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ORIGINAL INVESTIGATION

Effects of fluoxetine on CRF and CRF1 expression in rats exposed to the learned helplessness paradigm

Georgina Valeria Fernández Macedo · María Laura Cladouchos · Laura Sifonios · Pablo Martín Cassanelli · Silvia Wikinski

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Abstract
Rationale Stress is a common antecedent reported by people suffering major depression. In these patients, extrahypothalamic brain areas, like the hippocampus and basolateral amygdala (BLA), have been found to be affected. The BLA synthesizes CRF, a mediator of the stress response, and projects to hippocampus. The main hippocampal target for this peptide is the CRF subtype 1 receptor (CRF1). Evidence points to a relationship between dysregulation of CRF/CRF1 extrahypothalamic signaling and depression.
Objective Because selective serotonin reuptake inhibitors (SSRIs) are the first-line pharmacological treatment for depression, we investigated the effect of chronic treatment with the SSRI fluoxetine on long-term changes in CRF/CRF1 signaling in animals showing a depressive-like behavior.
Methods Male Wistar rats were exposed to the learned helplessness paradigm (LH). After evaluation of behavioral impairment, the animals were treated with fluoxetine (10 mg/kg i.p.) or saline for 21 days. We measured BLA CRF expression with RT-PCR and CRF1 expression in CA3 and the dentate gyrus of the hippocampus with in situ hybridization. We also studied the activation of one of CRF1's major intracellular signaling targets, the extracellular signal-related kinases 1 and 2 (ERK1/2) in CA3.
Results In saline-treated LH animals, CRF expression in the BLA increased, while hippocampal CRF1 expression and ERK1/2 activation decreased. Treatment with fluoxetine reversed the changes in CRF and CRF1 expressions, but not in ERK1/2 activation.
Conclusion In animals exposed to the learned helplessness paradigm, there are long-term changes in CRF and CRF1 expression that are restored with a behaviorally effective antidepressant treatment.

Keywords Major depression · Stress · CRF · CRF1 · ERK1/2 · Antidepressant drug · Basolateral amygdala · Hippocampus

G. V. Fernández Macedo (✉) · M. L. Cladouchos · L. Sifonios · P. M. Cassanelli · S. Wikinski
Instituto de Investigaciones Farmacológicas (ININFA-UBA/CONICET),
Junín 956, 5º piso,
1113, City of Buenos Aires, Argentina
e-mail: georginafm@ffyb.uba.ar

S. Wikinski
Primera Cátedra de Farmacología, Facultad de Medicina,
Universidad de Buenos Aires,
Paraguay 2051, 15º piso,
1113, City of Buenos Aires, Argentina

Introduction

Major depression is a debilitating syndrome that forms part of several mood disorders affecting more than 120 million people all over the world (WHO 2011). Vulnerability to stress is a hallmark of depression. Both exposure to stressful events and high sensitivity to nonstressful threats are common antecedents reported by people suffering from a depressive episode (Nestler et al. 2002).

Corticotrophin-releasing factor (CRF) is an important mediator of the stress response both in the hypothalamic and extrahypothalamic systems (Aguilera 1998; Bao et al. 2008; Cratty et al. 1995; Dirks et al. 2002; Dunn and Berridge 1990; Dunn and Swiergiel 2008; Gallagher et al. 2008; Hauger et al. 2006; Holsboer 1999; Luo et al. 1994; Wang et al. 2011; Ziegler and Herman 2002). At the hypothalamic level, CRF released from the paraventricular nucleus (PVN) controls the hypothalamic–pituitary–adrenal (HPA) axis response to stress coordinating energy disposition and storage (Dallman et al. 1995). At the extrahypothalamic level, CRF is present in different neuronal circuits (Swanson et al. 1983) and participates

as a neuroregulator in the behavioral and emotional integration of environmental and endogenous inputs associated with stress.

The basolateral amygdala (BLA) that contains CRF-synthesizing neurons (Falco et al. 2009), where an increment in CRF immunoreactive neurons during stress is observed (Becker et al. 2007), and that participates during periods of stress regulating anxiety and affective responses (Shekhar et al. 2005) forms part of this extrahypothalamic circuit together with the hippocampus. These two regions have been found to be affected in depressed patients. On one hand, an abnormal activation in response to negative stimuli (Fu et al. 2004; Siegle et al. 2002) and increased volume of the amygdala in depressed patients (Frodl et al. 2002; Tebartz van Elst et al. 2000) have been consistently shown. On the other hand, decreased volume (Bremner et al. 2000; Neumeister et al. 2005; Sheline et al. 1996, 2003) and trophic and functional alterations in the hippocampus have been found in depressed patients and in animal models of the disease (Bergstrom et al. 2008; Bravo et al. 2009; Hageman et al. 2008; Hajszan et al. 2005; Magarinos et al. 1996, 1997; Neumeister et al. 2005; Reines et al. 2004, 2008; Soetanto et al. 2010; Watanabe et al. 1992), suggesting hypofunction of this structure.

In the hippocampus, the main CRF target is the subtype 1 receptor (CRF1, Refojo et al. 2005). Evidence points to a relationship between dysregulation of CRF/CRF1 extrahypothalamic signaling and depression. Transgenic mice with a conditional deletion of CRF1 in corticolimbic structures show normal HPA axis functioning but a reduced anxiety response (Muller et al. 2003), and the genetically induced overexpression of CRF in the entire brain induces an anxious phenotype (Kasahara et al. 2007). Accordingly, anxiolytic and antidepressant-like effects of CRF1 antagonists in different animal models of depression have been reported (Arborelius et al. 2000; Gutman et al. 2011; Hodgson et al. 2007; Mansbach et al. 1997; Overstreet and Griebel 2004; Overstreet et al. 2004; Yamano et al. 2000). However, recent findings on the therapeutic efficacy of these compounds in depressed patients are controversial, since after some evidence in favor of CRF1 antagonists antidepressant activity appeared (Zobel et al. 2000) further exploration rendered negative results (Binneman et al. 2008).

