



# Stress and glucocorticoids regulated corticotropin releasing factor in rat prefrontal cortex

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## ABSTRACT

Corticotropin releasing factor (CRF) is considered as the central driving force in the stress response and plays a key role in the pathogenesis of depression. CRF neurons have been identified to locate in most regions of the prefrontal cortex (PFC), a brain region that is highly associated with the control of emotion and cognition. However, little is known on the regulation of CRF in this region. In this study, we aimed to identify the regulatory effect of acute restraint stress and glucocorticoid on PFC CRF and characterize the possible function of CRF in the PFC. We found that acute restraint stress increased and glucocorticoid decreased PFC CRF mRNA expression. The expression of glucocorticoid receptor (GR) was found to colocalize with CRF neurons in the PFC. In addition, recruitment of GR by the CRF promoter was observed *in vivo*. Specific attention was paid to the effect of CRF on CRF receptor 1 (CRFR1) expression in primary PFC cultures. The results showed that CRF increased CRFR1 expression through the MEK-ERK1/2 pathway. In summary, this study may contribute to the better understanding of CRF functions in the PFC.

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## 1. Introduction

Corticotropin-releasing factor (CRF), a 41-amino acid peptide that is mainly produced in the hypothalamic paraventricular nucleus (PVN), plays a crucial role in stress response and is considered as the central driving force in the activity of hypothalamic-pituitary-adrenal (HPA) axis (Bissette, 1990; Swaab, 2003). In addition to the hypothalamus, CRF neurons are also located in most regions of the prefrontal cortex (PFC) (Swanson et al., 1983), a brain region that is highly associated with the control of emotion and cognition (Miller, 1999). These regions are part of the circuitry involved in modulating ACTH-cortosterone mediated responsivity to stress (Diorio et al., 1993). Previous studies indicate, in the neocortex, CRF appears to act as a neuromodulator, or possibly even a neurotransmitter, and is involved in mediating increased arousal, learning, and anxiety while simultaneously decreasing food intake and sexual behavior and altering locomotion (Koob and Heinrichs, 1999; Roozendaal et al., 2002; Smagin et al., 2001). Many of these effects may be mimicked with intraventricular administration of

CRF (Dunn and Berridge, 1990; Koob and Heinrichs, 1999; Sherman and Kalin, 1987; Takahashi et al., 1989). More recently, another study shows that CRF and acute stress can alter the function of 5-HT in PFC pyramidal neuron (Tan et al., 2004).

A wide variety of stressor has been found to increase the expression of the CRF gene in the PVN, such as restraint stress, foot shock, hypovolemia, and hypoglycemia (Harbuz et al., 1994; Herman et al., 1998; Hsu et al., 1998; Imaki et al., 1995; Kalin et al., 1994; Ma et al., 1997b; Paulmyer-Lacroix et al., 1994; Tanimura et al., 1998). CRF gene expression in the amygdala has also been reported to increase in response to the restraint stress (Hsu et al., 1998; Kalin et al., 1994). In addition, a large body of literature also indicates CRF gene expression is regulated in a complex manner by glucocorticoids (Schulkin et al., 1998, 2005). Briefly, it is well known that glucocorticoids negatively regulate the expression of CRF gene in PVN (Keller-Wood and Dallman, 1984; Swanson and Simmons, 1989). On the other hand, chronic or repeated peripheral administration of corticosterone increases CRF mRNA expression in the central nucleus of the amygdala (CeA) and the bed nucleus of the stria terminalis (Makino et al., 1994a, b; Watts and Sanchez-Watts, 1995). Furthermore, adrenalectomy has opposite effects on central CRF gene expression in the CeA and PVN with a decreased CRF expression in the CeA and increased expression in the PVN (Palkovits et al., 1998; Viau et al., 2001). However, it is not yet known whether stress and glucocorticoid regulate expression of CRF in the PFC.

In this study, we seek to further our understanding of the regulation of CRF mRNA expression in the PFC under condition of the

**Abbreviations:** ADX, adrenalectomized; CeA, central nucleus of the amygdala; ChIP, chromatin immunoprecipitation assay; CRF, corticotropin releasing factor; CRFR1, CRF receptor 1; GR, glucocorticoid receptor; HPA, hypothalamic-pituitary-adrenal; ir, immunoreactivity; PFC, prefrontal cortex; PVN, paraventricular nucleus.

