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ORIGINAL ARTICLE

The anxiolytic effect of environmental enrichment is mediated via amygdalar CRF receptor type 1

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Environmental enrichment (EE) is known to have an anxiolytic effect in several animal models; however, the molecular mechanisms underlying these behavioral changes are not understood. In this study, we have shown that the anxiolytic effect of EE is associated with alterations in the corticotropin-releasing factor receptor type 1 (CRFR1) expression levels in the limbic system. We found that the decrease in anxiety-like behavior after housing in enriched conditions was associated with very low levels of CRFR1 mRNA expression in the basolateral amygdala of C57BL/6 mice. We further showed using a lentiviral-based system of RNA interference, that knockdown of CRFR1 mRNA expression in the basolateral amygdala induces a significant decrease in anxiety levels, similar to those achieved by EE nurture. Our data strongly suggest that reduced expression of CRFR1 mRNA levels in the basolateral amygdala mediates the effect of EE on anxiety-like behavior.
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Keywords: anxiety; CRF receptor type 1; environmental enrichment; amygdala; CRFR1 knockdown; lentiviruses

Introduction

Anxiety disorders are considered to be the most prevalent class of mental disorders in the United States,[1] and Europe.[2] However, the precise mechanisms leading to anxiety disorders are not completely understood. Much evidence indicates that the complex balance between environmental contingencies and genetic factors have a crucial role in the development of stress-related psychopathologies.

Research has increasingly indicated that environmental stimulation is beneficial for the physiological and psychological well-being of the animals. Animals maintained under enriched conditions have been shown to express reduced levels of emotionality-related measurements such as defecation and freezing, less stereotyped behaviors and reduced anxiety levels.[3] An enriched environment is a combination of complex inanimate and social stimulation[4] and generally consists of housing conditions that facilitate enhanced sensory, cognitive, motor and social stimulation relative to standard housing conditions (SC) that severely restrict natural behavior and the animal's control over its environment. Recent studies have shown that investigations of gene–environment interactions might reveal molecular targets for the development of therapeutic agents that mimic or enhance the beneficial effects of environmental stimulation.[5,6] Although the beneficial effects of environmental enrichment (EE) on the general psychological and physical well-being of the animals are well documented, the molecular mechanisms underlying the anxiolytic effect of EE are not understood.

Among the various peptide and neurotransmitter systems that have been implicated in the regulation of stress, the corticotropin-releasing factor (CRF) system has a critical role in initiating the cascade of biological events during the stress response.[7–9] Dysregulation of the stress response can have severe psychological and physiological consequences[10,11] and chronic hyperactivation of the CRF system has been linked to stress-related emotional disorders such as anorexia nervosa, depression and anxiety.[10–17] In addition to CRF, the mammalian CRF-peptide family contains urocortin 1, urocortin 2 and urocortin 3. The effects of the CRF-peptide family are mediated through activation of two known receptors, CRFR1 and CRFR2.[18] Data from experiments using animal models showing anxiogenic-like behavioral effects of CRF administration and anxiolytic-like activity of CRFR1-selective antagonists led to the suggestion that CRF may be involved in the pathophysiology of anxiety-related disorders.[10–17] In addition, mice deficient in CRFR1 show decreased anxiety-like behavior and have an impaired neuroendocrine stress response, showing the requirement of CRFR1 for both the behavioral and neuroendocrine responses to

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stress.[19,20] It is noteworthy that the anxiolytic phenotype of a limbic-specific conditional CRFR1 knockout mouse line suggests that CRFR1 modulates anxiety-like behavior independently of the hypothalamic-pituitary-adrenal system.[21] Given the above considerations, we hypothesized that changes in limbic CRFR1 expression may have a crucial role in mediating the anxiolytic effect of EE.

In this study, we show that the anxiolytic effect of EE is correlated with a significant downregulation of CRFR1 mRNA expression in the basolateral complex of the amygdala (BLA), a limbic CRFR1 expressing structure[22,23] that has been implicated as a major anatomical site for the regulation of anxiety-like states and reactions.[24–27] To genetically mimic the anxiolytic effect of EE, we designed and constructed a lentiviral vector expressing small interfering RNA targeted against the CRFR1 mRNA (lenti-siCRFR1), and used it for specific *in vivo* knockdown of BLA CRFR1 of adult mice. We have shown that similar to the EE effect, knockdown of CRFR1 mRNA in the BLA significantly reduced anxiety-like behavior.

Materials and methods

Animals

Adult female C57BL/6J mice (Harlan Laboratories, Jerusalem, Israel) were used for the EE experiments. Adult male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, Maine, USA) were used for lentiviral stereotaxic injections. Throughout the experiments, the animals were maintained in a temperature-controlled room (22 °C ± 1) on a reverse 12 light–dark cycle. 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.

Environmental enrichment

At the age of weaning (4 weeks), mice were randomly distributed into two different environmental conditions. Half of the group was housed (12 mice per cage) in a stimuli enriched cage (the enriched environment) and the other half was housed (4 per cage) in standard condition cages without any stimulation. The EE cages consist of a 85 × 75 × 20 cm transparent perspex box containing the following enrichment items: polyvinyl chloride tubes; running wheels; Nestlets (Ancare, Bellmore, NY, USA), as a nesting material; the Mouse House (Tecniplast, Buguggiate, Varese, Italy), a red transparent perspex nest box; the Refuge (Ottoenvironmental, Milwaukee, WI, USA), a paper-based nest box; the 'Mplex' (Ottoenvironmental) a red transparent polycarbonate hammock; wood blocks; and plastic toys. Some enrichment items such as the running wheel were not removed, and other items such as the nesting material and the wood blocks were replaced once a week. The order of the items was changed twice a week to increase the sense of novelty. The housing density of the EE cages is 1.8 mouse dm^{-2}. The standard condition cages consist of a 36.5 × 20.7 × 14 cm, polysulfone box (Tecniplast), with three or four animals per cage, with bedding material, food and water *ad libitum*, without any enrichment items. The housing density of the SC cages is 5.3 mouse dm^{-2}. The bedding material used in both the EE and standard condition cages was Teklad Sani Chips (Harlan Laboratories).

Behavioral studies

All behavioral studies were performed during the dark period (07:00–19:00). Mice were habituated to the test room for 2 h before any test. For the assessment of anxiety-like behavior, the open field, the light–dark transfer and the elevated plus maze tests were used, in this order. Two recovery days were given between the tests. The battery of anxiety-like behavior tests was performed within the same group of mice. The parameters measured in the three tests were quantified using an automated video tracking system (VideoMot2; TSE Systems GmbH, Bad Homburg, Germany). For the assessment of general locomotor activity, mice were single housed in standard cages and their activity was tracked during 3 consecutive days by an infrared-based automated system (Infra-Mot; TSE Systems GmbH). The open field consists of a plexiglas box (50 × 50 × 22 cm). The arena was illuminated with 120-lx. 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 number of entries to the center, the latency to enter the center and the total distance traveled were measured. The light–dark transfer test consists of a polyvinyl chloride box divided into a black dark compartment (14 × 27 × 26 cm) and a white 1050-lx illuminated light compartment (30 × 27 × 26 cm) connected by a small passage. Mice were placed in the dark compartment to initiate a 5-min test session. The time spent in the light compartment and the number of entries to the light compartment were measured. The elevated plus maze apparatus consists of a gray polyvinyl chloride maze, comprising a central part (5 × 5 cm), 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-lx. Mice were placed in the center, facing an open arm to initiate a 5-min session test. The time spent in the open arms, the number of entries to the open arms and the number of entries to the close arms were measured.

