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Differential activation of CRF receptor subtypes removes stress-induced memory deficit and anxiety

Cedomir Todorovic,[1,2] Jelena Radulovic,[2] Olaf Jahn,[2] Marko Radulovic,[2] Tessi Sherrin,[1] Cathrin Hippel[2] and Joachim Spiess[1,2]

[1]John A Burns School of Medicine, SNRP2, 651, Ilalo St, Honolulu, Hawaii-96813, USA

[2]Molecular Neuroendocrinology Laboratory, Max Planck Institute for Experimental Medicine, Hermann Rein Str. 3, 37075 Goettingen, Germany

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Abstract

The objective of this study was to investigate the role of corticotropin-releasing factor receptors 1 (CRF_1) and 2 (CRF_2) in anxiety-like behavior and learning of C57BL/6J mice after exposure to a stressful stimulus. When C57BL/6J mice were exposed to immobilization (1 h) serving as stressful stimulus, context- and tone-dependent fear conditioning were impaired if the training followed immediately after immobilization. The stress-induced impairment of context-dependent fear conditioning was prevented by specific blockade of CRF_2 of the lateral septum (LS) with anti-sauvagine-30. Immobilization did not only affect conditioned fear, but also enhanced, through CRF_2 of the LS, anxiety-like behavior determined with the elevated plus maze. Recovery from stress-induced anxiety and impairment of context-dependent fear conditioning was observed after 1 h delay of training and required hippocampal CRF_1, as indicated by the finding that this recovery was prevented by blockade of intrahippocampal CRF_1. It was concluded that exposure to a stressor initially affected both anxiety-like behavior and contextual conditioned fear through septal CRF_2, while the later activation of hippocampal CRF_1 resulted in the return to baseline levels of both processes. Intraventricular injection of mouse urocortin 2, a CRF_2-selective agonist, removed the stress-induced anxiety and learning impairment, but did not reduce the activation of the hypothalamic pituitary adrenal axis indicative of the hormonal stress response. We propose that the enhanced anxiety is the component of the stress response responsible for the memory deficit.

Introduction

Corticotropin-releasing factor (CRF), a 41-residue neuropeptide (Spiess *et al*., 1981), mediates many neuroendocrine and behavioral responses to stress (Vale *et al*., 1981; Koob & Heinrichs, 1999). CRF exhibits its actions through two distinctly distributed, G-protein-coupled CRF receptor subtypes, CRF_1 and CRF_2 (Van Pett *et al*., 2000). CRF_1 and CRF_2 are differentially involved in the modulation of fear and anxiety formation. Our previous results demonstrated that injection of human/rat CRF (h/rCRF) into the dorsal hippocampus (i.h.) enhances conditioned fear by activation of CRF_1. In contrast, conditioned fear is reduced by h/rCRF acting through CRF_2 of the lateral septum (LS; Radulovic *et al*., 1999). Thus, depending on the brain region and the receptor subtype involved, CRF enhances or reduces conditioned fear. Similarly, CRF can be anxiogenic or anxiolytic. Mice lacking either the CRF_1 (Smith *et al*., 1998; Timpl *et al*., 1998) or CRF_2 gene (Bale *et al*., 2000; Kishimoto *et al*., 2000) display reduced or heightened anxiety-like behavior, respectively, suggesting that CRF_1 mediates while CRF_2 predominantly attenuates anxiety-like behavioral responses. In addition, it has been described that urocortin 2 (Ucn2), a CRF_2-selective agonist (Reyes *et al*., 2001), exhibits delayed anxiolytic-like effects in the elevated plus maze (EPM; Valdez *et al*., 2002). However, other evidence indicates that LS CRF_2 is capable of inducing anxiety-like behavior (Radulovic *et al*., 1999) and increasing certain defensive behaviors, such as stress-induced freezing (Bakshi *et al*., 2002). Based on these results, it was hypothesized that during the early phase of the stress response CRF plays a stimulatory role in stress responsiveness through activation of CRF_1 and septal CRF_2, whereas a delayed activation of non-septal CRF_2 by Ucn2 and possibly urocortin 3 (Ucn3), another CRF_2-selective agonist (Lewis *et al*., 2001), may participate in reduction of the behavioral responsiveness to stress (Reul & Holsboer, 2002; Bale & Vale, 2004).

Our previous analysis of the time courses of stress-induced changes of anxiety-like behavior in the EPM and fear conditioning suggested a complex involvement of CRF_1 and CRF_2 in the stress response. In particular, exposure of Balb/c mice to 1-h immobilization results in an immediate LS CRF_2-mediated increase of anxiety measures in the EPM, whereas hippocampal CRF_1-mediated enhancement of conditioned fear occurs when training is delayed by 3 h after immobilization (Radulovic *et al*., 1999). These observations raise two important issues. Firstly, they disagree, at least on the level of CRF-mediated regulation of anxiety-like behavior and conditioned fear, with the general hypothesis that CRF_1 and CRF_2 act in an antagonistic manner, such that CRF_1 initially activates and CRF_2 later attenuates the stress response (Reul & Holsboer, 2002; Bale & Vale, 2004). Secondly, they raise the question whether the initial anxiety response was responsible for the subsequent modulation of conditioned fear, or whether these responses occurred independently from each other (Davis, 1998).

Correspondence: Dr C. Todorovic, as above.[1]
E-mail: cedomir@hawaii.edu

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Hence, the objectives of this study were: (a) to clarify in detail the roles of CRF receptor subtypes in the onset and offset of the stress reaction, using anxiety-like behavior and conditioned fear as measured behavioral variables; and (b) to elucidate the nature of the interrelationship between the CRF-mediated anxiety and fear formation and the role of the CRF receptor subtypes in this relationship.

Materials and methods

Animals

Nine-week old male C57BL/6J mice (Centre D'Elevage Janvier, Sultzfeld, France) were individually housed in macrolon cages and maintained on a 12 h light : dark cycle (lights on at 07.00 h) with access to food and water *ad libitum*. All experimental procedures were in compliance with the European Council Directive (86/609/EEC) and the Animal Section Law under the supervision of the District Government of Braunschweig (Lower Saxony, Germany). The number of mice per group was 10–12.

Synthesis and preparation of drugs

CRF and related peptides were synthesized as described previously (Rühmann *et al*., 1996; Jahn *et al*., 2001). The CRF agonist h/rCRF and the CRF antagonists astressin (Ast) and anti-sauvagine-30 (aSvg-30) were initially dissolved in 10 mM acetic acid and diluted 1 : 2 with twofold concentrated sterile artificial cerebrospinal fluid (aCSF). The final pH of the peptide solutions was 7.4. In contrast, the CRF_2-selective agonist mouse Ucn2 was dissolved in sterile saline at pH 5.5. By using saline as solvent, high concentrations of Ucn2 could be obtained (maximum solubility c_{max} > 1500 mM). The same procedure was used for the CRF_2-selective agonist mouse Ucn3, which also showed a high solubility in saline (c_{max} = 192 µM). Thus, the maximal dose of Ucn3 that could be applied per mouse was 400 ng (96 pmol) in a volume of 0.5 µL. Actual peptide concentrations of application solutions were determined by amino acid analysis using norleucine as internal standard (Rühmann *et al*., 1996). The maximum solubility was determined as described previously (Eckart *et al*., 2001). Either 10 mM acetic acid diluted with twofold concentrated aCSF or saline was used for control injections. The CRF_1-selective agonist DMP696 (Chemical and Physical Sciences Department, Bristol-Myers Squibb Company, Wilmington, DE, USA) was dissolved in dimethyl sulfoxide to a concentration of 4 mg/mL. For cannula injection the stock was diluted in aCSF to a final concentration of 100 ng/mL (total amount, 252 pmol). As the behavioral responses of aCSF-injected mice did not differ from those of saline-injected mice or vehicle-injected mice, these data were combined.

Cannulation and administration of drugs

The mice were deeply anaesthetized by intraperitoneal (i.p.) injection of 1.2% avertin (0.4 ml/mouse). Approximately 3 min after injection, narcosis and paralysis were tested by lack of paw reflexes to gentle pressure. Following this the injection system (C235; Plastics One, Roanoke, VA, USA) consisting of a double-guided cannulae, dummy and a cap were implanted and affixed to the skull of the mice using dental cement. The cannulae were placed in both lateral brain ventricles, anteroposterior (AP) −0.5 mm, lateral 1 mm, depth 2 mm; dorsal hippocampus, AP −1.5 mm, lateral 1 mm, depth 2 mm; or in the lateral intermediate septal (LSi) area, AP +1 mm, lateral 0.5 mm, depth 3 mm (Franklin & Paxinos, 2001). The animals were allowed to recover for 7–8 days before the experiments started. On the day of the experiment, mice were exposed to a light isoflurane anesthesia, the cap and the dummy were removed, and peptide solutions were delivered through an injector linked to two Hamilton microsyringes with plastic tubing. CRF receptor agonists and antagonists were injected 30 min before training, unless differently specified. The drugs were administered bilaterally by a microinjector (CMA/Microdialysis, Sweden) over a 15-s period so that a volume of 0.25 µL was injected in each side. The volumes for local injections were selected on the basis of histological analysis of methylene blue injections covering the targeted brain region. The cannula placement was verified for each mouse immediately after the behavior experiments. Methylene blue was administered (0.25 µl/site), and this was followed by cervical dislocation and removal of the brain for histological examination (Fig. 1). The number of mice per group at the beginning of the experiments was 10–12. The average loss of mice due to cannula misplacement did not exceed 10%. Only data obtained from mice with correctly inserted cannulae were included in the statistical analysis. The total doses of CRF receptor agonists and antagonists were selected on the basis of the minimal requirements for changes in anxiety-like behavior as determined in previous experiments (Behan *et al*., 1995; Radulovic *et al*., 1999; Eckart *et al*., 2001; Li *et al*., 2003).