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acute restraint stress and glucocorticoids. In addition, the effects of CRF on mRNA expression of CRF receptor 1 (CRFR1) in PFC primary cell cultures were also analyzed.

## 2. Materials and methods

### 2.1. Animals

Adult Sprague–Dawley male rats, weighing 250–300 g (Anhui Experimental Animal Center, Hefei, Anhui, PR China) were used. Rats were kept in animal facilities under a 12 h light/dark cycle, with lights on at 0800 h and food and water *ad libitum*. The use and care of animals in the present study followed international guidelines and protocols approved by the Institutional Animal Care and Use Committee of the University of Science and Technology of China. All efforts were made to minimize animal suffering and to reduce the number of animals used.

### 2.2. Acute restraint stress

The rats were handled in the colony room once every day for 7 days prior to initiating experimental stress procedures. Handling comprised picking up each rat for a short period of time and then returning it to its home cage. Immediately following the handling phase, the experimental stress manipulations began. Rats were randomly assigned to weight matched groups that received one of the following four treatments: handling ( $n = 5$ ), restraint stress for 0.5 h ( $n = 6$ ), restraint stress for 1 h ( $n = 6$ ), or restraint stress for 3 h ( $n = 6$ ). Prior to the restraint stress, rats were transported out of the colony room to an adjacent, brightly lit, quiet room, and were placed into a transparent Plexiglas tube (23.5 cm in length and 7 cm in diameter) with tails protruding. The size of the tube restricted both lateral and forward/backward movement but did not interfere with breathing. All the stress experiments began at 0830 h in the morning. The three groups of rats which receive acute restraint stress were killed by decapitation at 0.5, 1, 3 h after initiation of restraint, respectively. Control rats were rapidly removed from the cages and decapitated. All experiments were performed in the morning, and rats were killed between 0900 and 1130 h.

### 2.3. Adrenalectomy and corticosterone treatment

Adult male Sprague–Dawley rats were anesthetized with 7% chloral hydrate solution (1 ml/100 g) and either bilaterally adrenalectomized (ADX) or sham ADX ( $n = 6$ ) using flank incisions. Following ADX, rats were provided with 0.9% saline in place of water to maintain electrolyte balance. The ADX group was then subdivided into two further groups that receive either vehicle (10% ethanol in saline, *sc*,  $n = 7$ ) or corticosterone treatment (10 mg/kg in 10% ethanol in saline, *sc*,  $n = 7$ ) twice daily (9:00 a.m. and 17:00 p.m.). On the morning of day 7, all the rats were killed approximately 1 h after their last injection.

### 2.4. Mifepristone treatment

Administration of the glucocorticoid receptor antagonist mifepristone (Beijing Zizhu Pharmaceutical Company, PR China) (50 mg/kg, *sc*), also known as RU486 and has been used in our previous studies (Wu et al., 2007), took place 20 and 2 h before the acute restraint stress. RU486 were dissolved in saline. The solutions were prepared immediately before use and saline was used as the vehicle in the experiment.

### 2.5. Prefrontal cortex dissection and sample preparation

All the rats were decapitated and the blood samples were collected in tubes containing heparin sodium as an anticoagulant and centrifuged at 4 °C, and after separation the plasma was stored at –80 °C until assayed with corticosterone ELISA kit (Assay Designs Inc, Ann Arbor, MI). The brain was removed immediately, and a prefrontal cortex block was dissected. To dissect the rat PFC, the frontal parts of the hemispheres were dissected between bregma 2.7 and bregma 5.2 according to coordinates of Paxinos and Watson (Paxinos and Watson, 1998) and collected after the removal of their basal parts at the level of rhinal fissures. The total dissection time was less than 2 min from decapitation per rat. Tissue samples were placed immediately in liquid nitrogen and later kept at –80 °C until further analysis. Frozen tissues were homogenized with a Glas-Col's variable-speed homogenizer in cold Trizol Reagent (Invitrogen, La Jolla, CA, USA) for further RNA and protein extraction according to the manufacturer's instructions.