Corticosterone assay

Tail blood samples from SC and EE mice were collected before (basal), immediately after 15 min of restraint stress, and 30, 60 and 90 min from stress initiation. The restraint stress was induced using a cut 50-ml plastic conical tube. Plasma samples were immediately centrifuged and stored at −20 °C until assays for hormone measurement were conducted. Corticosterone was quantified with specific corticosterone antibody kindly provided

by Dr Kenyon and Dr Al-dujaili (Endocrinology Unit, Centre for Cardiovascular Science, The Queen's Medical Research Institute, Edinburgh) using the enzyme-linked immunosorbent assay method.

Microdissection and RNA preparation

The BLA and the bed nucleus of the stria terminalis (BNST) were microdissected, using the Palkovits technique as described earlier,[28] for the quantification of CRFR1 mRNA levels. Immediately after the decapitation, the brain was removed and placed into a 1-mm metal matrix (Stoelting Co., Wood Dale, IL, USA, cat# 51386). The brain was sliced using standard razor blades (GEM, Personna American Safety Razor Co., Cedar Knolls, NJ, USA; cat# 62–0165) into 2-mm slices that were quickly frozen on dry ice. The amygdala and BNST were punched using a microdissecting needle of an appropriate size and stored in −80 °C. RNA extraction was made using PerfectPure RNA Tissue kit (5 PRIME GmbH, Hamburg, Germany). Extracted RNA was treated with DNAse to avoid false-positive results caused by DNA contamination. The RNA samples were reverse transcribed to generate complementary DNA pools that were later used as templates for semiquantitative and quantitative real-time PCR analysis.

Semiquantitative and quantitative reverse transcriptase (RT)-PCR

The complementary DNA products were used as templates for semiquantitative and quantitative RT-PCR analysis using specific primers for CRFR1, the ribosomal protein S16, and hypoxanthine phosphoribosyl-transferase. In the semiquantitative RT-PCR, the expression of ribosomal protein S16 served as internal control. The PCR conditions were as follows: complementary DNA equivalent to 50 ng of total RNA was amplified by PCR for 40 cycles at an annealing temperature of 58 °C. The final $MgCl_2$ concentration was 2 mM, and each reaction contained 0.5 U of Taq DNA polymerase (Hy Labs, Rehovot, Israel). In the quantitative real-time PCR the expression of hypoxanthine phosphoribosyl-transferase served as internal control. The real-time PCR reaction was performed in a Rotor-Gene 6000 thermocycler (Corbett Research, Sydney, Australia) using fluorescent SYBR Green technology (ABgene, Blenheim Road, UK). The PCR conditions were as follows: complementary DNA equivalent to 10 ng of total RNA was amplified by PCR for 45 cycles at an annealing temperature of 61 °C. Each quantitative PCR reaction contained 10 µl 2× SYBR Green Mastermix, and final primer concentration of 250 nM. The specificity of the amplification products was verified by melting curve analysis.

Oligonucleotide primers

Sense and antisense primers were selected, when possible, to be located on different exons to avoid false-positive results caused by DNA contamination. The specific primers were designed using Primer Express software (PE Applied Biosystems, Perkin Elmer, Foster City, CA, USA). The following primers were used in the semiquantitative PCR reactions: for CRFR1 5′-TGCCAGGAGATTCTCAACGAA-3′ (sense) and 5′-AAAGCCGAGATGAGGTTCCAG-3′ (antisense) corresponding to nucleotides 495–515 and 656–676, respectively; for S16 5′-TGCGGTGTGGAGCTCG TGCTTGT-3′ (sense) and 5′-GCTACCAGGCCTTTGAG ATGGA-3′ (antisense). The following primers were used in the quantitative real-time PCR reactions: for CRFR1 we used the same primer as in semiquantitative PCR; for hypoxanthine phosphoribosyl-transferase 5′-GCAGTACAGCCCCAAAATGG-3′ (sense) and 5′-GGTCCTTTTCACCAGCAAGCT-3′ (antisense) corresponding to nucleotides 599–618 and 630–650, respectively.

Lenti-shCRFR1 design and production

Four different short hairpin RNA (shRNA) target sequences from the open reading frame of mouse CRFR1 were designed using http://sfold.wadsworth.org software and cloned into shRNA expression cassettes driven by the H1 promoter in the p156RRLsinPPTCMV-GFP-PREU3Nhe lentiviral construct (kindly provided by Dr Inder Verma, the Salk Institute for biological studies). The recombinant pseudotyped lentiviral vectors were generated by cotransfection of four plasmids into HEK293T cells, as described earlier.[29] The shRNA constructs were generated by synthesis of four different 83-mer oligonucleotides containing a 5′ end containing a unique XbaI restriction site, a stretch of five adenosines as a template for the Pol III promoter termination signal, a 19-nucleotide sense and antisense strands, separated by a 9 nucleotide loop and 20 nucleotides complimentary to the 3′ end of the Pol III H1 promoter. The following shRNA oligonucleotides were used (in italics the sense and antisense strands; in bold the nine nucleotide loop):

shCRFR1 #1: 5′-CTGTCTAGACAAAAA*TGAAGGC CCTTCTCCTTCT***TCTCTTGAA** *AGAAGGAGAAGGGCCTTCA*GGGGATCTGTGGTC TCATACA-3′

shCRFR1 #2: 5′-CTGTCTAGACAAAAA*CCACCTCC CTCCAGGATCA***TCTCTTGAA** *TGATCCTGGAGGGAGGTGG*GGGGATCTGTGGTC TCATACA-3′

shCRFR1 #3: 5′-CTGTCTAGACAAAAA*GCTACAA CACCACAAACAA***TCTCTTGAA** *TTGTTTGTGGTGTTGTAGC*GGGGATCTGTGGTCT CATACA-3′

shCRFR1 #4: 5′-CTGTCTAGACAAAAA*TCTTTCTC TTCAACATTGT***TCTCTTGAA** *ACAATGTTGAAGAGAAAGA*GGGGATCTGTGGTC TCATACA-3′

Validation of lenti-shCRFR1 constructs

The ability of lenti-shCRFR1 vectors to knockdown the CRFR1 expression was assessed by western blot analysis. HEK293 T cells infected with the four different shRNA viruses or with a non-related virus