Fear conditioning

Context- and tone-dependent fear conditioning were performed as described previously (Stiedl & Spiess, 1997; Stiedl *et al*., 2000) using a computer-controlled fear conditioning system (TSE, Bad Homburg, Germany). Fear conditioning was performed in a Plexiglas cage (36 × 21 × 20 cm) within a fear conditioning box constantly illuminated (12 V, 10 W halogen lamp, 100–500 lux). In the conditioning box, a high-frequency loudspeaker (KT-25-DT; Conrad, Hirschau, Germany) provided constant background noise [white noise, 68 dB sound pressure level (SPL)]. The training (conditioning) consisted of a single trial. The mice were exposed to the conditioning context (context 1) (180 s) followed by a tone [conditioned stimulus (CS), 30 s, 10 kHz, 75 dB SPL, pulsed 5 Hz]. After termination of the tone, a footshock [unconditioned stimulus (US), 0.7 mA, 2 s, constant current] was delivered through a stainless steel grid floor. The fear conditioning chamber was thoroughly cleaned with 70% ethanol before each animal. Memory tests were performed 24 h after fear conditioning. Contextual memory was tested in the fear conditioning box (context 1) for 180 s with background noise, but without tone-CS or -US presentation. Subsequently, without delay, the tone-dependent memory test was performed in a novel context (context 2). Context 2 represented an identically sized cage with a plain floor in a white-surrounding environment (350–500 lux) outside the fear conditioning box that was cleaned with 1% acetic acid before each animal. No background noise was provided. In the tone-dependent memory test, a 180-s pause without stimulation (pre-CS phase) preceded a 180-s period of auditory stimulation by exposure to the CS. Freezing, defined as a lack of movement besides heartbeat and respiration, was simultaneously recorded by two unbiased observers in 10-s intervals and was used as an index of conditioned fear. The activity burst produced by the electric shock was automatically detected by an infrared beam system and analysed by a software developed in collaboration with TSE.

Immobilization stress

Acute immobilization of the mice was carried out by taping their limbs to a Plexiglas surface for 1 h (Smith *et al*., 1995).

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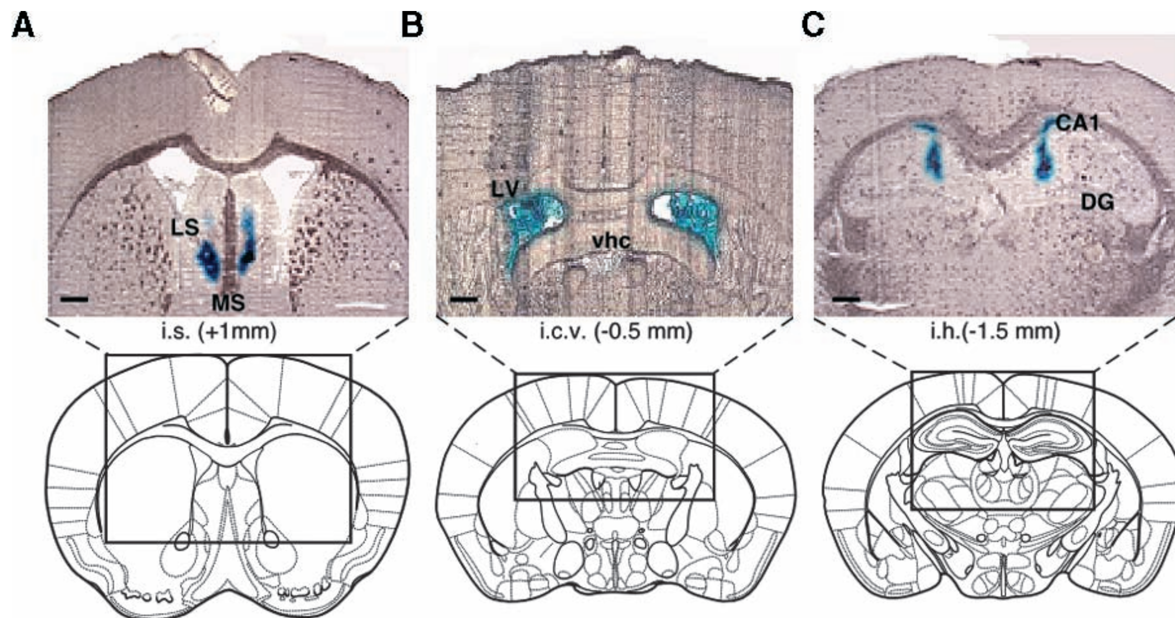

FIG. 1. Anatomical localization of the injection sites for CRF receptor agonists and antagonists according to the coronal sections from the Mouse Brain Atlas (Franklin & Paxinos, 2001). Native brain sections of mice injected with methylene blue. Scale bars: 400 μm [injection into the lateral septum site (i.s.), A]; 800 μm [injection into the lateral ventricles (i.c.v.) and dorsal hippocampus (i.h.) sites, B and C]. CA1, hippocampal subfield; DG, dentate gyrus; i.c.v., intracerebroventricular; i.h. intrahippocampal; i.s. intraseptal; LS, lateral septum; LV, lateral ventricle; MS, medial septum; vhc, ventral hippocampal commisura.

EPM

Anxiety-related behavior was investigated using the plus-maze test (Radulovic *et al*., 1999). The behavior of mice was recorded by a video camera connected to a PC computer and analysed by TSE software (VideoMot 2). The time spent, distance crossed and number of entries into the open arms, closed arms and center were recorded for 5 min. The light intensity in the plus-maze was 650 lux in the open arms and center, and 350 lux in the closed arms. Selective change in the preference for the open arms, as measured by percentage of the time spent in the open arms and number of entries into the open arms of the plus maze, was interpreted as a measure of anxiety. The traveled distance (cm) was taken as a measure for locomotor activity.

Hormone measurements

Blood samples were collected by retro-orbital eye bleeding from mice immediately after the end of immobilization. Blood samples from non-stressed mice were collected within 30 s after their removal from the home cage. Each mouse was bled only once. Adrenocorticotropic hormone (ACTH) and corticosterone levels were determined by competitive RIA assay (MP Biomedicals, Solon, OH, USA). The inter- and intra-assay coefficients of variance for ACTH were 7% and 5%, respectively, with a detection limit of 2 pg/mL (0.44 pM). For corticosterone, the inter- and intra-assay coefficients of variance were 7% and 4%, respectively, with a detection limit of 0.4 ng/mL (0.86 nM).

Ex vivo *autoradiographic analyses*

For *vivo* autoradiography analysis, C57BL/6J mice were exposed to a light isoflurane anaesthesia followed by injection into the lateral ventricles (i.c.v.) with CRF_2 receptor ligands Ucn2 or Ucn3. The brains were then removed following cervical dislocation and frozen in chilled 2-methylbutane (−20 to −30 °C). Then, they were mounted onto a cryostat block with Tissue-Tek and sectioned using a Leica cryostat. Twenty-micrometer sections were thaw-mounted onto Fisher Scientific 'plus-charged' slides, allowed to air-dry and stored at −80 °C until use. On the day of assay, slides were thawed to room temperature and allowed to dry for 20 min. The sections were preincubated for 1 min in incubation buffer [phosphate-buffered saline (PBS) supplemented with 10 mM $MgCl_2$, 2 mM EGTA and 0.1% bovine serum albumin, pH 7.0] and then incubated for 40 min in incubation buffer containing a final concentration of 200 pM [^{125}I]-aSvg-30 (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) at room temperature. Non-specific binding was determined under the same conditions in adjacent sections by the addition of 1 μM aSvg-30 (final concentration). Following incubation, the slides were dipped (2 s) into Millipore water, then washed for 2 min in ice-cold wash buffer [PBS supplemented with Triton X-100 (0.01%), pH 7.0], and dipped again into Millipore water for 2 s. They were then dried under a stream of cold air for 5–10 min and then exposed to Biomax MR X-ray film (Kodak) for 3 days at −80 °C. Autoradiograms were digitized on a Microtek ScanMaker 8700 (Microtek, Hsinchu, Taiwan) for optical density readings and quantification on a Macintosh computer using the public domain NIH Image program (version 1.63).

Behavioral procedures

Experiment 1: modulation of fear conditioning by immobilization stress: involvement of septal CRF_2

Male C57BL/6J mice were exposed to 1-h immobilization. Immediately, 0.5, 1 or 24 h after the end of the exposure to this stressful stimulus the mice were trained for context- and tone-dependent fear conditioning. Memory tests were performed 24 h after fear conditioning (Fig. 2A). For the pharmacological part of the experiment, the same design as described above was applied except for additional injection into the LS (i.s.). One dose (400 ng/mouse) of the

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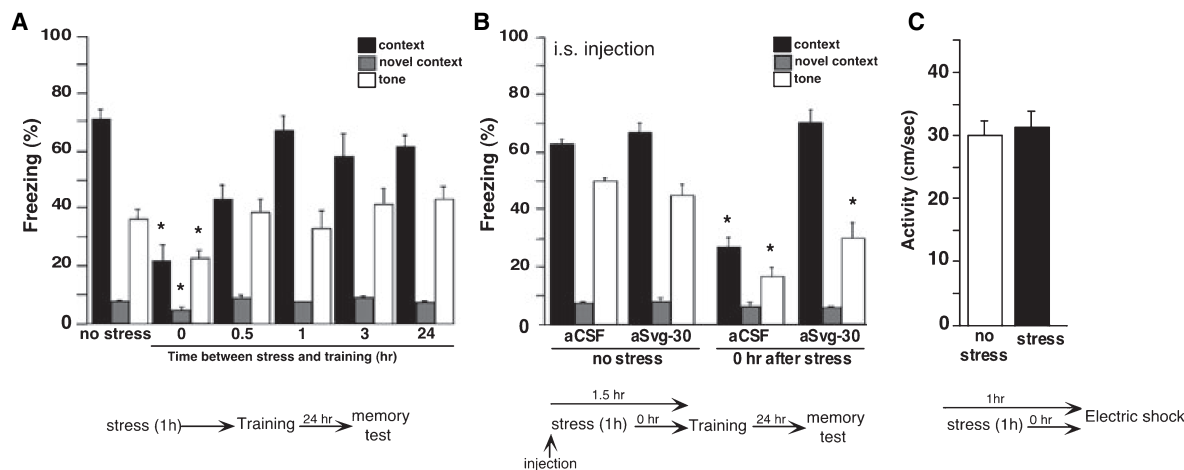