### 2.6. Primary culture of PFC cells

Rat prefrontal cortex cultures were prepared as described previously (Wang et al., 2003). Briefly, prefrontal cortex was dissected from 18 day rat embryos, and cells were dissociated by incubating with 0.25% trypsin-EDTA (Invitrogen, Carlsbad, CA, USA) for 30 min and subsequent trituration through a Pasteur pipette. The cells were plated on precoated 6-well plates (coated with poly-L-lysine, Sigma-Aldrich, St. Louis, MO) for mRNA analysis or on coverslips (coated with poly-L-lysine, put in 6-well plates) for immunocytochemistry in phenol red free Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) at a density of  $1 \times 10^5$  cells/cm<sup>2</sup>. After 1 day, the medium was changed to phenol red free Neurobasal medium (1.5 ml, Invitrogen, Carlsbad, CA, USA) supplemented with 2% B27 Serum-Free Supplement (Invitrogen, Carlsbad, CA, USA), and replaced every 3–4 days. Treatment with 5-Fluoro-2'-deoxyuridine (20 µg/ml, Sigma-Aldrich, St. Louis, MO) on the fourth day after plating was used to block cell division of non-neuronal cells, which helped to stabilize the cell population. The cultures were maintained at 37 °C in a 5% CO<sub>2</sub> humidified atmosphere. All treatments were carried out after 10 days in culture.

To examine the effect of glucocorticoid on CRF expression, a quarter of the medium was replaced by fresh Neurobasal medium and various agents added: dexamethasone (Sigma-Aldrich, St. Louis, MO), RU486 (Sigma-Aldrich, St. Louis, MO) and forskolin (Sigma-Aldrich, St. Louis, MO) either alone or in various combinations. To examine the effect of CRF on CRFR1 expression, CRF (Sigma-Aldrich, St. Louis, MO) and U0126 (Cell Signaling Technology, Beverly, MA) were added. Dexamethasone and RU486 were prepared by dissolving them in ethanol followed by dilution in Neurobasal medium prior to their application. CRF was prepared by dissolving it in distilled water. Forskolin and U0126 were prepared by dissolving it in DMSO (Sigma-Aldrich, St. Louis, MO). Each culture had its own set of controls.

### 2.7. Real-time quantitative RT-PCR analysis

Real-time quantitative RT-PCR was performed to analyze CRF and CRFR1 mRNA expressions in the rat prefrontal cortex and the primary prefrontal cortex neurons. The  $\beta$ -actin mRNA expression was analyzed as the internal control. Generally, 1 µg of total RNA was reverse transcribed to obtain cDNA. Reaction conditions for reverse transcription were as follows: 100 pmol/L oligo-dT, 1 mmol/L each deoxynucleoside triphosphate, 200 unit RNAsin, and 200 units Murine Moloney Leukemia Virus Reverse Transcriptase (Promega,

Madison, WI, USA). The reaction was run at 42 °C for 1 h. The mixture was then heated at 72 °C for 10 min and quickly chilled on ice.

Primers used in the real-time quantitative PCR were shown as follows. For CRF (accession no. NM\_031019), the sense primer was 5'-AAAGGGGAAAGGCAAAGAAAAGG-3' and the antisense primer was 5'-AAGGCAGACAGGGCGACAGAG-3'. For CRFR1 (accession no. NM\_030999), the sense primer was 5'-TGCCTGAGAAACATCATC-CACTGG-3' and the antisense primer was 5'-TAATTGTAGGCGGCTGTCACCAAC-3'. For  $\beta$ -actin (accession no. NM\_031144), the sense primer was 5'-TTGCTGACAGGATGCAGAA-3' and the antisense primer was 5'-ACCAATCCACACAGAGTACTT-3'. All the primers were designed with program Primer Premier 5.0. Q-PCR was performed on cDNA products using the SYBR Premix Ex Taq (Takara, Dalian, China) and 0.5  $\mu$ M of each primer in a 30  $\mu$ l reaction. Samples were amplified with the ABI Prism 7000 (Applied Biosystems, Foster City, CA, USA) as follows: 2 min at 50 °C, 10 min denaturation at 95 °C followed by 40 cycles of denaturation for 15 s at 95 °C and annealing and elongation for 1 min at 64 °C.