as a negative control, were transfected with 100 ng myc-tagged CRFR1 expression vector per 35-mm well plates using polyethylenimine (Sigma-Aldrich, St Louis, MO, USA). Forty hours after transfection, cells were washed and lysed in a buffer containing: 20 mM Tris (pH-8), 137 mM NaCl, 2.7 mM KCl, 10% glycerol, 1% NP-40, 1 mM phenylmethylsulphonyl fluoride, 1 mM dithiothreitol, 0.5 μg ml^{-1} aprotinin and 1 μg ml^{-1} leupeptin. The lysate was gently mixed for 15 min in ice and centrifuged at 14 000 × *g* for 15 min at 4 °C. Protein concentration was determined using Bradford reagent (Bio-Rad, Richmond, WI, USA). Samples were boiled for 5 min, and proteins were electrophoresed on 10% SDS-polyacrylamide gel (Bio-Rad), and then transferred onto nitrocellulose membranes. Membrane was then blocked with 5% skim milk powder in Tris-buffered saline Tween 20 (TBST) (10 mM Tris pH-8.0, 150 mM NaCl, 0.05% Tween 20), probed with antibodies specific for myc raised in mouse (Abcam, Cambridge, UK), washed with TBST, and incubated with peroxidase labeled anti-mouse antibody (Amersham Biotech Amersham Pharmacia, Piscataway, NJ, USA). Immunoreactive proteins were visualized using Super Signal West Pico Chemiluminescent substrate (Pierce, Rockford, IL, USA) and developed using RX Fuji film (Fuji, Dusselsorf, Germany). Membrane was stripped by incubating in stripping buffer containing 100 mM β-mecaptoethanol, 2% SDS in 62.5 mM Tris-HCl pH-6.8 for 30 min at 50 °C and reprobed with antibodies directed against the protein kinase PAK, raised in rabbit (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The relative protein level was determined using the NIH Image 1.63 densitometry software (http://rsb.info.nih.gov/ij/).

The ability of the lenti-shCRFR1 vectors to attenuate the CRFR1 functionality was assessed by measuring the CRF-induced cyclic adenosine monophosphate (cAMP) signaling. HEK293T cells infected with the different shRNA viruses or with a non-related virus as a negative control were used for cyclic AMP responsive element (CRE) activation studies. Cells were plated in 60-mm well plates and transfected with 0.13 μg CRFR1 expression vector, 0.78 μg luciferase (*luc*) reporter containing a fragment of the *EVX1* gene, which contains a potent CRE site (kindly provided by Dr Marc Montminy, The Salk Institute), and 32 ng renilla luciferase (*ruc*) under the promoter of elf2. Twenty-four hours after transfection cells were harvested using trypsin and replated in a 96-wells plate. Forty hours after transfection cells were treated for 4 h with vehicle or with different concentrations (6.25–1000 pM) of CRF. The cells were harvested and the luciferase reporter activity was assayed as follows: cells were washed with phosphate-buffered saline and lysed by resuspending in 100 μl of 100 mM KPO_4 buffer (pH-7.8) containing 1 mM dithiothreitol and 0.5% (vol vol^{-1}) Triton X-100. The lysate was divided in two: half was used to measure CRE-luciferase activity and the other half to measure elf2-luciferase activity. The *luc* assay buffer contains 100 mM Tris-acetate (pH-7.8), 10 mM MgOAc, 100 mM ethylene-diaminetetraacetic acid, 2 mM adenosine triphosphate (pH-7.0) and 74 μM luciferin. The *ruc* assay buffer contains 100 mM KPO_4 (pH-7.5), 100 mM NaCl, 1 mM ethylene-diaminetetraacetic acid and 1.43 μM coelenterazine (BIOSYNTH AG, Staad, Switzerland). Luminescence was measured in a luminometer (Veritas, Turner BioSystems Sunnyvale, CA, USA) for 5 s immediately after addition of luciferin assay buffer. Transfections were performed at least three times (in 4–6 replicates) for each construct or treatment tested. To correct for variations in transfection efficiencies, luciferase activities were normalized to renilla activity.

Intracerebral injections of lentiviral vectors

Twenty-four adult (8 weeks) C57BL/6J male mice (Jackson Laboratory) received bilateral stereotaxic injections of lentivirus to the BLA (1 μl lentivirus each side, 0.3 μl min^{-1}). Twelve mice received the lenti-shCRFR1 and 12 mice the control virus, which consist of the same lentiviral construct containing a scramble non-relevant shRNA sequence, which ensure that the cellular microRNA machinery will be activated to provide the appropriate control.

Preliminary studies were performed to examine the green fluorescent protein (GFP) distribution in mice injected with 0.5, 1.0 or 2.0 μl of lentiviruses into the BLA. These studies have shown that for this brain structure and using our viral preparation, 1 μl volume would give the desirable spread. However, using this methodology, individual differences in the distribution between mice can be observed, including infected neurons, which are extending the borders of the defined anatomical structures.

Mice were anesthetized with a mix of 25% Ketamine; 25% Xylazine; 10% Acepromazine in saline solution, and placed on a computer-guided sterotaxic instrument (Angle Two Stereotaxic Instrument, myNeurolab, Leica Microsystems Inc., Bannockburn, IL, USA), which is fully integrated with the Franklin and Paxinos mouse brain atlas through a control panel. The lentiviral vectors were delivered using a Hamilton syringe connected to a motorized nanoinjector. To allow diffusion of the solution into the brain tissue, the needle was left for an additional 2 min after the injection (coordinates, relative to bregma: AP = −1.34 mm, L = ± 3.75 mm, H = −5.5 mm, based on a calibration study indicating these coordinates as leading to the BLA in C57BL/6 strain).

The mice were allowed to recover from the surgery for a period of 2 weeks and then assessed for anxiety-like behavior. They were then anesthetized with chloral hydrate (1.4 gr kg^{-1} body weight, intraperitoneal) and transcardially perfused with 10 ml phosphate-buffered saline followed by 100 ml 4% parafolmadehyde (PFA) in borate buffer, pH 9.5. The brains were removed and postfixed overnight in 4% PFA + 30% sucrose at 4 °C. Free-floating 30 μm coronal BLA sections were collected on a microtome (Leica Microsystems GmbH, Wetzlar,

Germany) and stored in phosphate-buffered saline at 4 °C until use.

Immunohistochemistry
For GFP immunostaining, we used a biotinylated anti-GFP antibody raised in rabbit as primary antibody (Abcam) and streptavidin Cy2 conjugated as secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA, USA).

Data analysis
Behavioral parameters and the levels of CRFR1 mRNA expression in brain tissues quantified by real-time PCR were analyzed by two-tailed Student's *t*-test. Plasma concentrations of corticosterone were analyzed by two-factorial multivariate analysis of variance with repeated-measures, with times of blood sampling considered as within-subjects factors, and enriched and standard housing as between-subjects factors. Contrast analysis was used to test the locus of significant main effects and interactions when present. The results are presented as means ± s.e.m.

