; center panels).

Discussion

The current study shows that prolonged site-specific CRF OE in the CeA of male mice attenuates anxiety-like behaviors, predominantly after exposure to stressful stimulation; and that such OE in the BNSTdl promotes depressive-like behavior, while not affecting anxiety levels. Although the CRF is known to mediate the activation of the HPA axis, the lack of variance in the corticosterone levels between the test groups in this study rules out HPA as a mediator of the observed results.

Chronic exposure to stressors alters the individual's ability to mount a healthy stress response, through the habituation of the HPA axis and cellular and structural changes in both central and peripheral structures.[13,37] Failure to properly regulate the response to prolonged exposure to stressors ('chronic stress') enhances the risk of stress-related mental illnesses, such as anxiety disorders and depression.[8–13,19]

Studies using animal models of fear and anxiety implicated the CeA and BNST; highly connected brain regions of the extended amygdala, as having key functions in the anxiety-like states and reactions. Each of these structures appeared as the dominant mediator in a different anxiety paradigm,[28–30,38–42] and exposure to chronic stress was shown to cause up-regulation of CRF expression in both the CeA and BNST nuclei.[43,44] Depression was also associated with dysregulation of the CRF system,[8–12] and was shown to follow dysfunction of several brain sites, such as hypothalamus, hippocampus, amygdala, and striatum.[45]

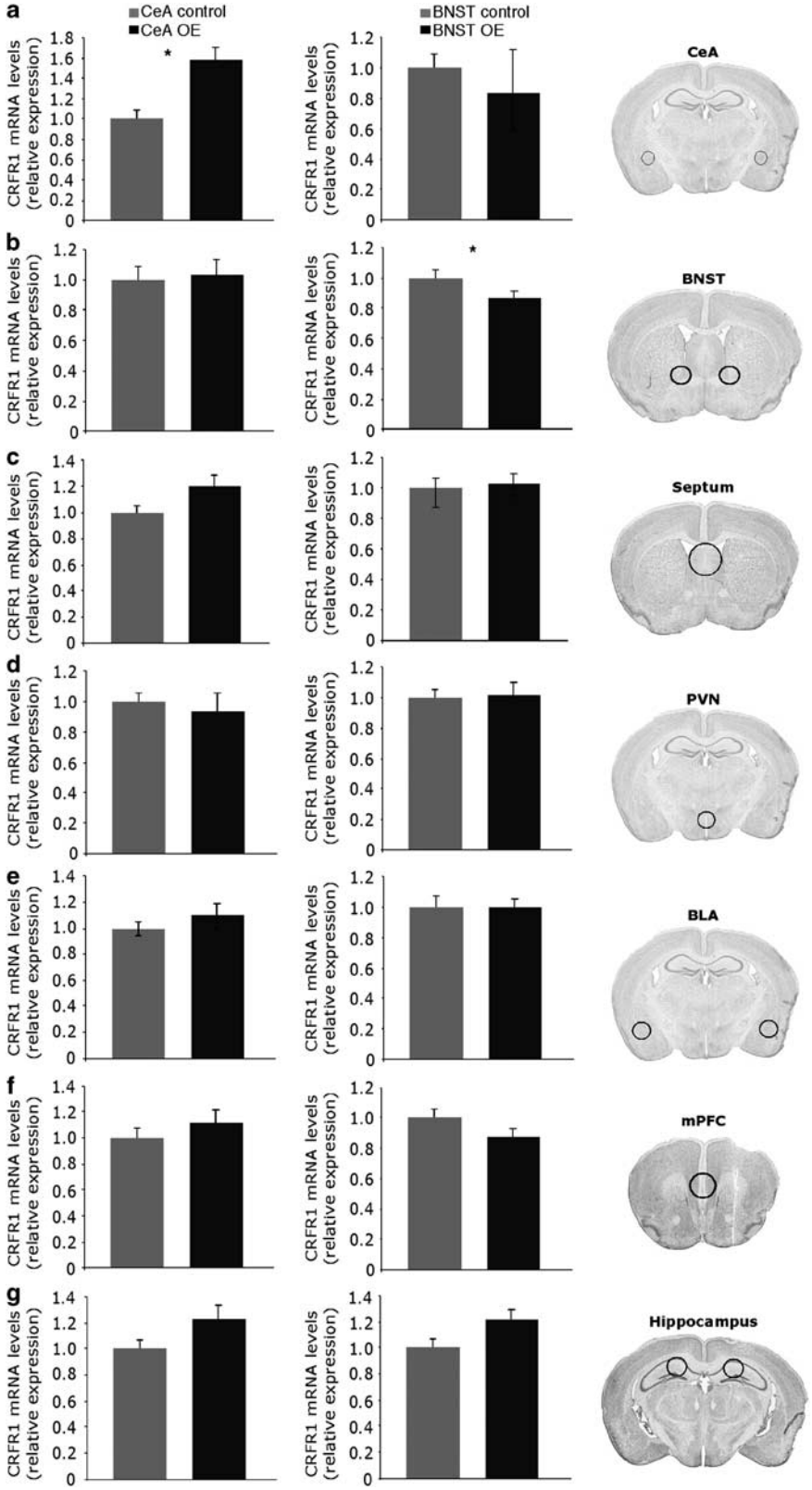
Several transgenic mice lines over-expressing CRF have been established earlier. Stenzel-Poore *et al*.[46,47] produced the first mouse line, broadly over-expressing CRF. In this line, unrestricted OE of CRF resulted in anxiogenic phenotype, elevated adrenocorticotropic hormone and elevated corticosterone levels, accompanied by symptoms of Cushing-like syndrome. A central nervous system-restricted CRF OE mouse model[48,49] showed a hyperactive HPA axis, but with a delayed appearance of symptoms for Cushing-like syndrome. More recently, Lu *et al*.[50] used the Cre/LoxP system generating transgenic mice lines OE CRF in a spatio-temporally regulated manner. Using this method, they showed stress-induced hypersecretion of stress hormones by mice over-expressing CRF in the entire central nervous system using the Nestin-Cre mouse line, but not by mice with CRF OE restricted to forebrain neurons using the Camk2a-Cre mouse line. In addition, they showed that central nervous system-restricted CRF OE promoted increased struggle in the FST and tail suspension test, whereas the forebrain-restricted CRF OE failed to affect behavior in these tests.