FIG. 2. Stress-induced impairment of context-dependent fear conditioning is mediated by septal CRF_2. In mice subjected to 1-h immobilization and trained immediately afterwards fear conditioning to context and tone was significantly impaired. Note that freezing behavior did not differ between the groups after exposure to novel context. Statistically significant differences: Scheffe test, $*P < 0.05$ vs control (non-stressed mice) (A). Stress-induced impairment of context-dependent, but not tone-dependent, fear conditioning was fully antagonized by 400 ng (110 pmol) anti-sauvagine-30 (aSvg-30) per mouse injected into the LS (i.s.) 30 min before immobilization. I.s. injection of aSvg-30 alone 1.5 h prior to the training did not produce any significant effect on fear conditioning. Statistically significant differences: Scheffe test, $*P < 0.05$ vs control [non-stressed mice + artificial cerebrospinal fluid (aCSF)] (B). Footshock reactivity during fear conditioning training did not significantly differ between the mice exposed to 1-h immobilization and the naïve control group (C).

CRF_2-selective antagonist aSvg-30 was injected 30 min prior to 1-h immobilization. An injection of vehicle lacking aSvg-30 served as control. The mice were trained for fear conditioning immediately after the end of immobilization. The effect of aSvg-30 on fear conditioning in the absence of stress was tested at the same dose. For this control, aSvg-30 was injected i.s. 1.5 h prior to the training for fear conditioning. After injection and before training, the mice were kept in their home cages. The training and memory tests 24 h later were performed as described under the 'Fear conditioning' section above (Fig. 2B).

Experiment 2: stress-induced anxiety-like behavior and specific inhibition

Male C57BL/6J mice were exposed to 1-h immobilization and 0.25, 0.5, 1 or 24 h after the end of immobilization tested in the EPM. After immobilization, the mice were kept in their home cages prior to testing (Fig. 3A–C). For the pharmacological part of the experiment, the same design was applied except for i.s. injections and time delay after immobilization (30 min) (Fig. 3D–F). Male C57BL/6J mice were injected i.s. with aSvg-30 (400 ng/mouse) or vehicle as control 30 min prior to immobilization, and were tested with EPM 30 min after the end of immobilization. An injection of vehicle lacking aSvg-30 served as control (Fig. 3D–F). The effect of aSvg-30 in the EPM in the absence of stress was tested at the same dose. For this control, aSvg-30 was injected i.s. 2 h prior to the testing in the EPM. After injection and before testing the mice were kept in their home cages. Details of the EPM test are described under the 'EPM' section above.

Experiment 3: recovery from stress-induced anxiety and learned fear deficit

For the recovery from learned fear deficit, male C57BL/6J mice were injected (i.h.) with Ast (300 ng/mouse), aSvg-30 (400 ng/mouse), DMP696 (50 ng/mouse) or vehicle as control 30 min prior to immobilization, and trained for context- and tone-dependent fear conditioning 60 min after the end of immobilization (Fig. 4A). The experimental design contains control experiments to examine the action of all drugs at the doses used on fear conditioning in the absence of immobilization. To this end, they were injected i.h. 2.5 h prior to training for fear conditioning. After injection and before training the mice were kept in their home cages. The training and memory test 24 h later were performed as described under the 'Fear conditioning' section above (Fig. 4A).

Recovery from stress-induced anxiety was determined in an experiment using the same experimental design as described in the previous paragraph (Fig. 4A), except that mice were tested with EPM 60 min after the end of immobilization (Fig. 4B–D). Details of the EPM test are described in the 'EPM' section above.

Experiment 4: prevention of the learned fear deficit by non-septal CRF_2 activation reducing anxiety-like behavior

Anxiolytic properties of the CRF_2-selective agonists were tested by i.c.v. injection of male C57BL/6J mice with Ucn2 (100, 200, 400 ng/mouse), Ucn3 (100, 200, 400 ng/mouse) or vehicle as control 30 min prior to test with EPM (Fig. 5A–C).

In the next experiment, C57BL/6J mice were injected i.c.v. with the CRF_2-selective agonist or vehicle as control 5 min prior to immobilization. One group of mice was tested in the EPM 30 min after the end of immobilization (Fig. 6A–C). A second group of mice was trained for context- and tone-dependent fear conditioning immediately after the end of immobilization (Fig. 7A). Details of the EPM test are described in the 'EPM' section above. The training and memory test 24 h later were performed as described in the 'Fear conditioning' section above.

Statistics

Statistical evaluation (StatView 5.0.1 software; SAS Institute, Cary, NC, USA) was performed by two- and one-way analysis of variance (ANOVA), with Scheffe test applied, *post hoc*, for

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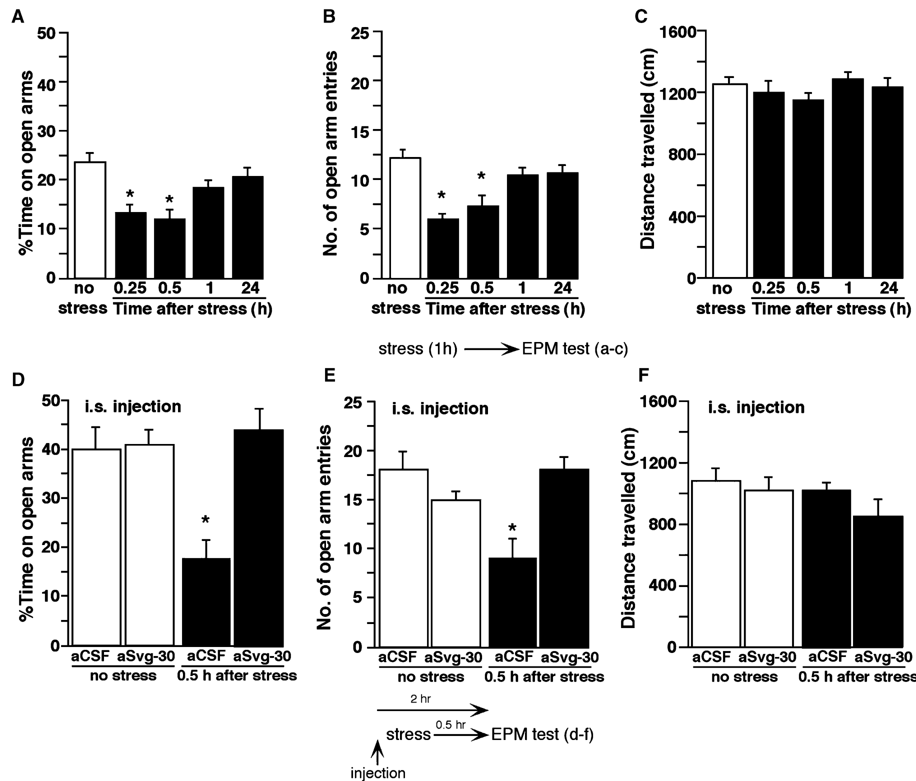

FIG. 3. Stress-induced enhancement of anxiety-like behavior is mediated by septal CRF_2. Mice subjected to 1-h immobilization exhibited transient enhancement of anxiety-like behavior 15 and 30 min after the end of immobilization, as indicated by significantly decreased time spent on the open arms (A) and number of entries into the open arms (B) of the elevated plus maze (EPM). Locomotor activity as indicated by total distance travelled (cm) was not affected (C). Statistically significant differences: Scheffe test, $*P < 0.05$ vs control (non-stressed mice). Injection into the LS (i.s.) of 400 ng (110 pmol) anti-sauvagine-30 (aSvg-30) per mouse, 30 min before immobilization stress, prevented the stress-induced decrease of the time spent on the open arms (D) and number of entries into the open arms of the EPM (E), without affecting locomotor activity (F). The non-stressed control group injected with aSvg-30 i.s. alone 2 h before the EPM test did not display any significant changes in plus-maze behavior (D–F). Statistically significant differences: Scheffe test, $*P < 0.05$ relative to control [non-stressed mice + artificial cerebrospinal fluid (aCSF)].

individual between-group comparisons at the $P < 0.05$ level of significance. Data are expressed as mean ± SEM.

Results

Experiment 1: modulation of fear conditioning by immobilization stress: involvement of septal CRF_2

When C57BL/6J mice were subjected to 1-h immobilization, trained immediately afterwards and tested 24 h later for their memory, they showed significant impairment of both context- ($F_{5,55} = 8.92$; $P < 0.05$; Scheffe test, $P < 0.05$ vs non-stressed controls) and tone-dependent fear conditioning ($F_{5,55} = 4.55$; $P < 0.05$; Scheffe test, $P < 0.05$ vs non-stressed controls; Fig. 2A).