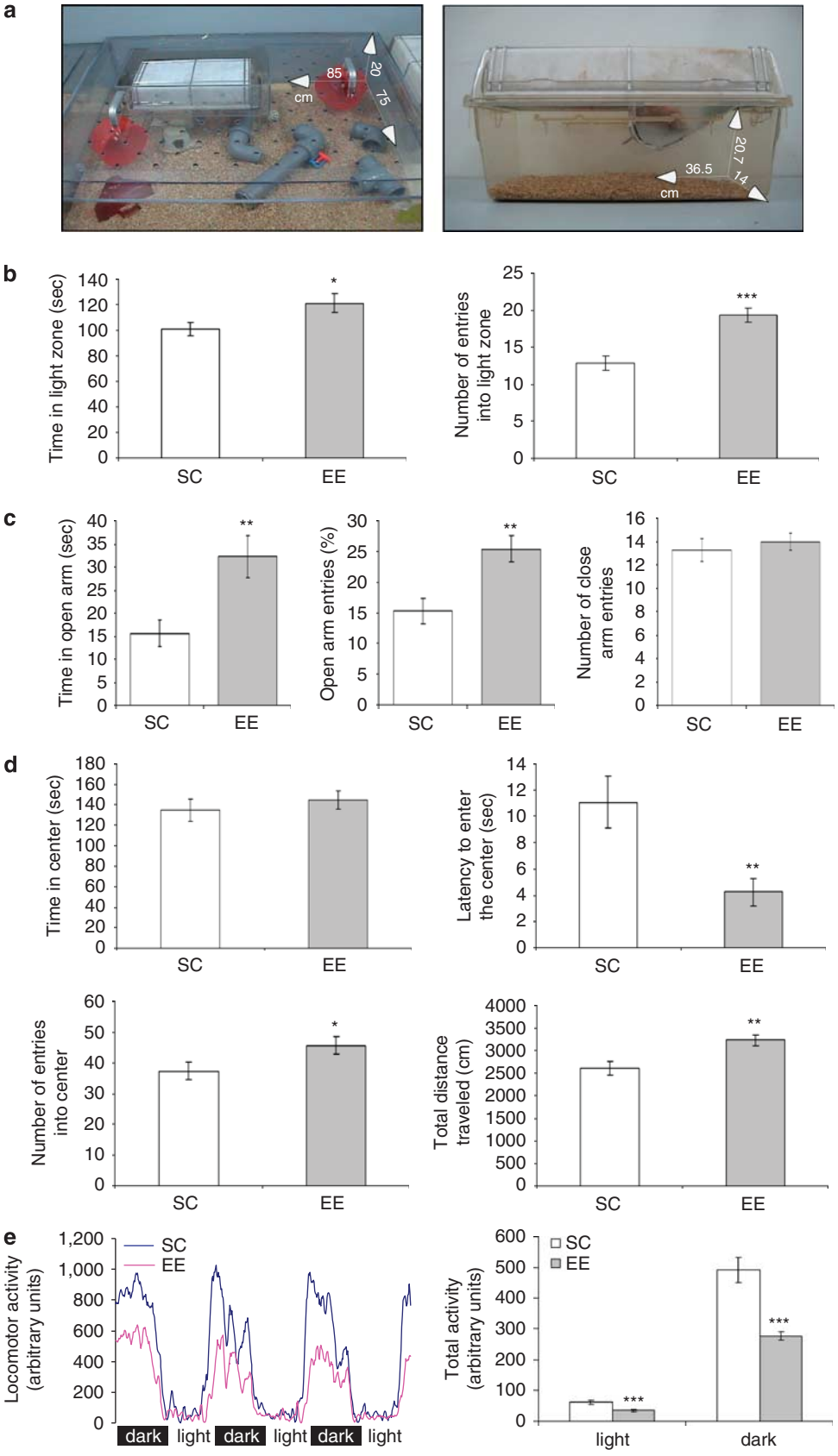
Results

Reduced anxiety-like behavior in mice exposed to EE
To examine the effect of EE on anxiety-like behavior, animals housed for 6 weeks in specially designed enriched cages or SC (Figure 1a), were tested in three different anxiety paradigms based on the natural avoidance behavior of mice: the light–dark transfer, the elevated plus maze and the open field tests ($n = 12$ per group). In the light–dark transfer test, EE mice spent significantly more time in the light zone ($P = 0.039$) and showed a significant increase in the number of entries to the light zone ($P < 0.001$, Figure 1b), compared with SC mice. In the elevated plus maze test, EE mice spent significantly more time in the open arms ($P = 0.006$) and had significantly higher percentage of entries into the open arms ($P = 0.002$, Figure 1c). No difference in the number of entries to the close arms, an internal measure of locomotor activity, was found between the groups. In the open field test, EE mice showed a significant decrease in the latency to enter the center of the arena ($P = 0.007$), and a significant increase in the number of entries to the center ($P = 0.05$) and the total distance traveled in the arena, a parameter of exploratory behavior ($P = 0.003$, Figure 1d). However, no difference in the time spent in the center of the arena was found between the groups. To assess home cage locomotion, general locomotor activity was determined during 3 consecutive days using an automated system. EE mice showed a significantly decrease in locomotor activity (Figure 1e), both in the light and the dark phase of the day, thus ruling out the possibility that the increase in exploratory behavior results from an effect of the housing conditions on locomotor activity.

Modified corticosterone profile in mice kept in EE conditions
Basal and stress-induced corticosterone levels were measured in plasma of EE ($n = 12$) and SC ($n = 12$) mice (Figures 2a and b). A two-factorial multivariate analysis of variance (repeated-measures) indicated a significant main effect of time ($F_{4,88} = 22.48$, $P < 0.001$), and a significant 'time × housing' interaction ($F_{4,88} = 11.53$, $P < 0.001$); No significant effect for housing condition ($F_{1,22} = 0.01$, $P < 0.915$) was found. Further *t*-test comparisons revealed that EE mice have lower resting levels of plasma corticosterone ($P < 0.001$, Figure 2a) and an attenuated corticosterone response, 15 min ($P \leqslant 0.001$) and 30 min ($P < 0.01$) after restrain stress, relative to SC mice (Figure 2b). Interestingly, although plasma corticosterone levels of SC mice returned to basal levels 90 min after stress initiation, the EE mice plasma corticosterone remained significantly elevated (Figure 2b).

Low CRFR1 mRNA expression in the BLA of EE mice
To test our hypothesis that changes in limbic CRFR1 expression have a crucial role in mediating the anxiolytic effect of EE, we assessed the CRFR1 mRNA levels in the BLA and the BNST, two limbic CRFR1-expressing nuclei associated with anxiety-like behavior.[22] The BLA and the BNST of 12 EE and 12 SC mice that were housed in the different conditions for a period of 6 weeks, were microdissected (Figures 3a and d), and pooled into two groups of six mice for each treatment. A densitometry analysis of semiquantitative RT-PCR revealed a marked decrease in CRFR1 mRNA expression levels in the BLA of EE mice compared to SC mice (Figure 3b), whereas in the BNST were only slightly decreased (Figure 3e). The semiquantitative RT-PCR results were confirmed by two additional experiments, in which the microdissected BLA and BNST of each mouse were not pooled,