The relative target gene mRNA level was determined using the  $\Delta$ Ct method. This was done by firstly normalizing the resulting threshold cycle (Ct) values of the target gene mRNAs to the Ct values of the internal control  $\beta$ -actin in the same samples:  $\Delta$ Ct = Ct (the target gene) – Ct ( $\beta$ -actin). These values were further normalized with the control group:  $\Delta\Delta$ Ct =  $\Delta$ Ct (sample group) –  $\Delta$ Ct (control group). The fold change was then obtained ( $2^{-\Delta\Delta$ Ct). The relative target gene mRNA level represents an average fold calculated from separate experiments. PCR reactions were performed twice and similar results were observed.

## 2.8. Western blot

Western blotting was performed to analyze the ERK activity in the primary prefrontal cortex neurons. Generally, 20  $\mu$ g of primary neurons extracts for each sample were run on a 10% SDS polyacrylamide gel and then were transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membranes were then blocked for 1 h at 37 °C in PBS–tween buffer (0.01 M PBS and 0.05% Tween 20) containing 5% non-fat milk and incubated for 2 h at room temperature with the primary antibody diluted in PBS–tween buffer with 0.5% non-fat milk. Immunoblotting was performed using the anti-p-ERK (1:2000, Cell Signaling Technology, Beverly, MA) and anti-ERK antibody (1:2000, Cell Signaling Technology, Beverly, MA). After washing, membrane was incubated for 1 h at room temperature with goat anti-rabbit IgG conjugated with horseradish peroxidase (1:2000, Promega, Madison, WI, USA). After rinsing, subsequent detection was performed using the ECL Western blot system (SuperSignal West Pico chemiluminescent Substrate, Pierce, Rockford, IL, USA) according to the manufacturer's instruction. Western blotting was performed twice and similar results were observed.

Western blot was also performed on the rat PFC tissues to test the specificity of the GR antibody (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) used in the immunofluorescence staining and Chromatin immunoprecipitation assay (ChIP) following the protocol described above.

## 2.9. Immunofluorescence staining

For the brain slice, the adult Sprague–Dawley rats were anaesthetized with 7% chloral hydrate solution (1 ml/100g) and perfused through the ascending aorta with PBS followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. After perfusion, brains were removed and post-fixed by immersion in the same fixative overnight. Then the brains were dehydrated through a graded ethanol series and embedded in paraffin. Serial 6  $\mu$ m coronal sections were cut on a Leica microtome (Leica RM

2135). The sections were deparaffinized in xylene and then rehydrated in graded ethanol. Following TBS (0.05 M Tris buffer pH 7.4 containing 0.15 M NaCl) washes, the sections were treated with microwave (700 W) in 0.05 M citrate buffered saline (pH 6.0) for 2  $\times$  10 min for antigen retrieval and then cooled down at RT for 30 min.

After washing in TBS, sections were incubated for 30 min in 0.5% triton X-100 to permeabilize the tissue. Sections were blocked with 5% goat serum in TBST for 30 min at 37 °C to reduce nonspecific binding, then were incubated for 1 h at 37 °C with monoclonal mouse anti-GR (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and polyclonal rabbit anti-CRF (1:200, Bachem, Torrance, CA, USA) diluted in TBST with 5% goat serum and then incubated overnight at 4 °C. The next day the sections were washed and incubated with FITC-labeled goat anti-mouse antibody (1:200, Pierce, Rockford, IL, USA) and biotinylated anti-rabbit antibody (1:200, Vector, Burlingame, CA, USA) diluted in TBST with 5% goat serum for 1 h at 37 °C. After washing, the sections were incubated with Cy3-labeled streptavidin (1:500, Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) for 30 min at 37 °C. Then the sections were washed and coverslipped with glycerin.

For the primary cultures, rat PFC cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The following steps were performed as described above. To determined whether the primary culture cells are neurons, the monoclonal mouse anti-neuron specific nuclear protein (NeuN) antibody (1:100, Chemicon, Temecula, CA, USA), polyclonal rabbit anti-glial fibrillary acidic protein (GFAP) antibody (1:100, Dako, A/S, Denmark), polyclonal rabbit anti-MAP2 antibody (1:500, Chemicon, Temecula, CA, USA), monoclonal mouse anti-GFAP antibody (1:200, Chemicon, Temecula, CA, USA), and 4',6-diamidino-2-phenylindole (DAPI, 1:5000, Sigma–Aldrich, St. Louis, MO) nuclear stain were also used.