The receptor subtype specificity of this memory impairment was tested by administration of 400 ng (110 pmol) of the CRF_2-selective antagonist aSvg-30 into the LS (i.s.) 30 min before immobilization. The LS was selected because it had been demonstrated in an earlier study with Balb/c mice (Radulovic *et al.*, 1999) that specific blockade of septal CRF_2 reduces h/rCRF-induced memory impairment. A two-way ANOVA with treatment and stress as between-subject factors revealed significant main effects for treatment ($F_{1,34} = 11.6$; $P < 0.05$) and stress ($F_{1,34} = 8.09$; $P < 0.05$), as well as significant treatment–stress interaction ($F_{1,34} = 16.6$; $P < 0.05$) in context-dependent fear conditioning. A significant main stress effect ($F_{1,34} = 24.1$; $P < 0.05$) without treatment effect ($F_{1,34} = 0.4$; $P > 0.05$) and treatment–stress interaction ($F_{1,34} = 2.1$; $P > 0.05$) was found for tone-dependent fear conditioning. Significant treatment–stress interaction in context- but not tone-dependent fear conditioning was explained by analysis of simple effects of treatment showing that administration of 400 ng aSvg-30 i.s. 30 min before immobilization completely prevented stress-induced impairment of context- ($F_{1,17} = 23.73$; $P < 0.05$ vs aCSF-injected stressed mice) but not tone-dependent ($F_{1,17} = 1.2$; $P > 0.05$; Fig. 2B) fear conditioning of C57BL/6J mice trained immediately after the end of immobilization. In addition, i.s. injection of aSvg-30 alone was performed in a control experiment 1.5 h prior to training to compensate for the delay of injection and immobilization in the main experiment. This treatment did not produce any significant effect on fear conditioning ($F_{1,17} = 0.89$; $P > 0.05$ for context-dependent fear conditioning; $F_{1,17} = 1.73$; $P > 0.05$ for tone-dependent fear conditioning; Fig. 2B). These data suggested that aSvg-30 specifically blocked stress-induced impairment independently of its possible tonic effects on context-dependent fear. It was concluded on the basis of the specificity of aSvg-30 and the injection site that LS was at least one of the sites where CRF_2 mediated the impairing effect of stress on context- but not tone-dependent fear conditioning.

The possibility had to be considered that the exposure of mice to 1-h immobilization may have induced delayed sensitizing effects, which could lead to non-associative interference with conditioned freezing behavior 24 h later (Glazer & Weiss, 1976; Fanselow, 1980). We addressed this possibility by measuring freezing behavior in a

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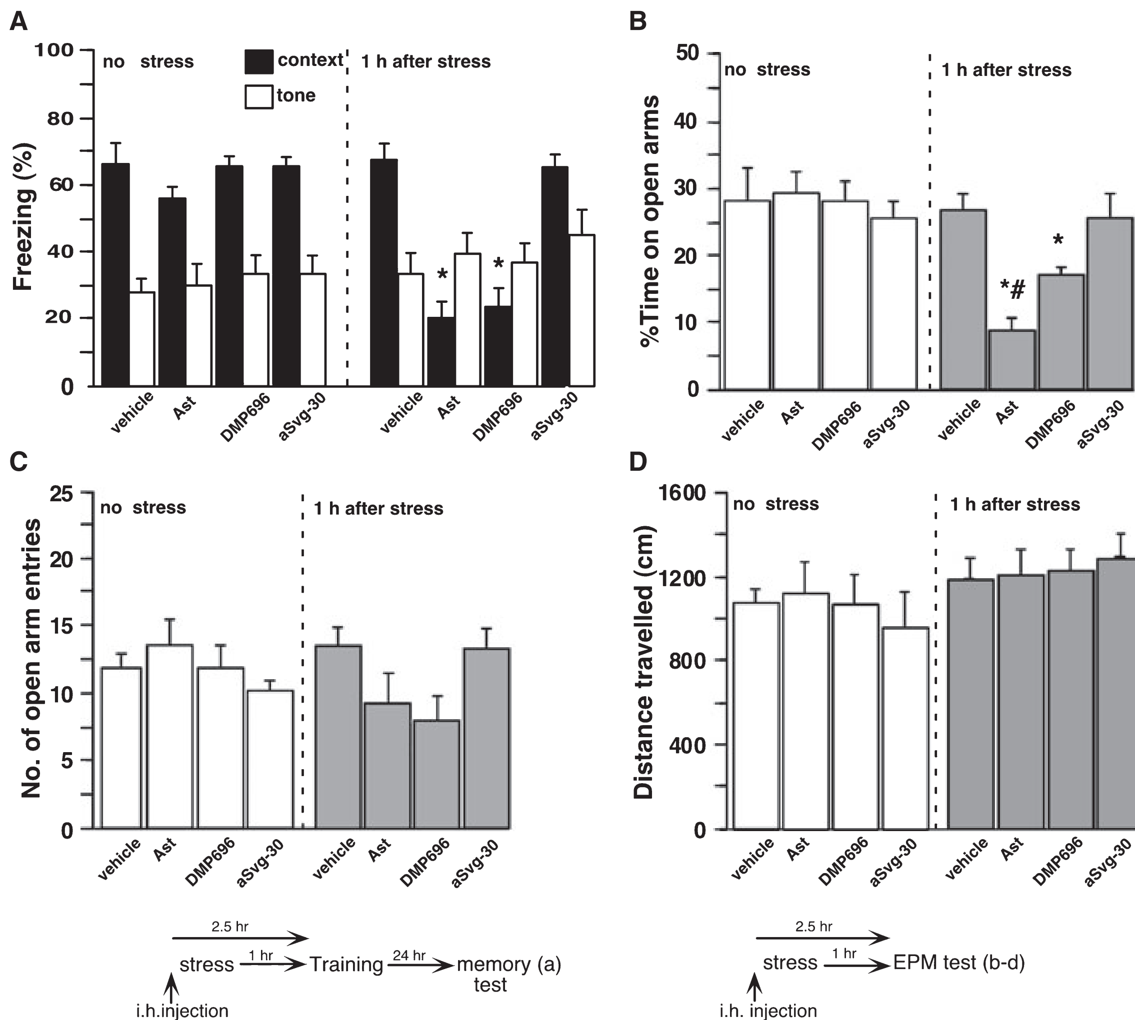

FIG. 4. Hippocampal CRF_1 is required for recovery from a stress-induced anxiety increase and conditioned fear decrease. Mice were injected into the dorsal hippocampus (i.h.) with 300 ng (85 pmol) astressin (Ast), 50 ng (126 pmol) DMP696 or 400 ng (110 pmol) anti-sauvagine-30 (aSvg-30) 30 min before immobilization and tested for anxiety-like behavior in the elevated plus maze (EPM) or trained for fear conditioning 1 h after immobilization. Under these conditions, stress-induced impairment of context- but not tone-dependent fear conditioning was prevented by 300 ng (85 pmol) Ast or 50 ng (126 pmol) DMP696, but not 400 ng (110 pmol) aSvg-30 per mouse (A). Recovery from stress-induced anxiety was prevented by treatment with Ast or DMP696, but not with aSvg-30, as indicated by decreased time spent on the open arms (B). The number of entries into the open arms of the EPM did not change significantly (C). The non-stressed control groups injected with Ast and DMP696 alone 2.5 h prior to the EPM test, or the training phase of the fear conditioning, did not exhibit significant differences compared with control groups (non-stressed mice + vehicle). Statistically significant differences: Scheffe test, $^*P < 0.05$ vs control (non-stressed mice + vehicle); $^\#P < 0.05$ vs stressed DMP696-injected mice.

novel context that served as a background stimulus during the tone-dependent memory test. A one-way ANOVA did not reveal any significant differences in freezing behavior between the experimental groups ($F_{5,55} = 0.98$; $P > 0.05$; Fig. 2A; $F_{1,17} = 0.21$; $P > 0.05$; Fig. 2B). These results indicated that the conditioned fear response was not generalized to context different from the conditioning context. Thus, the freezing response was specifically related to the CS. Absence of correlation between the measures of conditioned and unconditioned freezing was observed in all subsequent experiments employing fear conditioning (data not shown).

It was also considered that immobilization reduced the responsiveness of the mice to the foot shock serving as US. This consideration implied that the stress-induced learning deficit might be simply an artifact of lowered sensitivity to the foot shock. Therefore, we determined whether exposure to 1-h immobilization changed the footshock reactivity during training for fear conditioning. It should be noted that footshock reactivity reflects a very basic level of processing by the CNS (Shi & Davis, 1999; Sanders *et al.*, 2005). This procedure did not change the footshock reactivity during training for fear conditioning ($F_{1,18} = 0.94$; $P > 0.05$; Fig. 2C). It was concluded that 1-h immobilization affected the conditioned, but not unconditioned, fear response. Therefore, it was assumed that a change in the sensitivity to the US probably did not mediate the stress-induced impairment of fear conditioning.