Figure 1 Environmental enrichment (EE) reduces anxiety-like behavior. (**a**) Special cage for the enriched housing of mice compared with a standard cage. (**b**) EE mice showed significantly reduced anxiety-like behavior in the light–dark transfer test. EE mice spent more time in the light zone and showed an increase in the number of entries to the light zone compared with standard housing condition (SC) mice. The anxiolytic effect of EE could be confirmed in the elevated plus maze and the open field tests. (**c**) In the elevated plus maze test, EE mice spent more time in the open arms and showed an increase in the percentage of entries to the open arms. No difference was found in the number of entries to the close arms. (**d**) In the open field, EE mice show a shorter latency to enter the center of the arena, an increase in the number of entries to the center and an increase in the total distance traveled a parameter of exploratory behavior. No difference was found in the time spent in the center of the arena between the groups. (**e**) EE mice showed a decrease in general locomotor activity, measured during 3 consecutive days, both in the light and the dark phase of the day. Bars represent mean ± s.e.m., $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.


a
85
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Time in light zone (sec)
Number of entries into light zone
SC
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Time in open arm (sec)
Open arm entries (%)
Number of close arm entries
d
Time in center (sec)
Latency to enter the center (sec)
Number of entries into center
Total distance traveled (cm)
e
Locomotor activity (arbitrary units)
SC
EE
dark
light
Total activity (arbitrary units)
light
dark

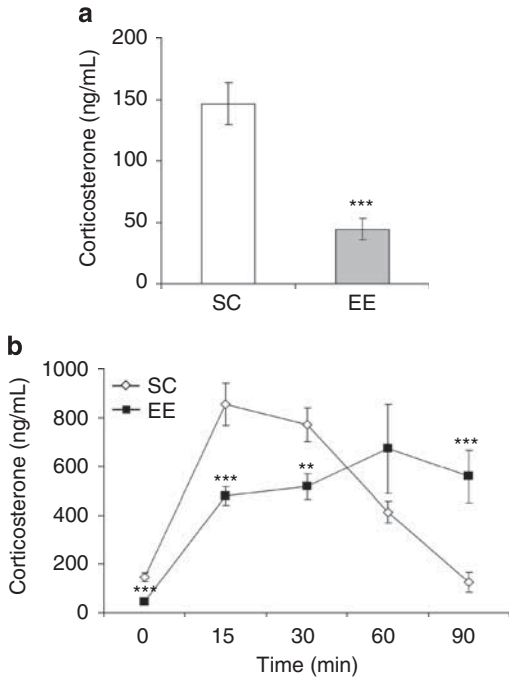

Figure 2 Modified corticosterone profile in mice kept in environmental enrichment (EE) conditions. (**a, b**) EE mice showed a decrease in basal corticosterone level (**a**) and a lower response 15 and 30 min after acute stress (**b**). However, although corticosterone levels of standard housing condition (SC) mice returned to basal levels 90 min after stress initiation, the EE mice corticosterone remained significantly elevated (**b**). Bars represent mean ± s.e.m., $^{**}P<0.01$; $^{***}P<0.001$.

but individually analyzed by quantitative real-time RT-PCR ($n=12$). One group of mice was housed in the different conditions for a period of 4 weeks and the other group for a period of 10 weeks. The analysis of the quantitative real-time PCR revealed that the CRFR1 mRNA expression was significantly reduced in the BLA of the EE group compared with their SC controls, both in the 4 and the 10 weeks EE protocols (Figure 3c). In contrast, the CRFR1 mRNA expression decrease in the BNST of the EE group could be confirmed only in the 10 weeks EE protocol (Figure 3f).

Establishment of lentiviral-based system for site-specific knockdown of CRFR1

To genetically mimic the anxiolytic effect of environmental stimulation, a lentiviral-based system expressing small interfering RNA against CRFR1 was designed and constructed to knockdown CRFR1 in the BLA. Four different shRNA target sequences from the open reading frame of the mouse *CRFR1* gene were designed and synthesized. shCRFR1 expression cassettes driven by the H1 promoter were constructed, and cloned into lentiviral vectors (Figure 4a). To confirm the ability of the shCRFR1 vectors to knockdown CRFR1 expression and to select the most efficient lentiviral construct, HEK293T cells were infected with lentiviruses expressing the different shRNAs, thus generating a stable cell line for each shCRFR1 expressing construct. These stable cell lines were transfected with plasmids expressing mouse CRFR1 carrying a myc tag, which was constructed for this purpose. Determining the levels of CRFR1 protein by western blot revealed that all four shCRFR1 showed reduced levels of CRFR1 expression compared with cells infected with a non-related virus (Figure 4b). The most efficient shCRFR1 vectors were shCRFR1#2 and shCRFR1#4 reducing CRFR1 protein expression by approximately 80%. To further determine whether knockdown of CRFR1 mRNA and protein expression is also reflected in decreased functionality of the receptor, we studied the activation of cAMP signaling in infected HEK293T cells. Cells with a stable expression of shCRFR1#2 and 4 were transfected with a EVX1–CRE–luciferase construct and luciferase levels, as a function of cAMP signaling activation, were measured after 4-h treatment with different concentrations of CRF (Figure 4c). Both shCRFR1#2 and shCRFR1#4 showed significant inhibition of cAMP signaling, when compared with cells infected with a non-related virus. The shCRFR1#4 construct was further used for *in vivo* knockdown of CRFR1. To confirm the *in vivo* knockdown of CRFR1, four mice were bilaterally injected to the BLA with lenti-siCRFR1 or control virus. The mice were allowed to recover from the surgery for a period of 6 days. Then the BLA of each mouse was microdissected and the *in vivo* knockdown of the CRFR1 mRNA expression was confirmed by semiquantitative RT-PCR analysis (Figure 4e).

BLA-specific CRFR1 knockdown reduces anxiety-like behavior

High-titer lentiviruses expressing siCRFR1 and GFP control viruses, were bilaterally injected to the BLA of C57BL/6 mice ($n=12$ each group). As the lentiviral construct also contains the enhanced green fluorescent protein (eGFP) reporter, we could confirm the level and distribution of the shRNA expression by immunofluorescence staining using an anti-GFP antibody (Figure 4d). To evaluate the effect of BLA–CRFR1 knockdown on anxiety-like behavior, we used the same behavioral paradigms used in the different housing conditions experiment (Figure 1). In the light–dark transfer test, mice injected with lenti-siCRFR1 (KD mice) spent less time in the light compartment ($P=0.038$), and showed less entries to the light ($P=0.021$) (Figure 5a), compared with mice injected with control viruses (CV). In the elevated plus maze test, KD mice spent more time in the open arms, although this difference did not reach statistical significance ($P=0.15$; Figure 5b). No significant difference was found in the percentage of open arm entries and the number of entries to the closed arms, between the groups. In the open field test, KD mice spent more time in the center of the arena ($P=0.041$) and showed more visits to the center ($P=0.032$). However, no difference in the latency to enter the center of the arena and the total distance traveled