The effect of clinically active antidepressant drugs on CRF and CRF1 has been extensively investigated (Bonne et al. 2010; Kim et al. 2006; Lowry et al. 2009; Raone et al. 2007; Santibanez et al. 2006; Stout et al. 2002). Remarkably, although selective serotonin reuptake inhibitors (SSRI) are the first-line pharmacological treatment for this group of diseases and the hippocampus and BLA are important brain regions of CRF action in depressive disorders, SSRI activity on this signaling pathway has been scarcely investigated. For instance, it has been shown that preventive treatment with fluoxetine does not exert any effect on PVN CRF overexpression while treatment with venlafaxine restores PVN CRF levels in animals exposed to 15-min swim stress or to chronic unpredictable stress (Stout et al. 2002).

Thus, the goal of our work was to investigate the effect of chronic treatment with the SSRI fluoxetine on the expression of CRF in the BLA together with the expression of CRF1 in the hippocampus of animals showing a depressive-like behavior. For this purpose, we employed the learned helplessness paradigm (LH), a well-validated model for investigating the antidepressant-like effect of drugs and the association between depressive-like behavior and specific neurobiological changes. Additionally, many other features reminiscent of those present in depressive patients, such as disruption of feeding, diminished consumption of palatable solutions, alterations in dominance hierarchies, and lack of response to rewarding brain activation, have also been demonstrated in animals exposed to this paradigm (Anisman and Merali 2001; Vollmayr et al. 2004). Learned helplessness is induced by exposure to inescapable or uncontrollable stress and is operationalized by the measurement of the latency to escape to subsequent stressful stimuli (Seligman and Maier 1967). In our laboratory, employing this model, we were able to document the persistence of the helpless behavior in absence of treatment (i.e., 115 days), its reversion after chronic administration of the antidepressant fluoxetine, as well as the correlation of the behavioral efficacy of the treatment with plastic changes in brain areas relevant for the depressive-like behavior (Reines et al. 2008).

Methods

Animals

Male Wistar rats (Bioterio Central, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina) weighing at least 230 g at the beginning of the experiments were housed in an air-conditioned room (20±2 °C temperature) with a 12:12-h light/dark cycle (lights on at 8:00 a.m.) and food and water supplied ad libitum. To avoid stress evoked by social isolation, animals were held in groups of four in standard laboratory cages. Experiments were conducted according to the Guide for the Care and Use of Laboratory Animals first edition (1996) provided by the National Institutes of Health, USA and guidelines provided by local authorities (Administración Nacional de Medicamentos, Alimentos y Tecnología Médica, Argentina). The minimum possible number of animals was used to achieve statistical significance. All efforts were made to minimize animal suffering.

Learned helplessness paradigm

The LH paradigm was performed according to Anisman and Merali (2001) with modifications previously described (Ferrero et al. 2005, 2007; Reines et al. 2004, 2008; Sifonios et al. 2009). Experimental conditions for the LH paradigm had been previously established in our laboratory by a set of experiments performed with the aim of carefully balancing the success of obtaining animals with helpless behavior that lasted at least 25 days; the suffering the animals were subjected to and the total number of animals needed. The efficacy of shocks of 0.3, 0.6, and 0.9 mA, 10 or 15 s duration, and 30 or 60 (in 30 or 60 min) total shocks were compared by Kruskal–Wallis test followed by Dunn's multiple comparison test. Ultimately, sixty 0.6-mA shocks lasting 15 s each were significantly different from the other conditions ($p<0.05$). This protocol resulted in 55 % of animals expressing the LH behavior (escape latencies ≥15 s on the test session, as described below) and was employed thereafter. Interestingly, these experimental conditions are intermediate regarding the least (Mallei et al. 2011) and the most (Nakagawa et al. 1999; Saade et al. 2003; Song et al. 2006; Takamori et al. 2001b) intense conditions recorded in the literature. Because previous studies (Stewart et al. 1990) failed to find any effect of lighting on escape performance after inescapable foots hocks, experiments were performed in the morning between 8 and 11 a.m.

Briefly, the animals were randomly assigned to LH or control groups. An acquisition session was performed to induce helplessness behavior; the animals were individually placed in a shuttle box and subjected to 15-s foot shocks (0.6 mA) delivered every 60 s for an hour. Control animals were exposed to the same conditions but without the electric shocks. At the end of this session, animals were returned to their cages. On day 4, acquisition of the helplessness behavior was evaluated through an active task that measured the latency to escape from foot shocks. Animals were placed in a shuttle box that consisted of two equal-sized compartments divided by a Plexiglas partition with a door. The test consisted of 15 trials performed in 15 min. Twenty-second shocks (0.6 mA) were delivered on each trial. The door was opened to let the rat escape to an electric shock-free compartment immediately after starting the shock in the first five trials. In the other 10 trials, the door was opened 3 s after starting the shock. By this procedure it is possible to differentiate a transient motor activation from a true and persistent escape behavior (Anisman and Merali 2001).

Mean escape latency was calculated over the 15 trials. A total of 51 rats were employed. As previously published (Ferrero et al. 2005, 2007; Reines et al. 2004, 2008; Sifonios et al. 2009), animals submitted to inescapable shocks ($n=33$) showing mean escape latencies of 15 s or more were considered to have acquired helpless behavior, and were classified as LH ($n=18$). Animals not showing behavioral despair were no longer evaluated in these experiments (15 rats). Control animals were also tested on day 4 and were classified as C ($n=17$). Occasionally some control animals (5 %, 1 rat) failed to escape from foot shocks on day 4. This animal was excluded from the protocol. On day 25, before the daily injection of fluoxetine, a test session similar to that performed on day 4 was carried out to evaluate the effect of the pharmacological treatment on LH behavior.