Acute administration of CRF peptide specifically into the amygdala resulted in increased anxiety-like behavior.[51] In this study we showed that prolonged CRF OE by the CeA cells resulted in reduced levels of anxiety-like behavior in response to acute stressful stimulation. Unlike pharmacological administration of CRF peptide directly into the CeA, which will result in the activation of amygdalar CRFR1, infecting neuron at the CeA with lentiviruses that overexpress CRF may result in the activation of CRFR1 expressed at the BNST region, through the anatomical connection between these extended amygdala sub-regions. Therefore, comparing the current results with the extensive pharmacological studies carried out earlier, is problematic and should be done with caution.

In addition, the long duration of CRF OE (4 months) raises the possibility that different compensatory

Figure 8 Relative CRFR1 mRNA levels following prolonged CRF OE in the CeA or in the BNSTdl. (**a**–**g**) CRFR1 mRNA levels, relative to hypoxanthine-guanine phosphoribosyltransferase in the CeA (**a**), BNST (**b**), septum (**c**), PVN (**d**), BLA (**e**), medial prefrontal cortex (mPFC) (**f**) and hippocampus (**g**) of mice with prolonged CRF OE in the CeA (left panels) and of mice with prolonged CRF OE in the BNSTdl (center panels). The exact loci taken for assessment are circled on images in the right panels (modified from Paxinos and Franklin's mouse brain atlas). Prolonged CRF OE in the CeA led to a significant increase in CRFR1 mRNA levels in the same nucleus (**a**, left), whereas prolonged CRF OE in the BNSTdl led to a reduced CRFR1 mRNA levels in the BNST (**b**, center). CRFR1 mRNA levels were not affected in all others examined loci. Values are expressed as the mean ± s.e.m. ($n = 8$–9 mice per group).

mechanisms may be involved. Korosi *et al*. have observed down-regulation of CRFR1 mRNA in several brain regions, in mice with central CRF OE, and an up-regulation of CRFR2.[33] In wild-type mice, stress was shown to cause a decrease of CRFR1 mRNA in the frontal cortex of rats, and up-regulation of this



receptor's mRNA in the hippocampus.[52] Here, we have observed an up-regulation of CRFR1 mRNA in the CeA of mice over-expressing CRF at the CeA, and down-regulation of the receptor in the BNST of mice over-expressing the CRF in the BNSTdl. These results do not provide a direct explanation for the observed phenotype; however, a mismatch between mRNA levels and actual receptor availability (binding capability), as shown earlier by Brunson *et al.*,[53] is a possibility.

Several studies have examined the regulation of CRFR1 desensitization and internalization after exposure to CRF.[54–56] A gradual loss of internalized CRFR1 was shown along 24 h of stimulation with CRF, indicating down-regulation of the receptor on prolonged exposure to its ligand.[56] Earlier studies have shown an up-regulation of the CRF-binding protein in the BLA, in response to acute stress [57] and after intracerebroventricular administration of CRF.[58] Further studies, focusing on possible changes in the CRF-binding protein and other relevant monoaminergic systems, known to be involved in mediating stress-linked behaviors, are required to reveal the mechanisms responsible for the observed phenotype.