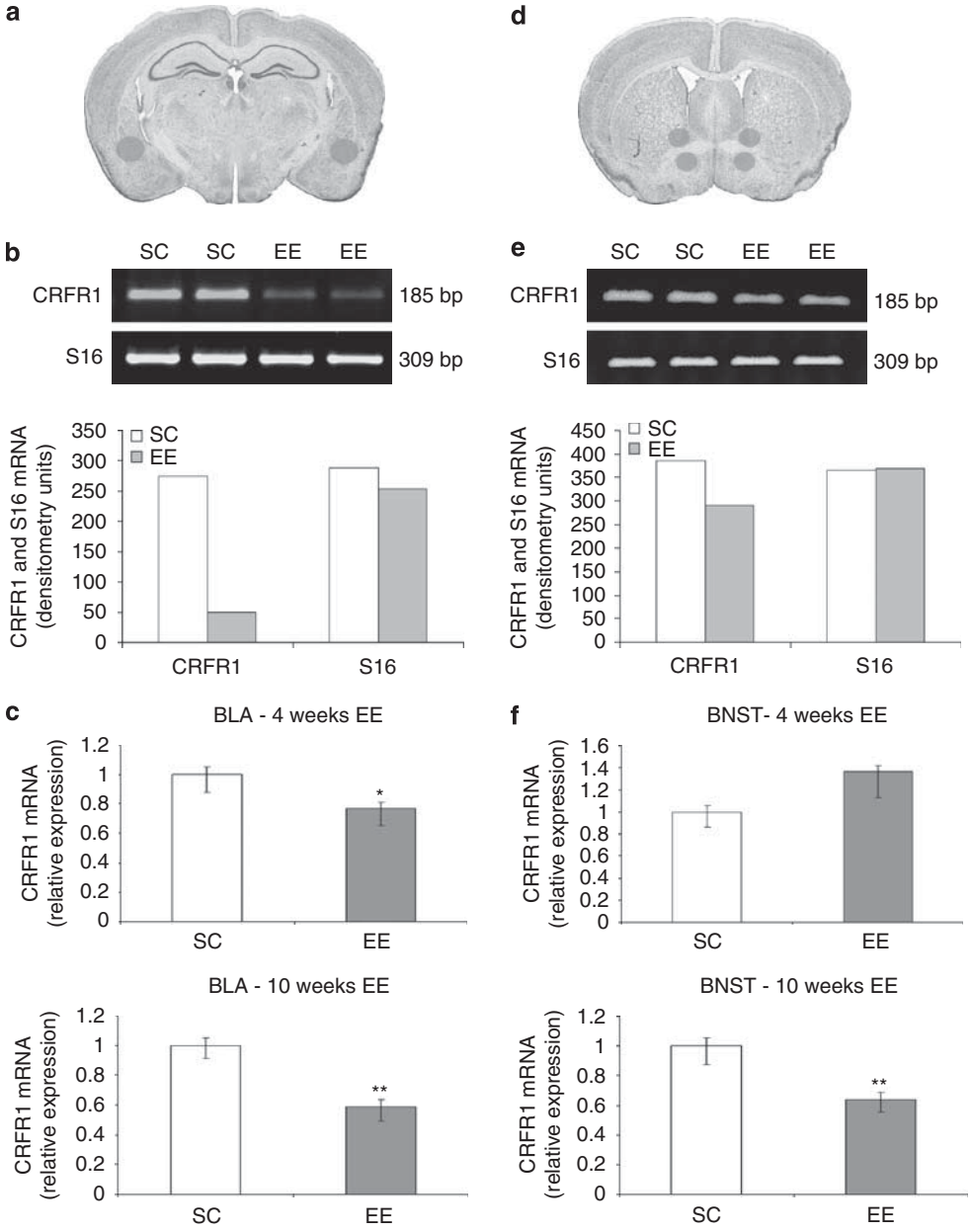

Figure 3 Low corticotropin-releasing factor receptor type 1 (CRFR1) mRNA expression in the basolateral complex of the amygdala (BLA) and bed nucleus of the stria terminalis (BNST) of environmental enrichment (EE) mice. (**a, d**) Brain sections adapted from the Paxinos & Franklin mouse brain atlas, showing the sites of BLA (**a**) and BNST (**d**) microdissection. (**b, e**) Semiquantitative RT-PCR and densitometry analysis of the CRFR1 mRNA expression in the BLA (**b**) and the BNST (**e**). Each band represents a pool of bilateral microdissected nuclei from six mice. (**c, f**) Quantitative real-time RT-PCR analysis of the CRFR1 mRNA expression in the BLA (**c**) and the BNST (**f**) obtained from mice kept for 4 or 10 weeks on EE protocol. Bars represent mean ± s.e.m., $*P<0.05$; $**P<0.01$. SC, standard housing condition.

were found between the groups (Figure 5c). Two consecutive days of monitoring general home cage locomotor activity showed no significant differences in locomotion between the groups, neither in the light nor the dark phases (Figure 5d).

Discussion

Understanding the molecular mechanisms underlying the anxiolytic effect of EE may improve the ability to design therapeutic interventions for affective and stress-related disorders. In this study, we have shown that the anxiolytic effect of EE is correlated with a marked downregulation of CRFR1 mRNA expression in the BLA of adult mice. We further show that lentiviral-mediated small interfering RNA knockdown of CRFR1 in the BLA has a similar anxiolytic effect.

EE has repeatedly been shown to have an anxiolytic effect in different tests measuring confrontation with novel environments. Animals reared in an enriched environment show less anxiety-like behavior, as