Pharmacological treatment

To evaluate the effect of the antidepressant fluoxetine on the parameters under study, animals classified as LH on day 4 were randomly assigned to receive daily (at 12 p.m.) intraperitoneal injections (i.p.) of 1 ml/kg of a 10-mg/ml fluoxetine solution (LH-F, $n=10$) or the same volume of saline (LH-S, $n=8$) for 21 days (from days 5 to 25). Control animals were randomly assigned to the same treatment (C-F, $n=7$or C-S, $n=10$). To avoid the acute effect of the test session on day 25 (Greetfeld et al. 2009; Luo et al. 1994), animals were sacrificed 24 h after the second test (on day 26). The dose of fluoxetine employed in this work was selected based on previous reports (Mato et al. 2010; Reines et al. 2008; Rodriguez-Gaztelumendi et al. 2009). Fluoxetine was kindly provided by Laboratorios Gador (Argentina).

Tissue preparation

Coronal sections or BLA punches were randomly obtained from animals from the four experimental groups. There were no differences between the mean escape latencies of the animals assigned to RT-PCR and ISH or IF experiments (Mann–Whitney test, $p=0.7910$). To obtain the tissues of interest for RT-PCR, rats were anaesthetized i.p. with a mixture of ketamine hydrochloride (50 mg/kg) and xylazine (2 mg/kg) and decapitated. Brains were removed, and bilateral BLA tissue punches were taken from coronal brain slices (1 mm tissue puncher, 1-mm-thick Brain Slicer Zivic Instruments, Pittsburgh, USA) delimited at the beginning of the slice by plate 25 of the atlas of Paxinos and Watson (1998; coordinates bregma −2.80 mm, dorsoventral and lateral references corresponding to the mentioned plate). The punches included mainly the BLA nucleus and some part of the lateral amygdala. Considering the position of the punch (see Fig. 2a), the central amygdala (CeA) was excluded. The BLA punches were stored at −80 °C until use.

To obtain the tissues of interest for in situ hybridization and immunofluorescence experiments, coronal brain sections were collected as previously described (Sifonios et al. 2009). Only tissue sections corresponding to plates 32 to 34 of the atlas of Paxinos and Watson (1998; coordinates

from bregma −3.14 to −3.60 mm, dorsoventral and lateral references corresponding to the mentioned plates) were employed in the experiments.

mRNA CRF real-time RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) followed by DNase digestion (Promega Corporation, Madison, WI). The real-time RT-PCRs were performed using the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The primers and probes have been previously described (Floyd et al. 2003; Lack et al. 2005). The probes were TAMRA probes labeled with 6-FAM for CRF and VIC for the housekeeping gene GAPDH.

The RT-PCR was performed using TaqMan® One-Step RT-PCR Master Mix Reagents (Applied Biosystems). The expression of CRF mRNA was analyzed by the $\Delta\Delta C_T$ method (Floyd et al. 2003; Livak and Schmittgen 2001). Experimental mRNA abundance relative to control (relative quantity, RQ) was calculated as $2^{-\Delta\Delta Ct}$.

CRF1 probe generation

cRNA digoxigenin (DIG)-labeled probes were generated under RNAse-free conditions from a pBluescript plasmid (Stratagene, CA) containing a fragment of cDNA encoding rat CRF1 donated by Dr. W. Vale (Salk Institute, CA, USA). The plasmid was linearized with *HindIII* or *XbaI* (Promega Corporation, Madison, WI) for transcription of antisense or sense probes, respectively. The linearized plasmid was purified using the high pure PCR purification kit (Roche, Mannheim, Germany), and the in vitro transcription was performed with the Dig RNA Labeling kit (Sp6/T7, Roche). DIG-labeled cRNA probes were analyzed on agarose gels (1 %) and quantified by absorbance at 260 nm.

In situ hybridization

The CRF1 in situ hybridizations (ISH) were performed under RNAse-free conditions, and one section per animal (four animals per group) was employed in each experiment. Six separate experiments were performed. Coronal sections containing two hippocampi each were mounted onto gelatin-coated slides and air-dried for 2 h. They were washed in 2× saline sodium citrate (SSC) and treated with proteinase K (5 μg/ml) for 30 min at 37 °C. The sections were then washed with 2× SSC and incubated with 0.25 % acetic anhydride in 0.1 M triethanolamine. The tissues were washed again in 2× SSC and incubated for 2 h at 60 °C with prehybridization solution (50 % formamide, 0.3 M NaCl, 0.001 M EDTA, 1× Denhart solution,10 % dextran sulfate, in 2× SSC). Next, they were incubated for 16 h at 60 °C with the hybridization solution (20 μg/ml tRNA, antisense probe 3 ng/μl in prehybridization solution). After hybridization, the sections were washed with 4× SSC and treated with RNAse A (20 μg/ml RNAse A, 0.5 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 8) for 1 h at 37 °C. The sections were rinsed in descending concentrations of SSC (2×, 1×, 0.5×, 0.1×). The detection of the labeled probe was performed with a DIG Nucleic Acid Detection Kit (Roche) according to manufacturer's instructions. The slides were covered with 70 % glycerol in 0.1 M phosphate buffer saline (PBS). The specificity of the labeling was assessed in three different experiments: specificity of the probe (using the sense probe instead of the antisense probe), specificity of the detection of RNA (treating the slides with 40 μg/ml RNAse A before hybridization), and specificity of the colorimetric detection (without labeled probe). Neither control experiment demonstrated a detectable hybridization signal.

ERK1/2 and pERK1/2 immunofluorescence

For the immunofluorescence (IF) staining of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) and their phosphorylated forms (pERK1/2), one section per animal (four animals per group) was employed in each experiment. Three experiments were performed. To remove the cryoprotectant, the free-floating sections were washed in 0.01 M PBS. For pERK1/2 immunofluorescence only, the slices were permeabilized with 0.1 M boric acid pH 8.5 for 10 min at room temperature. After that, tissues were incubated with the blocking solution (0.01 M PBS, 0.3 % Tritón X-100, and 5 % normal goat serum) in a shaker for an hour at room temperature. Next, the slices were incubated with the following primary antibodies: anti-ERK1/2 (rabbit monoclonal anti-p44/42 MAPK ERK1/2; Cell Signaling, Danvers, MA, USA) or anti-pERK1/2 (mouse monoclonal anti-phospho-p44/42 MAPK ERK1/2 Thr202/Tyr204 IgG1, Cell Signaling), diluted 1:200 or 1:100, respectively, in 0.01 M PBS, 0.3 % Tritón X-100, and 5 % normal goat serum, for 16 h at 4 °C. Then, brain sections were washed three times and incubated, in agitation, with the secondary antibody rhodamine red™-X-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:1,200 or rhodamine red™-X-conjugated goat anti-mouse IGg1 (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:1,000 in 0.01 M PBS, 0.3 % Tritón X-100, and 1 % normal goat serum in agitation in the dark for an hour. Finally, the tissues were washed three times and mounted onto gelatin-coated slides, cover-slipped with Vectashield® Mounting Medium (Vector Laboratories, Burlingame, CA) and stored at 4 °C in the dark. No immunofluorescent labeling was observed when the primary antibody was omitted.