It is noteworthy that Keen-Rhinehart *et al*.[59] recently reported that female rats continuously expressing CRF at the CeA-exhibited increased ASR, indicating an increase in baseline anxiety. Using the same behavioral test, we did not detect a difference in the ASR measurements between CeA CRF OE mice and controls; however, we observed a reduction in the reaction time to the startle stimuli in CRF OE mice, consistent with the rest of our results. The discrepancy between Keen-Rhinehart *et al.*, and our results may be attributed to the shorter duration of CRF OE, gender differences, species differences and differences in behavioral indices.

In this study, no significant differences in anxiety-like behavior were observed after prolonged OE of CRF in the BNSTdl. Several studies have compared the role of CeA and BNST in mediating anxiety-like behavior. Using lesions and chemical inactivation of CeA, BLA or BNST, it was suggested that the BNST, and not the CeA, is the primary mediator of anxiety.[28–30] Testing for light-enhanced startle after the inactivation of the different brain areas, Walker and Davis[29] reported that while inactivation of BLA or the BNST significantly decreased light enhancement of the startle response, inactivation of the CeA had no effect. However, when tested using the fear-potentiated startle, the inactivation of the CeA or the BLA, but not of the BNST, blocked this potentiation. Furthermore, lesions to the BNST, but not to amygdala, blocked CRF-enhanced startle after CRF central administration. As fear-potentiated startle is considered to be a paradigm representing fear, while light-enhanced and CRF-enhanced startle were suggested to relate more closely to anxiety, these findings suggested that the BNST responds to signals more akin to anxiety, whereas the CeA is involved in fear, and perhaps less in anxiety.[29] The OF, the DLT and the ASR tests assess anxiety-like behaviors. Interestingly, our results show decreased stress-induced anxiety after prolonged CRF OE in the CeA but not in the BNSTdl. Pharmacological administration of CRF (locally or intracerebroventricular) and site-specific CRF OE, which was used in this study, are fundamentally different. CRF expressed by infected neurons acts not only locally, but may activate neurons in other loci through the CRFR1. Projections of CRF-expressing neurons from the CeA reach BNST, and can activate CRFR1 located at this nucleus.[60–62] Prolonged CRF OE at the CeA is therefore suggested to affect CRFR1 regulation in the BNST, potentially leading to the attenuation of anxiety and to stress-induced anxiety reported in this study. CRF at high levels in the CeA are likely to defuse to the BLA.[63] In addition, virus infections in this study were not restricted to CRF-expressing neurons. Infected cells within the CeA or the BNSTdl could therefore have released CRF in the brain regions not normally innervated by these nuclei (e.g. the septum), and therefore activate receptors located in these regions. Although CRF binds CRFR2 at a lower affinity than to CRFR1, we cannot exclude the possibility that CRFR2 was activated in such brain regions. Similar experiments using the CRFR1 and CRFR2 knockout mice could provide further insight into the involvement of these two receptors in mediating the observed phenotype.

In the FST, mice with prolonged OE of CRF in the BNSTdl spent significantly more time floating immobile and less time swimming than control mice. Such behavior is thought to represent a depression-like state. Chronic stress is closely linked with depression;[64] however, the mechanism and circuitry underlying depression onset are complex and varied (for review, see ref. 65). Our results show that prolonged OE of CRF in the BNSTdl, but not in the CeA, is sufficient to promote depressive-like behavior. These findings complement the results of several other studies. Stout *et al*.[44] applied the chronic mild stress paradigm for the induction of the anhedonic symptoms of depression in rats, which were accompanied by a significant increase in CRF peptide levels in the BNST, but not in the amygdala. Furthermore, no significant changes in plasma ACTH and corticosterone levels were detected, suggesting an extra-HPA axis mechanism underlying the observed effects. In accordance with this study, Kim *et al*.[66] reported an increase in CRF mRNA levels in the dorsal BNST following a similar paradigm, and no effect on CRF mRNA levels in ventral BNST, CeA or the PVN of the hypothalamus. Although projections from the BNST do reach the PVN,[67] no differences in HPA axis activity were observed in this study, suggesting that the increased depressive-like behavior reported in this study was not because of the subsequent activation of the HPA axis.

To conclude, this study showed the different effects of prolonged CRF OE in two distinct regions of the

extended amygdala. Infecting discrete brain nuclei with lentiviruses resulted in prolonged and site-specific CRF OE. These two different CRF OE models produced distinct effects on behaviors, suggesting separate roles for CRF, expressed by different loci, in the regulation of the central stress response and potentially in the underlying mechanisms of mental disorders. Understanding the contribution of a single gene product, expressed by a specific brain region, to the brain response to challenge, will assist us in dissecting this complex brain response and may pave the way for novel approaches for the treatment of a variety of stress-related psychiatric disorders.

Conflict of interest

The authors declare no conflict of interest

Acknowledgments

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