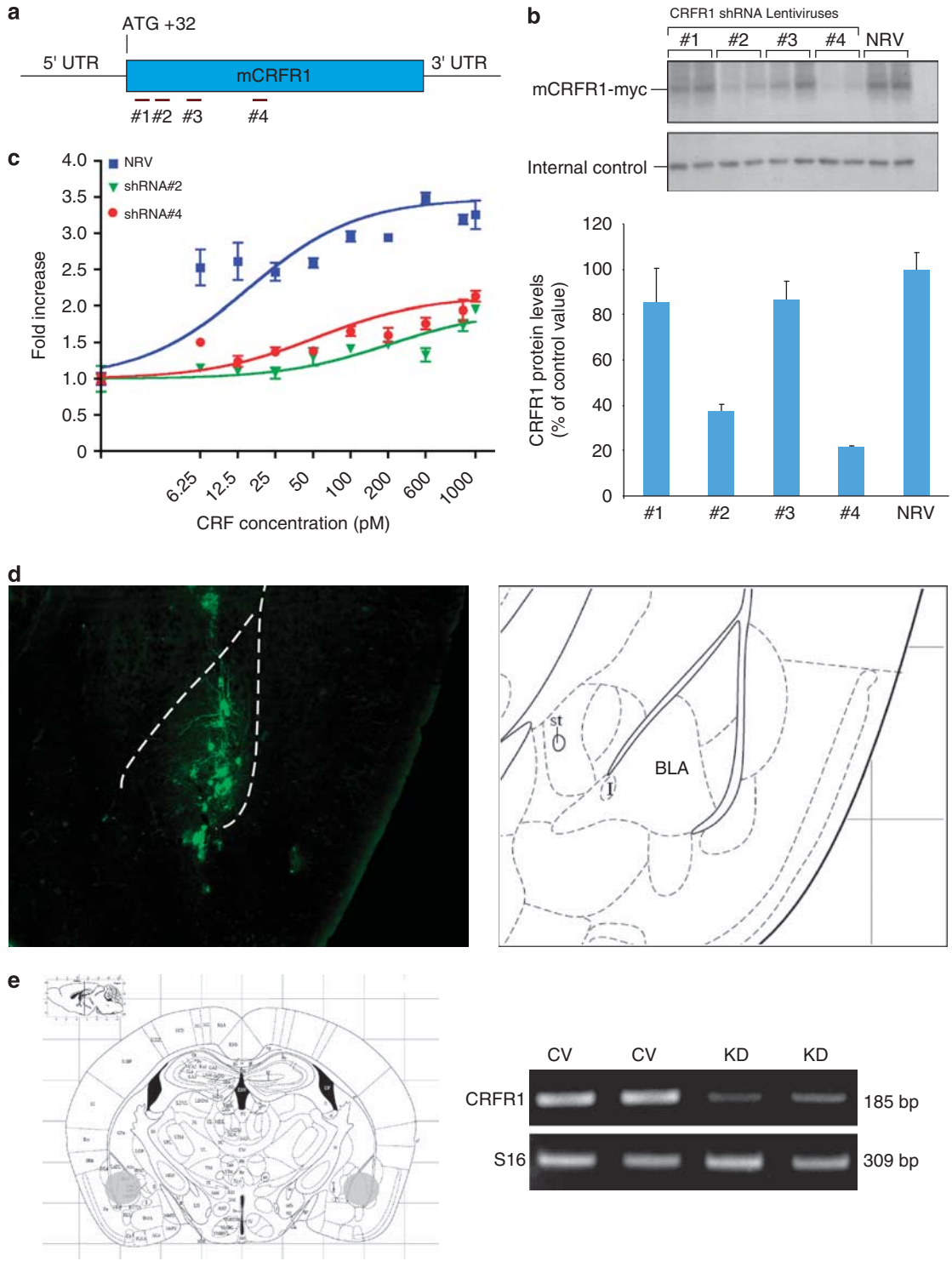

Figure 4 Lentiviral-based system for site-specific knockdown of basolateral complex of the amygdala (BLA)–corticotropin-releasing factor receptor type 1 (CRFR1). (**a**) Four different short hairpin RNA (shRNA) target sequences from the open reading frame of the mouse CRFR1 gene were designed. (**b**) Western blot analysis followed by densitometry measurements show the ability of viruses #2 and #4 to reduce levels of CRFR1 expression in CRFR1 HEK293T transfected cells. (**c**) The ability of viruses #2 and #4 to decrease the functionality of the receptor was confirmed by assessing cyclic adenosine monophosphate signaling activation, after 4-h treatment with different concentrations of CRF. (**d**) Green fluorescent protein immunostaining showing the specific delivery of lenti-siCRFR1 (KD) to the BLA. (**e**) Brain section showing the site of BLA microdissection and semiquantitative RT-PCR analysis showing the *in vivo* confirmation of the CRFR1 knockdown. CV, control virus.

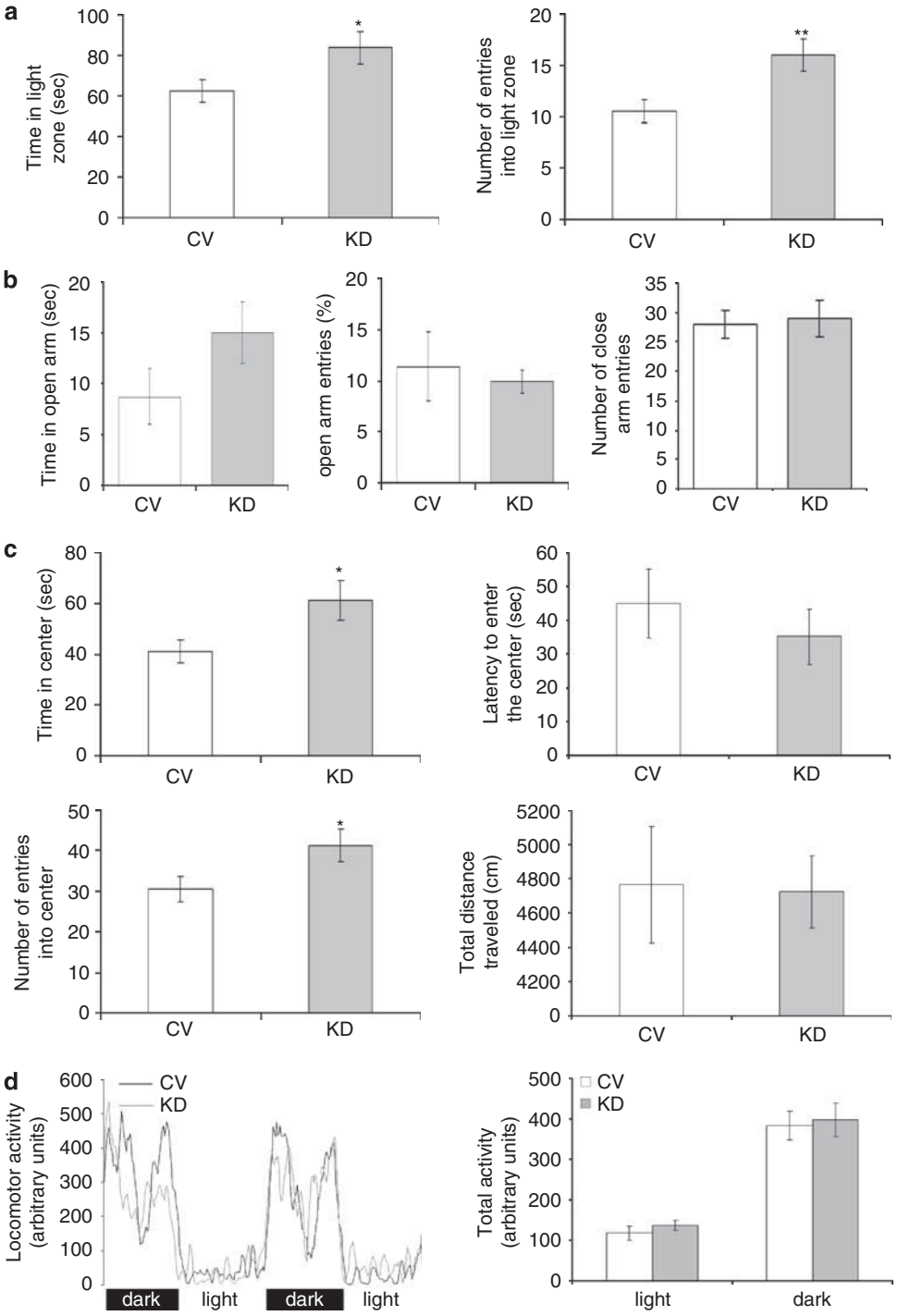

Figure 5 Knockdown of corticotropin-releasing factor receptor type 1 (CRFR1) in the basolateral complex of the amygdala (BLA) reduces anxiety-like behavior. After bilateral stereotaxic injection of lenti-siCRFR1 (KD) to the BLA, mice were tested in a battery of three tests for anxiety-like behavior. (**a**) In the light–dark transfer test, KD mice spent more time in the light zone and showed an increase in the number of entries to the light zone when compared with control virus (CV) mice. (**b**) In the elevated plus maze, KD mice showed a tendency to spend more time in the open arms. However, this difference did not reach statistical significance. No difference was found in the percentage of entries to the open arms and the number of entries to the closed arms, between the groups. (**c**) In the open field, KD mice spent more time in the center of the arena, and showed an increase in the number of entries to the center of the arena. However, no difference was found in the latency to enter the center and the total distance traveled between the groups. (**d**) General locomotor activity as measured during 2 consecutive days. No differences were found in the total activity between the groups, neither in the light nor the dark phase of the day. Bars represent mean ± s.e.m., $*P<0.05$; $**P<0.01$.