Image capture and analysis

CRF1 staining was analyzed in the Ammon's horn 3 (CA3) and dentate gyrus (DG) regions of the hippocampus. ERK1/2 and pERK1/2 staining were analyzed only in CA3.

CRF1 images were captured through a ×20 objective, while ERK1/2 and pERK1/2 images were captured through a ×40 objective on a Nikon ECLIPSE 50i light microscope equipped with a DS-5Mc cooled camera. A 510–560-nm excitation filter was used for rhodamine Red™-X-labeled ERK1/2 and pERK1/2 images. Homogeneous illumination of the microscopic field was kept constant by centering the light source. For every image, the entire *z*-axis (30 μm) was examined, and the focal plane chosen was that which allowed the best visualization of the specific labeling (i.e., typical labeling patterns for each protein and segmentation from background). For both immunofluorescent and colorimetric labeling, the analog images were digitized into an array of 512×512 pixels. The resolution of each pixel was 256 gray levels.

Optical density measurement

The staining intensity of all immunoreactive structures was calculated with ImageJ software (National Institute of Health, available at http://rsb.info.nih.gov/nihimage) based on optical density (OD) as previously described (Sifonios et al. 2009).

Statistical analysis

Behavioral data are expressed as the median±the interquartile range (from 7 to 10 animals per group). Since our experimental conditions do not allow a normal distribution of the data obtained (i.e., a maximum of 20 s was established for each escape trial), nonparametric statistical tests were employed for behavioral experiments. The data from the avoidance task performed on day 4 were analyzed with a Mann–Whitney test, and those from day 25 were analyzed with Kruskal–Wallis test followed by Dunn's multiple comparison test.

For ISH and immunofluorescence experiments, averages of OD in tissue sections (two hippocampi per section from 4FOUR animals per group) were obtained. Then, mean values (±SEM) of averages obtained in three (IF) or six (ISH) consecutive experiments for each group were calculated. RQs were individually obtained for each animal (three to six animals per group) in three consecutive experiments and averaged. Mean values (±SEM) of RQ per group were then calculated. Comparisons between experimental groups were carried out by two-way ANOVA followed by Bonferroni post hoc test. The correlation between escape latencies and CRF mRNA expression was analyzed by a Pearson's correlation test

All statistical analyses were performed with the GraphPadPrism 3.1 (GraphPad Software Inc.). Differences were considered significant when the p value was lower than 0.05.

Results

Fluoxetine reverses LH behavior

We used the learned helplessness paradigm as an animal model of depression (Fig. 1a). Depressive-like behavior was evaluated with an active avoidance task. On day 4, we characterized the behavioral impairment of rats submitted

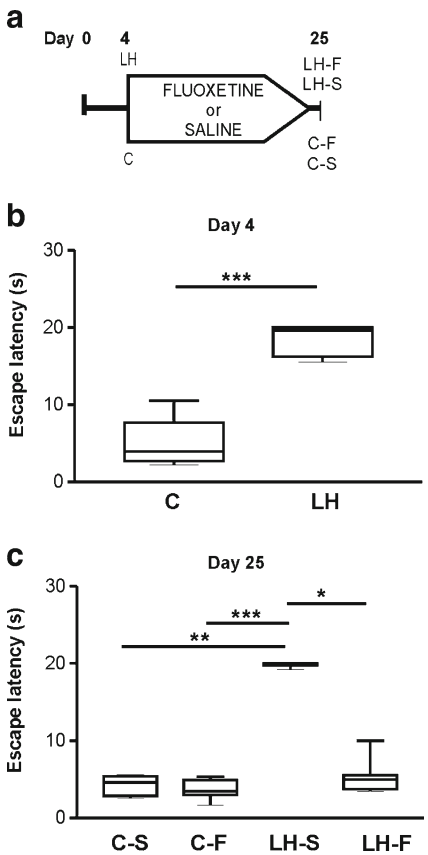

Fig. 1 Learned helplessness paradigm. **a** Schematic representation of the experimental procedure. On day 0, the animals were exposed to the LH paradigm. On day 4, the acquisition of helplessness behavior was evaluated. From days 5 to 25, the animals were treated with fluoxetine or saline. On day 25, the persistence of LH behavior after the pharmacological treatment was evaluated. **b** Behavioral performance on day 4 measured by escape latency. **c** Behavioral performance on day 25 measured by escape latency. *p<0.05, **p<0.01, ***p<0.001. Abbreviations: *C* control rats; *LH* rats that acquired the learned helplessness behavior; *C-S* control rats treated with saline; *C-F* control rats treated with fluoxetine; *LH-S* rats that developed the behavioral despair treated with saline; *LH-F* rats that developed the behavioral despair treated with fluoxetine

to the acquisition trial. The Mann–Whitney test showed that C animals had a lower mean escape latency compared to LH animals ($p<0.001$; Fig. 1b). LH and C animals received 21 daily injections of fluoxetine or saline. Rats that failed to develop the despair behavior on day 4 despite being subjected to the acquisition session were not further studied because, in clinical treatment, fluoxetine is ordinarily administrated if behavioral impairment is detected. To check the antidepressant-like effect of the treatment, animals were subjected to a test session on day 25. As previously published (Reines et al. 2008), fluoxetine administration reverted the despair behavior in LH rats (Fig. 1c). Escape latencies were analyzed by Kruskal–Wallis test followed by Dunn's multiple comparison test. Escape latencies of LH-F rats were similar to those observed in C-S or C-F groups. Moreover, despair behavior persisted in LH animals treated with saline, with escape latencies significantly higher than those shown by LH-F, C-S, or C-F animals ($p<0.05$, $p<0.001$, and $p<0.01$, respectively).