Experiment 2: stress-induced anxiety-like behavior and specific inhibition

We considered the possibility that the observed stress-induced memory deficit resulted from enhancement of anxiety generated by the stressful exposure to immobilization. Therefore, we investigated

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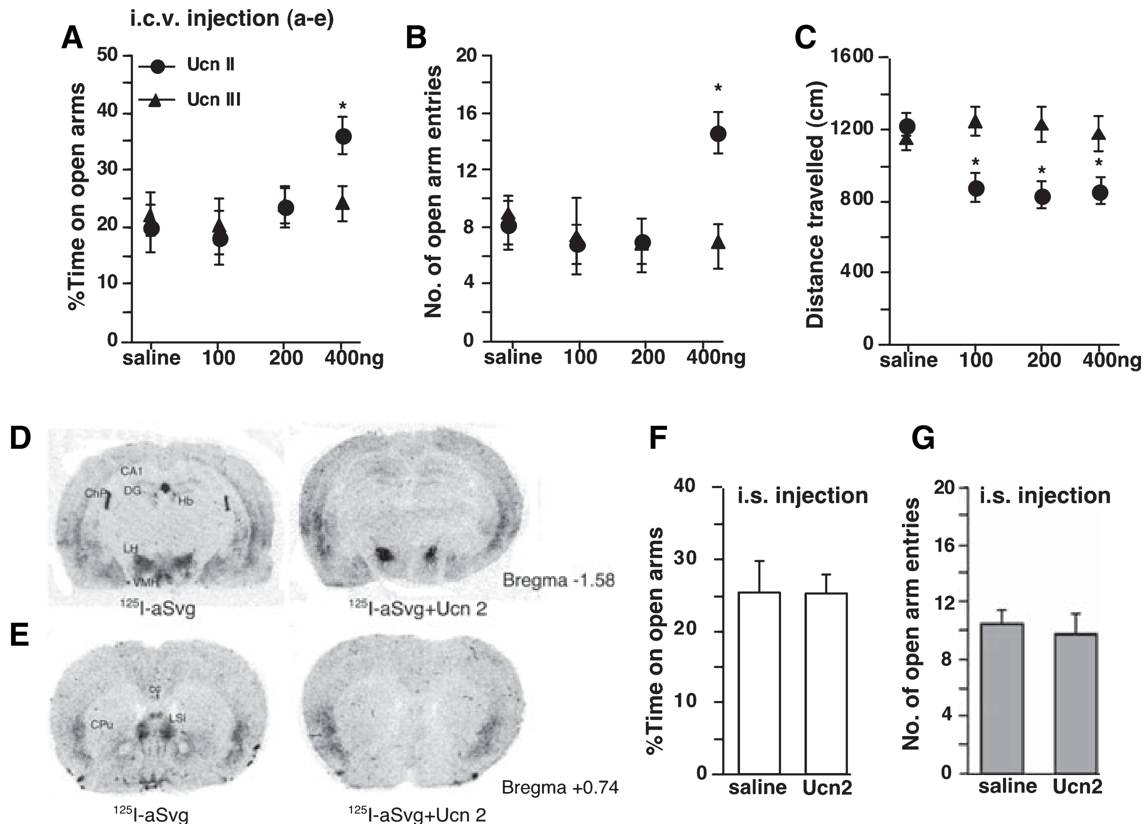

FIG. 5. Intraventricular administration of urocortin 2 (Ucn2) reduces anxiety-like behavior in the EPM test. Injection into the lateral ventricles (i.c.v.) of 400 ng (96 pmol) Ucn2, but not of Ucn3, significantly increased time spent on the open arms (A) and number of entries into the open arms (B) of the EPM, indicating an anxiolytic role for Ucn2. I.c.v. injections of Ucn2 (three doses used) elicited locomotor-suppressive effects (C). CRF_2 receptor occupancy of Ucn2 as determined by *ex vivo* receptor autoradiography following i.c.v. administration of 400 ng Ucn2 per mouse. The receptor occupancy as measured by inhibition of [^{125}I]-anti-sauvagine-30 (aSvg-30) labeling was evident in the various regions of these coronal sections of mouse brain (Bregma, −1.58 mm, level of the dorsal hippocampus; Bregma, +0.74 mm, level of the LS) (D and E). Abbreviations: CA1, CA1 region of Ammon's horn; cc, corpus callosum; ChP, choroids plexus; CPu, caudate putamen; DG, dentate gyrus; Hb, habenula; LH, lateral hypothalamic nucleus; VMH, ventromedial hypothalamic nucleus. Injection into the LS (i.s.) of 400 ng (96 pmol) Ucn2 did not significantly change the time spent on the open arms (F) and number of entries into the open arms (G) of the EPM. The agonist was injected 30 min before testing or death. Statistically significant differences: Scheffe test, *$P < 0.05$ relative to control (saline).

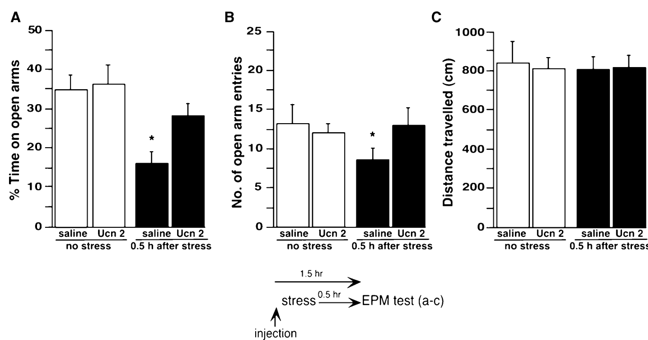

FIG. 6. Intraventricular administration of urocortin 2 (Ucn2) prevents stress-induced effects on anxiety-like behavior. Mice injected with 400 ng (96 pmol) Ucn2 per mouse i.c.v., 5 min before 1-h immobilization and tested 30 min afterwards did not exhibit stress-induced anxiety, as indicated by the increased time spent on the open arms (A) and the number of entries into the open arms (B) of the elevated plus maze (EPM). In the non-stressed control groups injected i.c.v. with Ucn2 alone 1.5 h prior to the EPM test, no effects on behavior were observed (A and B). Locomotor activity as indicated by total distance traveled (cm) was not affected by Ucn2 pretreatment (C). Statistically significant differences: Scheffe test, *$P < 0.05$ relative to control (non-stressed mice + saline).

the anxiety-like behavior after immobilization and its modulation by the CRF receptor subtypes. When C57BL/6J mice were exposed for 1 h to immobilization, and tested 15 and 30 min afterwards in the EPM, significantly enhanced anxiety-like behavior was observed as indicated by the time spent ($F_{4,54} = 16.12$; $P < 0.05$; Scheffe test, $P < 0.05$ vs non-stressed controls; Fig. 3A) and number of entries ($F_{4,54} = 4.51$; $P < 0.05$; Scheffe test, $P < 0.05$ vs non-stressed controls; Fig. 3B) into the open arms of the plus maze, without affecting locomotor activity as revealed by the total distance crossed ($F_{4,54} = 1.12$; $P > 0.05$; Fig. 3C). A return to basal anxiety levels was observed 1 h after the end of immobilization.

In our next experiment, mice were injected with 400 ng (110 pmol) of aSvg-30 into the LS (i.s.) 30 min before immobilization and exposed to EPM 30 min afterwards, to determine whether the stress-induced enhancement of anxiety-like behavior was generated by activation of CRF_2 of the LS. A two-way ANOVA with treatment and stress as between-subject factors revealed a significant main effect for treatment, a significant treatment–stress interaction, without significant main effect of stress on time spent ($F_{1,34} = 6.93$; $P < 0.05$ treatment; $F_{1,34} = 7.12$; $P < 0.05$ treatment–stress interaction; $F_{1,34} = 3.2$; $P > 0.05$ stress) and number of visits ($F_{1,34} = 5.22$; $P < 0.05$ treatment; $F_{1,34} = 4.14$; $P < 0.05$ treatment–stress interaction;

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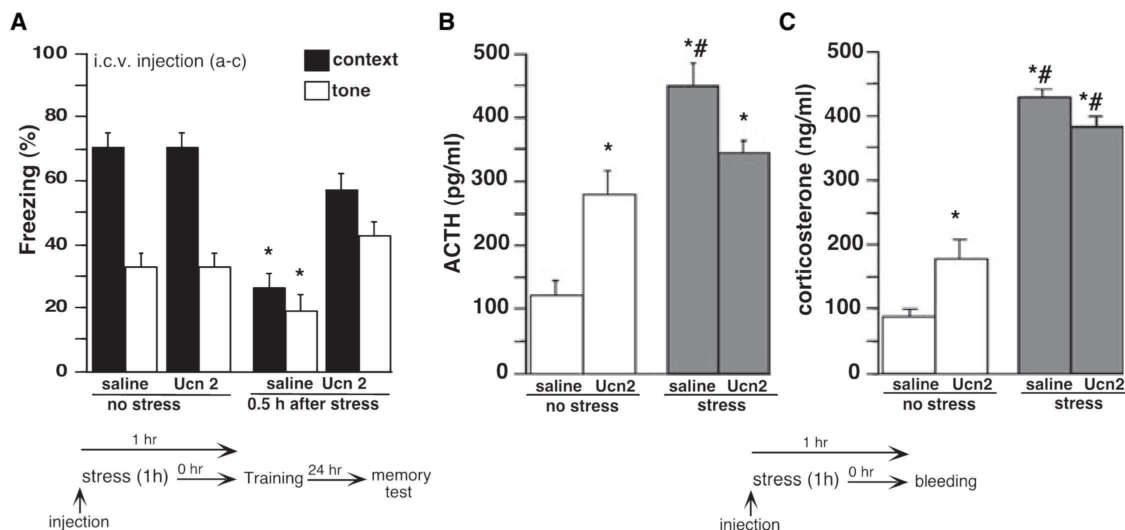

FIG. 7. Intraventricular administration of urocortin 2 (Ucn2) lowering anxiety-like behavior prevents stress-induced effects on conditioned fear without change of the corticosterone release. In mice injected with 400 ng (96 pmol) Ucn2 into the lateral ventricles (i.c.v.), 5 min before 1-h immobilization and trained for fear conditioning immediately after the end of immobilization, the stress-induced impairment of the conditioned fear was prevented (A). The same procedure did not alter adrenocorticotropic hormone (ACTH) and corticosterone levels compared with stressed saline-treated mice, as determined immediately after the end of immobilization. Both groups had significantly increased ACTH and corticosterone levels compared with the non-stressed control group (B and C). When compared with non-stressed saline-treated mice, ACTH and corticosterone levels were also increased in mice 1 h after i.c.v. injection of Ucn2 (B and C). Statistically significant differences: Scheffe test, *$P < 0.05$ relative to control (non-stressed saline-injected mice); $^{\#}P < 0.05$ vs non-stressed Ucn2-injected mice.