indicated by an increase in the number of open arm entries and the time spent in open arms of the elevated plus maze.[30–32] The anxiolytic effect of EE was also shown by a factor analysis using the open field test.[33] We first confirmed the anxiety-reduced phenotype of EE mice in three classical behavioral paradigms based on the natural avoidance behavior of mice, the light–dark transfer, the elevated plus maze, and the open field tests. In the light–dark transfer test, EE mice spent more time in the light zone and showed more entries to the light zone. In the elevated plus maze test, EE mice spent significantly more time in the open arms and had a higher percentage of entries into the open arms than the SC mice. In the open field test, EE mice showed a decrease in the latency to enter the center of the arena, an increase in the number of entries to the center of the arena and an increase in the total distance traveled; a measure of exploratory behavior. Overall, these findings reinforce the previously reported[30–33] anxiolytic effect of environmental stimulation. In addition, EE and SC mice were assessed for general home cage locomotor activity during 3 consecutive days using an automated system. Interestingly, EE mice showed a significant decrease in locomotor activity both in the light and the dark phase of the day. Therefore, we can rule out the possibility that the observed anxiolytic effect is a result of an increase in locomotor activity. The decrease in locomotor activity could possibly result from a reduction in stereotyped behaviors observed in EE mice.[34]

The effect of EE on stress can be also assessed by measuring stress hormone levels. To date, no consensus on the effect of EE on resting plasma corticosterone levels is available. Although some studies have reported that EE decreased basal corticosterone levels,[35] others have reported no difference[36–38] or an increase in basal corticosterone levels.[32,39] However, the majority of the studies concur that EE attenuates the hypothalamic-pituitary-adrenal response to acute stress[32,36] and is able to reverse the effect of past stressors on the hypothalamic-pituitary-adrenal reactivity.[37,38] In our study, EE decreased the basal and the stress-induced plasma corticosterone levels. However, although plasma corticosterone levels of SC mice returned to basal levels 90 min after stress initiation, the EE mice plasma corticosterone remained significantly elevated. Interestingly, in a previous study, the corticosterone levels of CRFR1 conditional knockout mice in which CRFR1 function is inactivated in the limbic system, remained significantly elevated 30 and 90 min after acute immobilization stress, suggesting that limbic CRFR1 is required for central control of hypothalamic-pituitary-adrenal-system feedback and hormonal adaptation to stress.[19] It is possible that the elevated level of corticosterone we measured 90 min after acute stress in EE mice, is a result of an impaired glucocorticoid negative feedback because of the EE-induced downregulation of CRFR1 mRNA expression in the amygdala, in which the glucocorticoid receptors and the CRF receptors co-express[40] and interact.[41] The elevated levels of corticosterone 90 min after exposure to acute stress can also reflect an EE-induced adaptation to stressful situations, as was shown that the treatment with a single high dose of corticosterone 1 h after exposure to psychogenic stress reduces the prevalence rate of posttraumatic stress disorder-like behavioral responses.[42]

The molecular mechanisms underlying the effects of EE have been largely studied in the context of learning, memory and neurological disorders.[43] Regarding the anxiolytic effects of EE, it has been suggested that the EE-induced increase in glucocorticoid receptors in the hippocampus[44,45] could enhance glucocorticoid sensitivity leading to an increase in the negative feedback loop from the hippocampus to the hypothalamus, suppressing the release of CRF.[46] However, one study has examined the effect of EE on *CRF* gene expression in the paraventricular nucleus of the hypothalamus and found no significant effect.[37] In this study, we found that EE significantly reduced the CRFR1 mRNA expression in the BLA, suggesting a novel molecular mechanism that could explain the anxiolytic effect of EE. Our finding is in accordance with a previous study showing that chronic administration of the anxiolytic benzodiazepine alprazolam, decreased CRFR1 mRNA expression in the BLA.[47] Our semiquantitative PCR analysis revealed that CRFR1 mRNA was also slightly downregulated in the BNST, supporting a role for the extended amygdala CRFR1 in mediating this effect. We further confirmed this result by real-time PCR, only when the mice were housed in EE conditions for a period of 10 weeks but not in the 4 weeks protocol. Although our efforts to specifically isolate the BLA and the BNST using the Palkovits technique,[28] the microdissected tissues could probably include additional surrounding structures that may express CRFR1, such as the lateral aspects of the central amygdala and the caudate-putamen, respectively.

Several behavioral and anatomical studies have shown that the BLA is an important site for CRF-induced anxiety-related physiological and behavioral responses. Injections of CRF or Urocortin 1 into the BLA result in dose-dependent and long-lasting anxiety-like responses[48–50] mediated through CRFR1.[51] To further identify the role of BLA CRFR1 in the anxiolytic effect of EE, we designed and constructed a lentiviral vector expressing small interfering RNA against CRFR1 to genetically downregulate its expression in the adult BLA. We found that knockdown of CRFR1 in the BLA induced an anxiolytic-like phenotype in the light–dark transfer and the open field tests. In the elevated plus maze test there was a trend toward an anxiolytic effect, although the results did not reach statistical significance. Our results are in accordance with the anxiolytic phenotype observed in the CRFR1 conditional knockout mice in the limbic system[19] and provide further support for the specific role of BLA CRFR1 in the anxiolytic effect of EE. To the best of our knowledge, this is the first study to

examine the behavioral effects of specific CRFR1 activity genetic inhibition in the BLA.

In the EE experiments female mice were preferred to male mice, in accordance to several previously published EE studies,[52–54] because males can show territorial behavior and aggression leading to stress that can affect the behavioral results. In contrast, male mice were preferred to female mice in stereotaxic injection studies, to avoid estrous cycle-induced within-group variability. Although, based on our current results and the public literature, we can conclude that the BLA–CRFR1 has a major role in mediating stress-induced anxiety by both genders, further detailed studies are needed to clarify the functional significance of the CRF type 1 receptors in the female BLA.

Given the results obtained in this study, we suggest CRFR1 in the BLA as a potential target for 'enviromimetics',[6] novel therapeutics that mimic or enhance the beneficial effects of environmental stimulation, in models of anxiety disorders. Further understanding of the contributions of the CRF system to the beneficial effects of environmental stimulation might provide not only mechanistic insights into the pathogenesis of environmentally modulated brain disorders, but could guide the development of a new class of therapeutics. Investigations of gene–environment interactions might reveal molecular targets for the development of therapeutic agents that mimic or enhance the beneficial effects of environmental stimulation.

Conflict of interest

The authors declare no conflict of interest.

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