Fluoxetine restores CRF mRNA expression in BLA from LH animals

The BLA is a brain area involved in the stress response and hypothesized to be hyperactive in depression. Even more, the BLA is involved in the expression of LH behavior (Hammack et al. 2011). The levels of BLA CRF mRNA were quantified by real-time RT-PCR 26 days after rats had been exposed to the acquisition trial. Figure 2a depicts the punch position in the coronal slice used to obtain the BLA. When the effect of fluoxetine on the levels of BLA CRF expression was evaluated by a two-way ANOVA (Fig. 2b), we found a significant interaction between fluoxetine treatment and behavioral performance ($F_{1,15}=6.895$, $p<0.05$). Therefore, the simple effects were analyzed. We found that the levels of expression in LH-S rats were higher than those in C-S animals ($p<0.05$). We observed that chronic treatment of LH rats with fluoxetine exerts a full corrective effect on increased CRF mRNA levels because values for LH-F and C-F groups were similar, while LH-F values were significantly lower than those for LH-S ($p<0.01$). Interestingly, when the correlation coefficient between CRF expression and escape latencies in the test session was assessed, a significant positive value was obtained ($r=0.8072$, $p<0.0001$; Fig. 2c).

Fluoxetine reverses the persistent decrease in CRF1 mRNA expression in LH animals

Measurement of CRF1 expression through colorimetric in situ hybridization was performed 26 days after the exposure to uncontrollable stress. The labeling pattern of CRF1 mRNA in various brain areas of naive animals, as shown in Fig. 3a, was similar to that reported by Van Pett et al. (2000). The hippocampus displayed a continuous and moderate signal in the cells of Ammon's horn. The quantity of signal in the dentate gyrus was smaller than that observed in the CA1 or CA3 areas.

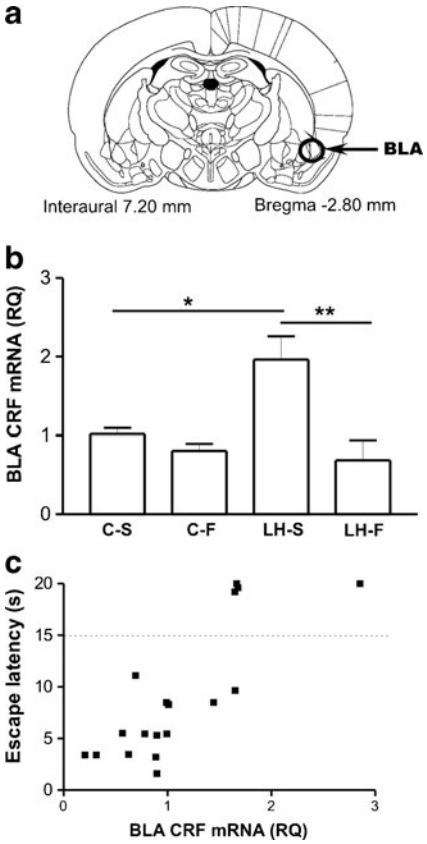

Fig. 2 **a**–**c** Real-time RT-PCR of mRNA CRF. **a** Schematic illustration of the punch position for extraction of the BLA, adapted from Paxinos and Watson (1998). **b** Quantification of mRNA CRF RQ in the BLA. **c** Correlation between mRNA CRF RQ and escape latencies. *$p<0.05$; **$p<0.01$, $p>0.05$ not shown. *RQ* relative quantity

The mRNA signal was measured in the CA3 pyramidal cells of the experimental groups, and the analysis with a two-way ANOVA test showed a significant interaction between fluoxetine treatment and behavioral performance ($F_{1,14}=7.261$; $p<0.001$). When the simple effects were examined, the LH-S group presented a persistent decrease in CRF1 mRNA OD values compared to the C-S group ($p<0.01$). Fluoxetine promoted the correction of CRF1 mRNA OD because the LH-F group showed similar values to the C-F group (Fig. 3b, c).

When we evaluated the CRF1 mRNA expression in the DG with a two-way ANOVA test, we found no significant interaction between fluoxetine treatment and behavioral performance ($F_{1,10}=1.459$, p=ns). LH-S animals showed a significant decrease in CRF1 mRNA OD compared to C-S animals ($p<0.05$). The chronic fluoxetine treatment caused a restoration of the OD levels because the LH-F animals had OD values similar to those of the C-S or C-F animals (Fig. 3d, e).