$F_{1,34} = 2.56$; $P > 0.05$ stress) in the open arm. An analysis of simple effects of treatment showed that the stressed mice injected i.s. with aSvg-30 spent a significantly increased time ($F_{1,16} = 11.83$; $P < 0.05$; Fig. 3D) in and increased the number of visits to ($F_{1,16} = 5.33$; $P < 0.05$; Fig. 3E) the open arms of the EPM when tested 30 min after immobilization in comparison with stressed mice injected i.s. with aCSF. In addition, no significant differences with the non-stressed control group injected with aCSF were found (Fig. 3D and E). Thus, the stress-induced anxiety was completely prevented by aSvg-30 injected i.s. It was concluded that LS was at least one of the major sites where CRF_2 mediated the anxiogenic effects of stress. A non-stressed control group was injected with aSvg-30 i.s. alone 2 h before testing in the EPM. Under these conditions, no effects on the anxiety-like behavior in the plus maze were observed ($F_{1,18} = 1.23$; $P > 0.05$ time spent in the open arm; $F_{1,18} = 1.48$; $P > 0.05$ number of visits in the open arm; Fig. 3D and E). Locomotor activity did not significantly differ among the groups (Fig. 3F).

Experiment 3: recovery from stress-induced anxiety and learned fear deficit

Recovery from learned fear deficit

Mice trained immediately after immobilization were impaired in context- and tone-dependent conditioned fear. The deficit in fear conditioning was not observed when training took place 1, 3 or 24 h after immobilization stress (Fig. 2A). Because we had observed in earlier experiments (Radulovic *et al*., 1999) that activation of hippocampal CRF_1 enhances fear conditioning, we investigated whether hippocampal CRF_1 was also involved in the recovery from stress-induced impairment, and thus enhancement of conditioned fear in C57BL/6J mice. On the same basis, it was hypothesized that antagonism to CRF_1 might lead to prolonged effects of stress on anxiety-like behavior and context-dependent fear conditioning.

For this purpose, mice were injected 30 min before immobilization into the dorsal hippocampus (i.h.) with 300 ng (85 pmol) Ast, a specific CRF receptor antagonist found to be non-selective for the CRF receptor subtypes (Gulyas *et al*., 1995), or 50 ng (126 pmol) of DMP696, a highly selective and potent non-peptidic CRF_1 antagonist (He *et al*., 2000). The mice were trained in the fear conditioning paradigm 1 h after the end of immobilization. At this time point, aCSF-treated stressed mice did not show stress-induced learning impairment (Fig. 2A). A two-way ANOVA revealed significant main effects for treatment ($F_{3,63} = 9.37$; $P < 0.05$) and stress ($F_{1,63} = 17.63$; $P < 0.05$) and treatment–stress interaction ($F_{3,63} = 14.35$; $P < 0.05$) for context-dependent fear conditioning. No significant main effects or interaction were found for tone-dependent fear conditioning. Analysis of simple effects of treatment revealed that both Ast ($F_{1,15} = 31.84$; $P < 0.05$ vs vehicle-injected stressed group) and DMP696 ($F_{1,18} = 26.37$; $P < 0.05$ vs vehicle-injected stressed group) application to the stressed group resulted in impaired context-dependent fear conditioning (Fig. 4A). In the control non-stressed group, injected i.h. with Ast ($F_{1,14} = 1.76$; $P > 0.05$ context-dependent fear conditioning) or DMP696 ($F_{1,18} = 1.24$; $P > 0.05$ context-dependent fear conditioning) alone 2.5 h before training, no effects were observed in the memory test 24 h later compared with the aCSF-injected non-stressed group (Fig. 4A). The 2.5 h time point was chosen to compensate for the duration of immobilization and the period after injection in the main experiment. These results indicated that Ast and DMP696 prevented the recovery from stress-induced impairment. This effect was mediated by CRF_1, as concluded from the observation that DMP696 binds specifically to CRF_1 and that injection of the CRF_2-specific antagonist aSvg-30 under the same conditions did not affect context- ($F_{1,15} = 1.13$; $P > 0.05$) or tone-dependent ($F_{1,15} = 0.78$; $P > 0.05$) fear conditioning (Fig. 4A).

Recovery from anxiety

We investigated whether activation of hippocampal CRF_1 was also involved in the recovery from stress-induced anxiety in C57BL/6J mice. For this purpose, the mice were injected i.h. with 300 ng

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(85 pmol) of the CRF receptor antagonist Ast or 50 ng (126 pmol) of the CRF_1-selective antagonist DMP696, 30 min before immobilization, and tested in the EPM 1 h after the end of immobilization. At this time point, aCSF-treated stressed mice had already recovered from stress-induced anxiety (Fig. 3A and B). A two-way ANOVA revealed significant treatment and stress main effects without treatment–stress interaction on time spent ($F_{3,65} = 4.15$; $P < 0.05$ treatment; $F_{1,65} = 7.21$; $P < 0.05$ stress; $F_{3,65} = 1.23$; $P > 0.05$ interaction) in the open arms of the EPM (Fig. 4B). The same treatment did not affect the number of visits into the open arm of the EPM ($F_{3,65} = 1.25$; $P < 0.05$ treatment; $F_{1,65} = 1.06$; $P < 0.05$ stress; $F_{3,65} = 4.55$; $P > 0.05$ treatment–stress interaction; Fig. 4C). Analysis of simple effects of treatment revealed that Ast ($F_{1,16} = 10.95$; $P < 0.05$ vs vehicle-injected stressed group) and DMP696 ($F_{1,18} = 4.62$; $P < 0.05$ vs vehicle-injected stressed group) injection prior to immobilization resulted in a significantly reduced time spent on the open arms (Fig. 4B) without significantly affecting the number of open arm entries ($F_{1,16} = 1.16$; $P > 0.05$ vs vehicle-injected stressed group for Ast; $F_{1,18} = 2.63$; $P > 0.05$ vs vehicle-injected stressed group for DMP696; Fig. 4C). Interestingly, the same analysis revealed that treatment with Ast under stressful conditions resulted in a significantly increased reduction of the time spent in open arms when compared with DMP696-treated mice under the same conditions ($F_{1,17} = 5.23$; $^{\#}P > 0.05$ vs DMP696-injected stressed group). In the non-stressed control groups injected with Ast or DMP696 i.h. alone 2.5 h before the EPM test, no effects on plus-maze behavior were observed compared with the vehicle-injected non-stressed group (Fig. 4B and C). Under the same conditions, the CRF_2-specific antagonist aSvg-30 did not interfere with the recovery process ($F_{1,16} = 1.94$; $P > 0.05$ time spent in the open arm; $F_{1,16} = 1.41$; $P > 0.05$ number of visits in the open arm; Fig. 4B and C). Locomotor activity did not significantly differ among the groups (Fig. 4D).

Experiment 4: prevention of the learned fear deficit by non-septal CRF_2 activation reducing anxiety-like behavior

Selection of anxiolytic CRF-like peptide

In view of the finding that the levels of anxiety-like behavior and conditioned fear were inversely affected by immobilization, we hypothesized that enhanced anxiety levels could interfere with the fear conditioning response. Next we designed experiments to modulate fear conditioning after exposure to immobilization by reduction of anxiety, and tested whether such a procedure would result in enhancement of fear conditioning.

Therefore, we initially tested the effects of i.c.v. injections of two CRF_2-selective agonists Ucn2 and Ucn3 in the EPM. Previous studies reported that such injections exhibit anxiolytic effects on EPM behavior (Valdez *et al*., 2002, 2003). Administration of the selective-CRF_2 agonist Ucn2 into the lateral ventricles (i.c.v.) 30 min before testing in the EPM significantly reduced anxiety-like behavior (Fig. 5A and B). A dose of 400 ng (96 pmol) Ucn2 significantly increased the time spent in the open arms ($F_{3,32} = 6.26$; $P < 0.05$; Scheffe test, $P < 0.05$ vs saline) and number of entries ($F_{3,32} = 10.51$; $P < 0.05$; Scheffe test, $P < 0.05$ vs saline) into the open arms of the EPM. Ucn2 also ($F_{3,32} = 5.67$; $P < 0.05$; Scheffe test, $P < 0.05$ vs saline) elicited a significant locomotor-suppressive effect (Fig. 5C). *Ex vivo* autoradiographic experiments revealed the overall distribution pattern of bound [^{125}I]-aSvg-30 in the brain sections of mice injected with 400 ng Ucn2 (Fig. 5D and E). CRF_2-binding sites specifically labeled by [^{125}I]-aSvg-30 were concentrated in the basolateral (BLA) and medial nucleus of the amygdala, the ventromedial and lateral nuclei of the hypothalamus, the choroid plexus, LSi and, to a lesser extent, in the dentate gyrus and the CA1 regions of the hippocampus. In the LSi, ventromedial nucleus of the hypothalamus, choroid plexus and hippocampal subregions with a known high production of CRF_2, i.c.v. injection of 400 ng Ucn2 resulted in a marked decrease of [^{125}I]-aSvg-30 binding (Fig. 5D and E). We found weaker *in vivo* binding of Ucn3 to CRF_2 (data not shown) and no observable effect on anxiety-like behavior in the EPM by Ucn3 ($F_{3,31} = 2.18$; $P > 0.05$ time spent in the open arm; $F_{3,31} = 1.21$; $P > 0.05$; number of entries into the open arms; $F_{3,31} = 2.59$; $P > 0.05$; locomotor activity; Fig. 5A–C).