Fluorescent signals were detected using a confocal laser scanning inverted microscope (LSM 510, Carl Zeiss, Maple Grove, MN, USA) with a 40 $\times$ /0.75 NA PlanApo objective and simultaneously collected using Zeiss image processing software (LSM 5, Carl Zeiss). Images were taken using a filter combination appropriate for the specific visualization of FITC (488 nm excitation, filter BP 500–530, displayed green) and Cy3 (543 nm excitation, filter LP 560, displayed red). Pinhole setting was 7.6 for the 488 nm and 8.4 for the 543 nm excitation laser lines. Laser intensity and gain were slightly adjusted to yield a distinct image of cells.

## 2.10. Chromatin immunoprecipitation assay (ChIP)

ChIP assays were performed to determine whether GR can bind to the rat CRF promoter *in vivo* according to our previous study (Chen et al., 2009). Briefly, Rat prefrontal cortex was removed from decapitated rats as described in the preceding text. Chromatin solutions from rat PFC were immunoprecipitated with the anti-GR antibody (Santa Cruz). For the negative controls, mouse IgG or no antibody were added. Final DNA extraction was amplified with primers designed to amplify the 320 bp rat CRF promoter region (forward, 5'-GAAAAGTTAGGTGGGAGTGGTG-3' and reverse, 5'-GAGGGACAGGGTTCTGCTATTT-3'). ChIP assays were performed twice and similar results were observed.

## 2.11. Statistical analysis

All the data are presented as the mean  $\pm$  SEM. Experimental data were subjected to statistical analyses using the one-way repeated measures ANOVA followed by LSD test or student's *t* test with a significance level of *P* < 0.05.

### 3. Results

#### 3.1. Effects of acute restraint stress on PFC CRF and CRFR1 mRNA expression

To study the effects of acute restraint stress on the PFC CRF expression, the acute restraint stress rat model was established. First, we detected the level of plasma corticosterone, which could be used as a marker of stress, in the stress rats and control subject. In our study, 0.5, 1 and 3 h restraint stress all significantly increased plasma corticosterone levels compared to the control level [ $222.6 \pm 29.5$  ng/ml,  $221.2 \pm 14.7$  ng/ml and  $180.3 \pm 37.6$  ng/ml vs.  $9.1 \pm 2.4$  ng/ml,  $F(3,19) = 13.654$ ,  $P < 0.05$ , Fig. 1A].

The changes in CRF mRNA expression in the PFC triggered by acute restraint stress at different time point were examined by real-time quantitative RT-PCR analysis. A single 0.5 h restraint stress was not sufficient to elevate the level of CRF mRNA expression. However, significant induction of CRF mRNA expression was observed in the PFC following 3 h restraint stress compared to the unstressed control [ $F(3,19) = 8.801$ ,  $P < 0.05$ , Fig. 1B]. In addition, 1 h restraint stress also caused the elevation of CRF mRNA level – a trend that did not reach significance [ $F(3,19) = 8.801$ ,  $P = 0.058$ , Fig. 1B]. We further detected the CRFR1 mRNA level in the PFC of the acute restraint rats. Acute restraint stress induced a time dependent increase in CRFR1 mRNA expression in the rat PFC. 3 h restraint stress significantly increased CRFR1 mRNA expression by about 60% compared to the unstressed control [ $F(3,19) = 4.372$ ,  $P < 0.05$ , Fig. 1C].