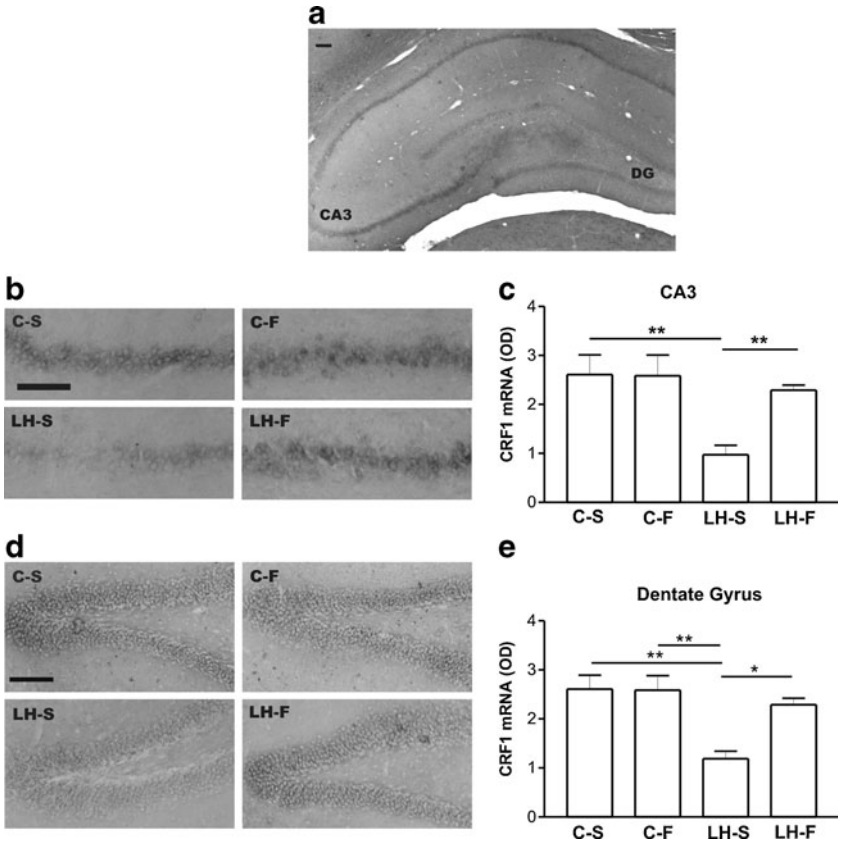

Fig. 3 In situ hybridization of CRF1 mRNA measured in the hippocampus. **a** Light photomicrograph of the general pattern of mRNA CRF1 expression in the hippocampus. **b** Representative photomicrographs of in situ hybridization in CA3 of experimental groups. **c** Quantification of CRF1 signal in CA3 measure by OD. **d** Representative photomicrographs of the in situ hybridization in the DG of experimental groups. **e** Quantification of CRF1 signal in the DG measure by OD. *$p<0.05$; **$p<0.01$; $p>0.05$ not shown. *Scale bar* 100 μm. *OD* optical density

Persistent decrease in ERK1/2 activity in LH animals is not reversed by fluoxetine treatment

One of the intracellular cascades triggered by CRF in the CA3 area of the hippocampus is the MAPK signaling pathway (Refojo et al. 2005). We performed an IF experiment of the MAPK ERK1/2 and its phosphorylated form to evaluate if the CRF1 decrease was observed in the LH group and if the effect of fluoxetine correlates with an alteration in this intracellular pathway. As usually reported (Corvol et al. 2005; Paul et al. 2007; Punn et al. 2006; Takagi et al. 2002), the ERK1/2 and the pERK1/2 labeling were quantified in the soma of the CA3 cells (Fig. 4a, b).

When we evaluated the ERK1/2 OD by a two-way ANOVA test, a strong trend towards a significant interaction between fluoxetine treatment and behavioral performance was observed for ERK1/2 ($p=0.0505$). When comparison among groups was performed, even though OD in LH-S rats did not differ from C-S ones, we found a significant effect of the treatment ($F_{1,8}=7.744$, $p=0.001$) because fluoxetine promoted an increase in ERK1/2 OD in C animals (C-F vs. C-S, $p<0.05$; Fig. 4c)

When the phosphorylated form was studied (Fig. 4b, d) using a two-way ANOVA test, we found no significant interaction between fluoxetine treatment and behavioral performance ($F_{1,8}=0.3856$, p=ns). A decrease in activation levels in LH-S rats was found in comparison with C-S ($p<0.05$). Fluoxetine treatment failed to reverse the changes in the activation of the MAPKs observed in LH-S animals; we found a persistent decrease in LH-F compared to C-F animals ($p<0.05$), and no differences were observed between LH-F and LH-S rats.

Discussion

The aim of this work was to investigate the effect of fluoxetine on the long-term changes in BLA and hippocampal CRF/CRF1 signaling pathways induced by exposure to the LH paradigm. The LH paradigm mimics several features observed in depressive disorders (Anisman and Merali 2001) and, as previously shown (Reines et al. 2008), in absence of treatment, the impairments can last at least 115 days, a picture that resembles the clinical presentation of a depressive episode that in absence of treatment could last 3 to 6 months (Posternak et al. 2006). Because the treatment was administered only to animals effectively showing depressive-like behavior, our experimental approach reproduces the clinical scenario in which treatment is provided once the behavioral and emotional impairment is present. Moreover, as we also evaluated the behavioral efficacy after chronic antidepressant administration, we can link the behavioral consequences of the acquisition session and the antidepressant treatment to the changes in