Ex vivo autoradiographic experiments revealed strong binding of the LS CRF_2 after i.c.v. administration of Ucn2. In view of the observation that CRF_2 of the LS mediates an increase of anxiety measures in the EPM, it was expected that i.c.v. administration of Ucn2 would balance anxiolytic and anxiogenic action of non-septal and septal CRF_2, respectively (Radulovic *et al*., 1999; Kishimoto *et al*., 2000). However, this treatment resulted solely in the anxiolytic action of the peptide. To address this apparent contradiction, we injected 400 ng (96 pmol) of Ucn2 i.s. 30 min prior to testing in the EPM. This treatment did not produce any significant changes in anxiety-like behavior ($F_{1,19} = 0.71$; $P > 0.05$, time spent; $F_{1,19} = 0.66$; $P > 0.05$, number of entries into the open arms of the plus maze; Fig. 5F and G). Absence of effects on anxiety-like behaviors observed after i.s. injection of Ucn2 explained thereby anxiolytic effects on EPM behavior obtained after i.c.v. administration of Ucn2.

Exposure to stress under anxiolytic conditions and control of hypothalamic pituitary adrenal axis (HPA) activity

In our next experiment, we tested whether i.c.v. injection of Ucn2, exhibiting an anxiolytic effect, would affect stress-induced anxiety. The underlying assumption of this experiment was that simultaneous activation of septal and non-septal CRF_2 would cancel each other in the manner that the activation of the non-septal CRF_2 by Ucn2 would lead to the removal of stress-induced anxiety. Two-way ANOVA revealed that i.c.v. administration of 400 ng (96 pmol) Ucn2 5 min before immobilization completely prevented stress-induced anxiety in mice tested 30 min after the end of immobilization when maximal anxiety was observed without Ucn2 pretreatment (treatment: $F_{1,33} = 4.21$; $P < 0.05$; stress: $F_{1,33} = 7.77$; $P < 0.05$; treatment–stress interaction: $F_{1,33} = 4.67$; $P < 0.05$ for time spent in the open arm; Fig. 6A; treatment: $F_{1,33} = 3.26$; $P < 0.05$; stress: $F_{1,33} = 4.19$; $P > 0.05$; treatment–stress interaction: $F_{1,33} = 5.61$; $P < 0.05$ for number of visits in the open arm; Fig. 6B). Pretreatment with Ucn2 did not affect locomotor activity (Fig. 6C). Analysis of simple effects of treatment showed that Ucn2-injected stressed mice exhibited reduced anxiety-like behavior in comparison with saline-injected (i.c.v.) stressed mice, as indicated by the increased time spent ($F_{1,18} = 14.13$; $P < 0.05$; Fig. 6A) and the number of visits ($F_{1,18} = 7.83$; $P < 0.05$) in the open arms (Fig. 6B). No significant differences in anxiety levels were observed between control non-stressed saline- and Ucn2-treated mice injected 1.5 h prior to the EPM test as indicated by the time spent ($F_{1,15} = 0.23$; $P > 0.05$) and the number of visits ($F_{1,15} = 0.31$; $P > 0.05$) in the open arms (Fig. 6A and B).

After we had established that i.c.v. injection of 400 ng Ucn2 before immobilization resulted in lower anxiety levels, we were able to directly test our hypothesis that stress impairs learning by its concomitant anxiogenic effect mediated by septal CRF_2, and that this impairment can be prevented by activation of non-septal CRF_2 accessible through the lateral ventricles. Indeed, administration of 400 ng Ucn2, i.c.v., 5 min before immobilization prevented stress-induced impairment of context- and tone-dependent fear

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conditioning of C57BL/6J mice trained immediately after the end of immobilization (Fig. 7A). A two-way ANOVA revealed significant treatment ($F_{1,36} = 6.15$; $P < 0.05$) and stress main effects ($F_{1,36} = 19.81$; $P < 0.05$), and treatment–stress interaction ($F_{1,36} = 9.57$; $P < 0.05$) for context-dependent fear conditioning. Significant stress main effect ($F_{1,36} = 12.88$; $P < 0.05$), treatment main effect ($F_{1,36} = 7.57$; $P > 0.05$) and treatment–stress interaction ($F_{1,36} = 8.37$; $P > 0.05$) were also found for tone-dependent fear conditioning. These effects were explained by an analysis of the simple effect of treatment showing that Ucn2 facilitated context- ($F_{1,19} = 14.38$; $P < 0.05$ vs saline-injected stressed group) and tone-dependent ($F_{1,19} = 8.18$; $P > 0.05$) fear conditioning, when given prior to immobilization. The saline-injected non-stressed group and the group that had received Ucn2 alone 1 h prior to training did not significantly differ in conditioned fear ($F_{1,17} = 0.21$; $P > 0.05$ context-dependent fear conditioning; $F_{1,17} = 0.10$; $P > 0.05$ tone-dependent fear conditioning; Fig. 7A). It was concluded that the activation of non-septal CRF_2 by Ucn2 prevented the stress-induced memory deficit. The results indicated a dissociation of the regulation of anxiety formation and fear conditioning by Ucn2 when applied i.c.v. Additionally, by preventing the stress-induced memory deficit with Ucn2 injection before immobilization that targeted mouse anxiety level, the possible interpretation that exposure to 1-h immobilization produced a change in the mouse behavior that interfered with performance during the training or testing, including non-specific changes in motor activity or impaired attention to a learning task, has become highly unlikely.

Additionally, the possibility had to be considered that the pretreatment with Ucn2 resulted in a changed stress response that could be responsible for the observed learning enhancement (Cahill & McGaugh, 1998). Therefore, we determined the ACTH and corticosterone levels indicating the activation of the HPA axis, which is generally accepted as an important measure of the stress response. The ACTH and corticosterone levels were assayed immediately after the end of immobilization. A two-way ANOVA showed that mice injected with 400 ng Ucn2 i.c.v. 5 min before immobilization (1 h) and stressed mice not treated with Ucn2 exhibited significantly increased ACTH ($F_{1,36} = 20.71$; $P < 0.05$ main effect of stress) and corticosterone levels ($F_{1,36} = 114.29$; $P < 0.05$ main effect of stress) compared with non-stressed control groups (Scheffe test, $^{*}P < 0.05$ relative to non-stressed saline-injected mice; $^{\#}P < 0.05$ relative to non-stressed Ucn2-injected mice; Fig. 7B and C). No main effects of treatment were observed. In view of these data, it was not probable that changes in the corticosterone action played a major role in the regulation of anxiety and memory formation after exposure to 1-h immobilization stress. Surprisingly, as indicated by treatment–stress interactions ($F_{1,36} = 10.26$; $^{*}P < 0.05$ for ACTH vs non-stressed saline-injected mice; $F_{1,36} = 8.42$; $^{*}P < 0.05$ for corticosterone vs non-stressed saline-injected mice), the ACTH and corticosterone levels were also increased in mice 1 h after i.c.v. injection of Ucn2 in the absence of a stressful stimulus (Fig. 7B and C). Because it has been demonstrated that human Ucn2 is unable to stimulate ACTH release (Hsu & Hsueh, 2001), the site of this delayed action might involve CRF_2 of the paraventricular nucleus of the hypothalamus (PVN) or of brain areas, such as the medial nucleus of the amygdala or the bed nucleus of the stria terminalis, providing afferent input to the PVN (Swanson & Sawchenko, 1980).

Discussion

The results of the present study demonstrate that exposure to a 1-h immobilization stressor initially increased anxiety and resulted in the impairment of context-dependent conditioned fear through LS CRF_2 activation. Subsequent delayed activation of hippocampal CRF_1 was required for both processes to return to baseline levels. We hypothesize that anxiety played a significant role in the generation of the observed memory deficit and the subsequent recovery process. The finding that the stress-induced memory deficit observed in the fear conditioning paradigm was prevented by intraventricular injection of Ucn2 supported this hypothesis. By Ucn2 application stress-induced anxiety was reduced, and conditioned fear was enhanced without removal of the HPA response. If the activity of the HPA axis is recognized as one of the major measures of the hormonal stress response, it can be concluded that hormonal stress and anxiety-like behavior were dissociated to a significant extent under these conditions. Thus, the HPA axis activation with the resulting cascade of responses finally leading to corticosterone release did probably not contribute significantly to the observed memory deficit.

The data presented here confirmed earlier observations with other mouse and rat strains (Radulovic *et al*., 1999; Bakshi *et al*., 2002) that LS CRF_2 contributes significantly to the stress-induced changes in anxiety-like behaviors. Additionally, our findings that activation of LS CRF_2 concurrently elevated anxiety-like behavior levels and impaired contextual fear conditioning are consistent with the described dissociation of unconditioned and conditioned fear responses mediated by the LS. In particular, it has been repeatedly found that lesion or pharmacological inhibition of this area reduces animal anxiety-related behaviors in the EPM. Therefore, it has been suggested that the LS normally plays an excitatory role in the control of anxiety (Menard & Treit, 1999). On the other hand, several lines of research suggest that activation of the LS inhibits the expression of fear conditioning (Desmedt *et al*., 1998). For example, exposure to conditional contextual aversive stimuli leads to inhibition of septal activity (Thomas *et al*., 1991) and a decrease in excitatory glutamatergic neurotransmission (Garcia & Jaffard, 1996). Moreover, h/rCRF and the non-selective natural CRF analog urocortin 1 (Ucn1) possess the ability to blunt excitatory glutamatergic transmission in the LS under both normal and stressful conditions. These effects are blocked by administration of a CRF_2, but not a CRF_1, antagonist (Liu *et al*., 2004). Thus, it is possible that LS CRF_2 mediates changes in anxiety-like behavior by interactions between the CRF and glutamate systems.