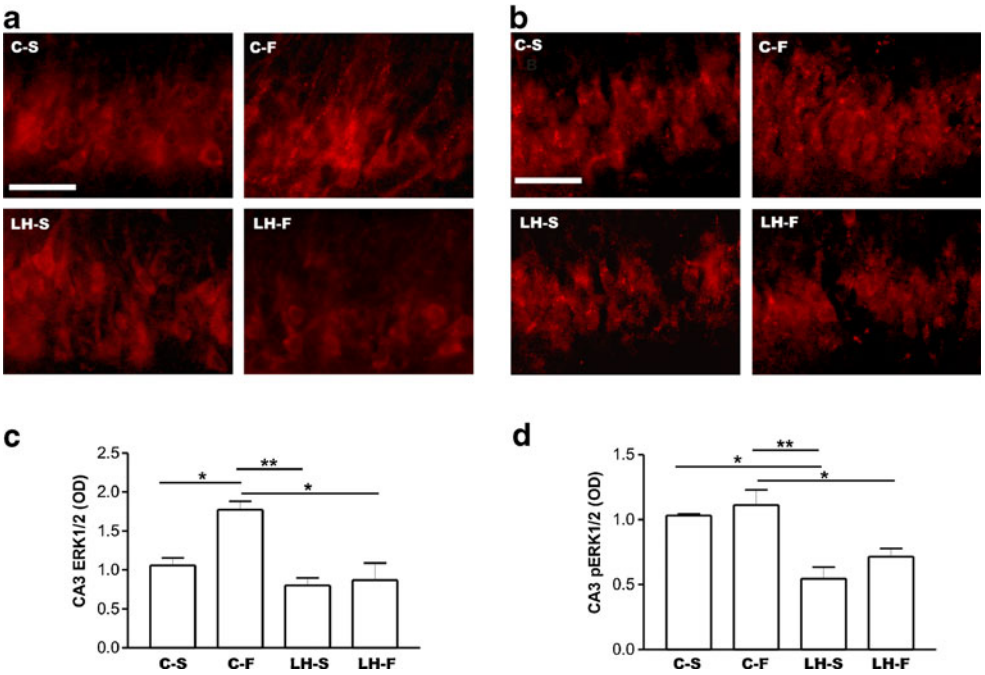

Fig. 4 Immunofluorescence of ERK1/2 and pERK1/2 in the CA3 region of the hippocampus. **a** Representative photomicrographs of ERK1/2 immunofluorescence of experimental groups. **b** Representative photomicrographs of pERK1/2 immunofluorescence of experimental groups. **c** Quantification of ERK1/2 OD. **d** Quantification of pERK1/2 OD. *$p<0.05$; **$p<0.01$, $p>0.05$ not shown. *Scale bar* 100 μm

CRF/CRF1 signaling. Our results show, for the first time, that exposure to one session of inescapable stress induces a long-lasting (i.e., more than 3 weeks) increase in CRF mRNA expression in the BLA and a decrease in CRF1 mRNA expression in the hippocampus and that chronic treatment with fluoxetine reverts both effects.

In LH saline-treated animals, we observed a persistent elevation of CRF mRNA in the BLA. The BLA has been shown to modulate the emotional response and the expression of LH behavior, and its role is different from that of the CeA, which participates in the acquisition of the behavioral impairment and in the regulation of the neuroendocrine response to stress (Hammack et al. 2011). The potential relationship between increased CRF expression in the BLA and behavioral despair seems to be reinforced by the positive and statistically significant correlation between CRF expression and escape latency in the test session with or without fluoxetine treatment. However, in a separate set of experiments performed in our laboratory in which we compared CRF expression in the BLA from animals sensitive or resilient to the induction of LH behavior, we found an increased expression of the peptide in both groups (Fernández Macedo, manuscript under revision). It seems that changes in CRF expression in forebrain structures, including BLA, are not enough for explaining complex behaviors (i.e., learned helplessness, swimming, social defeat, etc.). Instead, it is highly possible that concomitant changes in other brain areas occur. For example, when CRF is overexpressed in the forebrain, no differences were found in the forced swim test between transgenic and wild-type mice (Lu et al. 2008) but when animals are submitted to social stress significant differences between animals vulnerable and resilient were observed in PVN CRF expression (Elliott et al. 2010; Wood et al. 2010).

As expected from previous reports (Reines et al. 2008), chronic treatment with the antidepressant fluoxetine reverted the LH behavior induced by exposure to inescapable foot shocks. This treatment was also associated with a normalization of CRF expression in the BLA. As far as we know, this is the first evidence linking reversal of a depressive-like behavior with a normalization of CRF expression in the BLA. Aubry et al. (1999) had previously reported that chronic treatment with the tricyclic antidepressant amitriptyline elicited no effect on CRF expression in the BLA of naive rats. In addition, Stout et al. (2002) evaluated the effect of venlafaxine on CRF expression in animals submitted to chronic unpredictable stress and found that neither the behavioral procedure nor the pharmacological treatment induced changes in CeA CRF expression. These authors did not include evaluation of the CRF changes in the BLA as a consequence of chronic stress or pharmacological treatment. Employing a different approach, Lowry et al. (2009) demonstrated that fluoxetine prevents the anxiogenic effect of the infusion of CRF in two limbic brain areas, the nucleus accumbens and the entorhinal cortex, an observation that could be complementary to our results. As anxiety is both a predisposing factor to, and an intrinsic component of, depressive syndrome (Kessler et al. 2003; Lamers et al. 2011), it seems that the attenuation of behavioral and biochemical effects of CRF infusion (Lowry et al. 2009) and the decrease in CRF expression observed in our experiments in components of the limbic system could contribute to the antidepressant effect of fluoxetine. Additionally, because the antidepressant is administered

systemically, another possible brain region in which fluoxetine-mediated CRF modulation could result in behavioral improvement is the dorsal raphe nucleus where it has been shown that learned helplessness in rats is mediated by CRF2, but not CRF1 (Hammack et al. 2002, 2003).

When CRF1 expression was quantified in the hippocampus of saline-treated control and LH animals, we observed that CRF1 mRNA was lower in CA3 and the DG 26 days after the acquisition session. A decrease in CRF1 expression at approximately 1 week after the exposure to one session of mild or restraint stress has already been shown (Adlard et al. 2011; Greetfeld et al. 2009). Our observations provide additional information revealing that the CRF1 decrease persists at least for 3 weeks. Because CRF by itself may control the receptor expression (Korosi et al. 2006), it could be proposed that CRF1 downregulation may result from the increase in the release of its ligand from the BLA. However, it should be noted that although the hippocampus receives substantial input from the BLA (Pitkanen et al. 2000), and BLA contains CRF-synthesizing neurons (Falco et al. 2009), the amount of hippocampal CRF-mediated activity dependent on the BLA input is still unclear.

To corroborate that CRF1 downregulation reflects a true diminution in its activation, we also measured the levels of ERK1/2 activation, the main transducing pathway for CRF1 in CA3 (Refojo et al. 2005). In concordance with the above-mentioned results, no changes were observed in total ERK1/2 in saline-treated LH animals compared with saline-treated controls, but we observed a diminution of pERK1/2 in the first group. Although a putative cause for such diminution is the downregulation of CRF1, we cannot rule out other potential mechanisms. For example, ERK1/2 is also inhibited by CdK5 (Sharma et al. 2002), which has been shown to increase in different models of depression (Adzic et al. 2009; Cuadrado-Tejedor et al. 2011; Zhu et al. 2011). In addition, ERK1/2 phosphorylation is induced by the inhibition of glycogen synthase kinase 3β (Wang et al. 2006), an enzyme that we found to be activated in LH animals (Ferrrero A., PhD thesis manuscript; Universidad de Buenos Aires), and has been reported to increase in animals subjected to chronic mild stress (Silva et al. 2008), another well-validated experimental model of the disorder. However, in the DG, ERK1/2 is not activated by CRF1 (Refojo et al. 2005). In this area of the hippocampus, the transcription factor CREB mediates the CRF1 signal. In a separate set of experiments, CREB and pCREB levels in the DG were not concomitantly modified with CRF1 mRNA levels (Fernandez Macedo et al., manuscript under revision).

When LH and control animals were chronically treated with fluoxetine, along with the improvement on escape behavior from aversive stimuli and the normalization of CRF expression in BLA, a recovery of CRF1 expression both in CA3 and the DG of the hippocampus was observed. In the same way that high levels of CRF in saline-treated LH rats could explain the CRF1 downregulation, we suggest that normalization of CRF1 expression in the hippocampus could be a consequence of the reversion of CRF levels. Data about the effect of SSRIs on hippocampal CRF1 signaling are not yet available. Effects from different antidepressants on other brain areas are diverse. No effect has been shown for venlafaxine on CRF1 mRNA in the PVN, frontal cortex, or BLA. Similarly, no effect has been shown for venlafaxine, fluoxetine, reboxetine, or tranylcypromine on CRF1 density in the BLA, frontal cortex, or CeA of chronically stressed rats (Stout et al. 2002). Then it seems that the effect of antidepressants on CRF1 expression is area specific.

Basic research (Arborelius et al. 2000; Hodgson et al. 2007; Holsboer and Ising 2008; Ising et al. 2007; Jutkiewicz et al. 2005; Mansbach et al. 1997; Nielsen 2006; Overstreet and Griebel 2004; Takamori et al. 2001a) and some clinical trials (Zobel et al. 2000) have created some hope about the potential antidepressant efficacy of CRF1 antagonists. However, the phase II trials that have been performed have failed to reproduce these results (Binneman et al. 2008) indicating that the direct manipulation of CRF/CRF1 signaling would not be efficacious for all patients. In a recent review, Paez-Pareda proposed that a stratification of patients suffering depressive disorders according to their neuroendocrine profile could reveal a subpopulation with CRF hypersecretion who could benefit from CRF1 antagonists (Paez-Pereda et al. 2011). It is noteworthy that in our experiments in which LH behavior is paralleled by higher expression of CRF, the effective antidepressant-like action of fluoxetine is accompanied by a reversion of the altered expression of CRF and CRF1. Whether these effects are reproducible in other models of the disease in which CRF is not so strongly involved or with antidepressants with initially different mechanisms of action (i.e., dual antidepressants, MAO inhibitors, noradrenaline reuptake inhibitors) must be investigated.

In agreement with previous reports (Tiraboschi et al. 2004), when the levels of total ERK1/2 were quantified in the control animals injected with fluoxetine, significant increases were found. In addition, and in contradiction with what we expected after reversion of CRF1 downregulation, fluoxetine failed to restore pERK1/2 levels in LH animals. Failure to revert pERK1/2 decrease could indicate that other ERK1/2-coupled pathways aside from CRF1 may be altered in LH animals and are not restored by antidepressant treatment. For example, glutamate, a neurotransmitter shown to induce ERK1/2 phosphorylation, is decreased in LH rats and this is not reverted by chronic treatment with fluoxetine (Ferrero et al. 2005). In addition, this antidepressant could be activating MAPK phosphatases (Thiriet et al. 1998) which, in turn, may dampen pERK1/2 levels.

Several mechanisms of action have been proposed for fluoxetine, as well as for all antidepressant drugs. The

increased extracellular serotonin concentration subsequent to the inhibition of neuronal reuptake could be the first step in a cascade of events (Djordjevic et al. 2012; Reines et al. 2008, among others). CRF regulation of serotonin neurotransmission and signaling has been described. CRF1 activation in the dorsal raphe nucleus inhibits serotonin neuronal activity (Kirby et al. 2000) and accordingly also inhibits serotonin release in dorsal raphe targets (Price et al. 1998). Acute CRF (i.c.v.) increases, but chronic CRF (i.c.v.) decreases, the stress-induced serotonin release (Kagamiishi et al. 2003; Linthorst et al. 2002). Additionally, it has been shown from in vitro experiments that CRF increases serotonin signaling through CRF1, inducing recycling and cell surface expression of 5-HT_2R (Magalhaes et al. 2010).

Regarding serotonin's effect on CRF signaling, little information is available. Valentino et al. (1990) found that the SSRI sertraline interferes with CRF effects in the locus coeruleus, but in in vitro experiments Magalhaes et al. (2010) found that serotonin does not modify CRF signaling. In our experiments, administration of fluoxetine promotes a normalization of CRF expression and signaling in the BLA and hippocampus, respectively. These results seem to support the idea that serotonin decreases CRF's effects. The effect of inhibiting serotonin uptake on CRF expression has been investigated in animals lacking the serotonin transporter. Similar to what we found, CRF mRNA is decreased in these mice (Jiang et al. 2009). However, it should be noted that in these transgenic animals, the increase in serotonin levels during development causes a depressive-like phenotype (Wellman et al. 2007), which is rather different than what is observed with the antidepressant fluoxetine.

Finally, a dopamine-mediated effect of fluoxetine on the changes of CRF expression observed in our experiments cannot be excluded because, as observed by Refojo et al. (2011), other neurotransmitter systems, for example dopamine, that mediate fluoxetine's effect (Cao et al. 2010; Cuadra et al. 2001) could also regulate the CRF stress response.

In summary, our results demonstrate long-term correlative changes in CRF and CRF1 mRNA expression in an animal model of depression. Furthermore, we showed for the first time that a behaviorally effective treatment with the antidepressant fluoxetine can reverse the overexpression of CRF in the BLA, and can restore the hippocampal CRF1 expression.

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Conflict of interest The authors report no biomedical financial interests or potential conflicts of interest.

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