Interestingly, recovery from stress-induced anxiety and deficit of contextual fear conditioning was prevented by blockade of hippocampal CRF_1. The phasic anxiolytic action of hippocampal CRF_1 seemingly contrasts with results obtained in experiments with CRF_1-deficient mice. On the basis of these experiments, an anxiogenic role has been assigned to CRF_1 (Smith *et al*., 1998; Timpl *et al*., 1998; Muller *et al*., 2003). The results reported here were surprising, because we observed that the anxiety-like behavior of mice was not changed by direct injection of h/rCRF into the dorsal hippocampus (C. Todorovic & J. Spiess, unpublished observations). However, our results are compatible with the assumption that CRF_1 predominantly exhibits anxiogenic actions. Such an assumption is made in analogy to the observation that CRF_2 exhibits predominantly, but not exclusively, anxiolytic actions. It should be noted that although i.h. administration of the selective non-peptidic CRF_1 antagonist DMP696 resulted in delayed recovery from stress-induced anxiety, its effects were significantly weaker than the effects observed after i.h. administration of the peptidic CRF receptor subtype non-selective antagonist Ast. It is possible that recovery from stress-induced anxiety requires more complex mechanisms not only involving hippocampal CRF_1, but also hippocampal CRF_2. In particular, recent results from our laboratory demonstrate that exposure to 1-h immobilization leads to upregulation of CRF_2 mRNA within the hippocampal subregions CA1, CA3 and

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the dentate gyrus. (Sananbenesi *et al*., 2003). In view of these findings and our recent observation that activation of CRF_2 of the dentate gyrus decreased anxiety levels in the EPM (C. Todorovic & J. Spiess, unpublished observations), it is possible that delayed synchronous activation of both CRF receptor subtypes in the hippocampus is required for the observed return to baseline anxiety. As to the context-dependent fear conditioning, blockade of CRF_1 in the dorsal hippocampus before immobilization prevented recovery from stress-induced learning impairment of contextual fear, thus leading to a prolonged memory deficit. This finding was in accordance with previous results from our laboratory that h/rCRF increases the neuronal activity (Blank *et al*., 2002) and enhances context-dependent fear conditioning of Balb/c mice through CRF_1 (Radulovic *et al*., 1999).

Results from our study suggested dissociation between anxiety-like behavior and context-dependent conditioned fear, measured by freezing behavior. It is recognized that freezing behavior belongs to a broader class of defensive-related behaviors of the mouse (Blanchard *et al*., 2003). If it appears without prior relevant learning experience freezing is considered unconditioned behavior (Blanchard *et al*., 2003). Thus, it can be argued that our study provides evidence for interaction between different anxiety forms. It should be emphasized, however, that our study revealed a relationship between anxiety-like behavior in the EPM and freezing only when the latter was used as an indicator of a learned response. This view is confirmed by the findings that C57BL/6J mice did not exhibit generalization of freezing during the retention test in the context that was not employed for conditioning. Such CS-dependency of freezing indicated that the prior exposure to a stressful stimulus and subsequent increased anxiety levels did not lead to the non-specific sensitization of mice, which may be reflected in an increased freezing response to a novel stimulus. Therefore, the nature and/or associative history of the stimulus that elicits certain defensive responses, but not the identity of the response itself, may provide a solid base for differentiation between processes of anxiety and conditioned fear (Charney & Deutch, 1996; Davis, 1998).

In addition, our study demonstrated profound modulation of context-dependent but not tone-dependent conditioned fear after stress-induced activation of CRF_2 of the LS and delayed hippocampal CRF_1 activation. The finding that CRF_2 and CRF_1 of the LS and hippocampus, respectively, modulated only context- but not tone-dependent fear conditioning is consistent with the well-established role of these structures in this form of learning (Kim & Fanselow, 1992; Sparks & LeDoux, 1995). The differential time course of recovery from stress-induced impairment of context- and tone-dependent fear conditioning, as presented in our study, suggested that processing of these two types of stimuli did not follow the same pathways in C57BL/6J mice. Interestingly, i.c.v. application of the CRF_2-selective agonist Ucn2 reduced the stress-induced deficit of both context- and tone-dependent conditioned fear. Thus, we hypothesize the involvement of the non-septal CRF_2-dependent mechanism in the regulation of tone-dependent conditioned fear. Our study did not provide the receptor site of such action on tone-dependent conditioned fear. Recent results demonstrate that pharmacological inhibition of CRF receptor subtypes in the BLA impairs memory consolidation in an aversively motivated learning task (Roozendaal *et al*., 2002). Taking this finding into account together with the view that BLA is critical for conditioned fear responses to both contextual and discrete, explicit CS (Davis, 1998; Fanselow & LeDoux, 1999), one can hypothesize that Ucn2 may exert its effects on context- and tone-dependent fear conditioning through actions of BLA CRF_2. This requires further testing.

Work in our laboratory has already demonstrated a differential modulation of context- and tone-dependent fear conditioning of Balb/c mice through brain region- and CRF_1- and CRF_2-specific mechanisms (Radulovic *et al*., 1999). The observation from the present study that activation of septal CRF_2 impaired, whereas hippocampal CRF_1 enhanced fear conditioning suggested that the regulatory role of septal and hippocampal CRF receptors in fear conditioning is shared by Balb/c and C57BL/6J mice. The observed differences in response to a stressful stimulus between these two mouse lines could be attributed to a different extent and time course of CRF receptor subtype activation. For example, in C57BL/6J mice stress appears to exert immediate effects predominantly through septal CRF_2, thus producing learning impairment. Subsequent activation of hippocampal CRF_1 in both strains mediated recovery from the learning deficit for C57BL/6J mice, or improvement above the control values for Balb/c mice. Thus, the regional components of the CRF_1 and CRF_2 systems contribute differentially to the baseline and stress-induced modulation of fear conditioning in a strain-dependent fashion. Importantly, a recent study from our laboratory provided molecular insight into such a phenomenon. We find that the CRF system activates different intracellular signaling pathways in the hippocampus of Balb/c and C57BL/6N mice, and may have distinct effects on fear conditioning depending on the mouse strain investigated (Blank *et al*., 2003).

Intraventricular administration of Ucn2, but not of Ucn3, resulted in the reduction of anxiety-like behavior in the EPM as indicated here. In the same behavior test, CRF_2-deficient mice show increased anxiety-like behavior (Bale *et al*., 2000; Kishimoto *et al*., 2000). Thus, it was concluded that endogenous Ucn2 might mediate, probably via non-septal CRF_2, behaviors associated with a state of decreased anxiety. It is possible that the non-septal CRF_2-mediated decrease in anxiety limits the extent of the behavioral stress response mediated by septal CRF_2, and this may be beneficial by preventing excessive anxiety increase, which may limit the successful response to a stressful situation. This assumption was corroborated by our findings that intraventricular injection of Ucn2 prevented both elevated anxiety-like behavior and impairment of fear conditioning after exposure to a stressful stimulus.

The absence of behavioral effects of Ucn3 in the EPM test both contradicted and confirmed other studies. While one study reported that rats display a significantly increased preference for the open arms of the EPM following intraventricular injections of Ucn3 (Valdez *et al*., 2003), others did not observe behavioral effects elicited by Ucn3 in the EPM (Venihaki *et al*., 2004). The fact that intraseptal injection of Ucn2, close to the limit of its solubility using saline as solvent (96 pmol), did not lead to changes in anxiety-like behaviors in the EPM, confirmed the recent observation that, in the absence of a stressor, doses of Ucn2 below 240 pmol, injected into the LS, lack behavioral potency in various tests of anxiety (Henry *et al*., 2006). Consistently, i.c.v. injection of Ucn2 induces production of c-Fos protein in the bed nucleus of the stria terminalis, the central nucleus of the amygdala, the PVN, parabrachial nucleus and nucleus of the solitary tract, but not in CRF_2-rich regions such as the LS, raphe nuclei and the ventromedial nucleus of the hypothalamus (Reyes *et al*., 2001). The interpretation of behavioral effects by Ucn2 and Ucn3 is likely to be complicated by the identification of several splice variants of the CRF_2 gene expressed in the mouse forebrain (Kostich *et al*., 1998; Miyata *et al*., 2001; Catalano *et al*., 2003; Chen *et al*., 2005). It can be speculated that this variety of molecular species is responsible for the differential responsiveness of CRF_2 to stimulation by Ucn2 and Ucn3.

In summary, our data indicate that a stress-induced memory deficit probably results from the enhanced anxiety phase that is

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induced by the stressful experience rather than activation of the HPA axis. A new concept for the roles of CRF_1 and CRF_2 in both the acute and recovery phase of the stress-induced changes in fear-related behaviors is proposed. Exposure to a stressor appears to affect initially both anxiety-like behavior and contextual conditioned fear through septal CRF_2, while the later activation of hippocampal CRF_1 enables the CRF system to restore the baseline levels of conditioned fear and, probably, anxiety. As the levels of anxiety and conditioned fear were inversely affected by direct stimulation of CRF_2 in the LS and by i.c.v. application of Ucn2 prior to immobilization and fear conditioning, it was concluded that fear and anxiety formation represented dissociable, dynamically interacting, biological processes.

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Abbreviations

aCSF, artificial cerebrospinal fluid; ACTH, adrenocorticotropic hormone; Ast, astressin; aSvg-30, anti-sauvagine-30; BLA, basolateral nucleus of the amygdala; CRF, corticotropin-releasing factor; CRF_1, corticotropin-releasing factor receptor 1; CRF_2, corticotropin-releasing factor receptor 2; CS, conditioned stimulus; EPM, elevated plus maze; HPA, hypothalamic pituitary adrenal axis; h/rCRF, human/rat CRF; i.c.v., injection into the lateral ventricles; i.h., injection into the dorsal hippocampus; i.s., injection into the lateral septum; LS, lateral septum; LSi, lateral intermediate septum; PBS, phosphate-buffered saline; PVN, paraventricular nucleus of the hypothalamus; SPL, sound pressure level; Ucn1, urocortin 1; Ucn2, urocortin 2; Ucn3, urocortin 3; US, unconditioned stimulus.

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