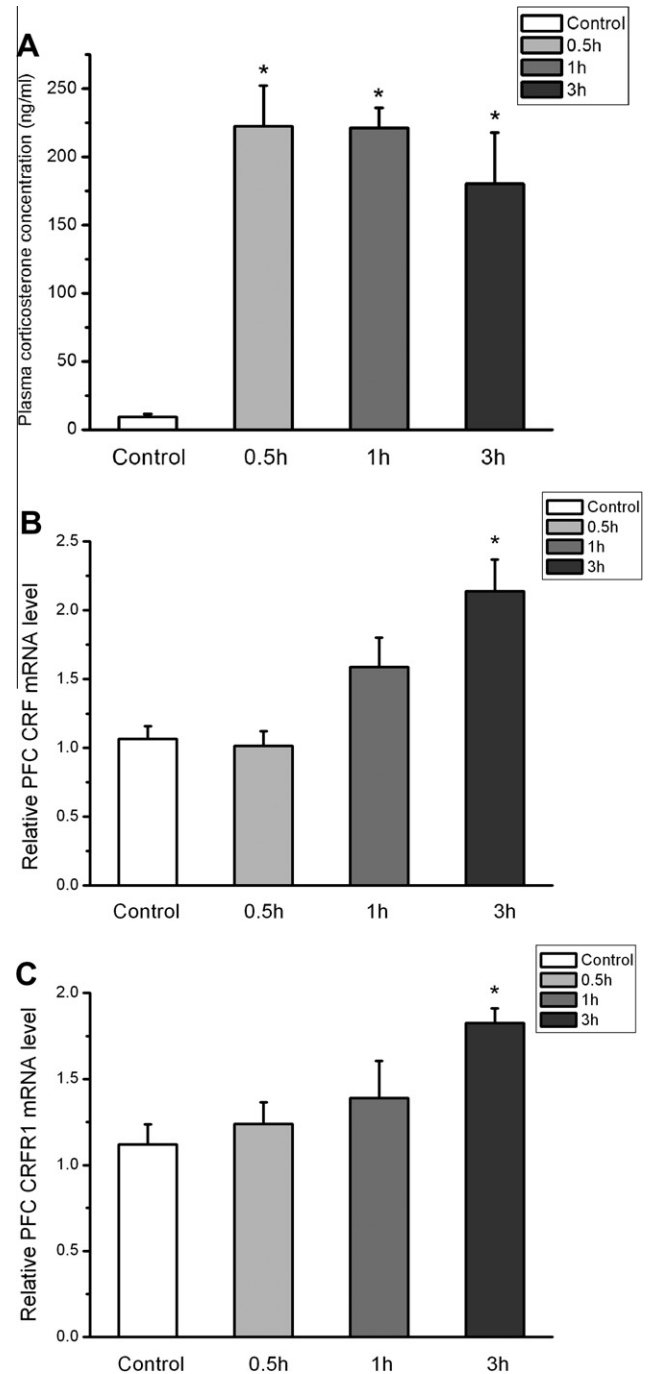
#### 3.2. Effects of corticosterone and RU486 on PFC CRF mRNA expression

To determine whether glucocorticoids involve in the PFC CRF expression, the adrenalectomized rats with and without corticosterone administration were used. 7 days after the surgery of adrenalectomy, the ADX rats showed significantly lower plasma corticosterone concentration compared to the sham group [ $2.9 \pm 0.3$  ng/ml vs.  $25.8 \pm 9.3$  ng/ml,  $F(1,11) = 176.184$ ,  $P < 0.05$ , Fig. 2A], which validated our ADX model. Meanwhile, the plasma corticosterone levels of ADX rats which received corticosterone treatment were dramatically higher than the ADX group [ $296.4 \pm 65.2$  ng/ml vs.  $2.9 \pm 0.3$  ng/ml,  $F(1,12) = 8.975$ ,  $P < 0.05$ , Fig. 2A]. According to the results of real-time quantitative RT-PCR, the ADX rats received corticosterone treatment showed significantly lower PFC CRF mRNA level compared to the ADX group [ $F(1,12) = 7.913$ ,  $P < 0.05$ , Fig. 2B].

We also measured CRF mRNA responses to acute restraint stress in the rats which were pretreated with RU486. The unstressed rats pretreated with saline or RU486 were used as the unstressed control. Following 1 h restraint stress, the rats pretreated with RU486 showed significantly higher PFC CRF mRNA level compared to the both unstressed control groups and the stressed ones pretreated with saline [ $F(3,29) = 5.545$ ,  $P < 0.05$ , Fig. 2C].

#### 3.3. Colocalization of CRF and GR in the rat PFC

A high proportion of PFC CRF cells were colocalized with GR in both the rat PFC brain slices (Fig. 3A–C) and the primary PFC cells (Fig. 3D–F). In the brain slices, CRF immunoreactivity (ir) within cell bodies showed red cytoplasmic staining (Fig. 3A), and green GR staining was mainly observed in the nucleus (Fig. 3B). In the primary cells, the pattern of colocalization showed a little difference, that is, red whole cell CRF staining (Fig. 3D), green nuclear GR staining (Fig. 3E), and yellow CRF-GR double staining (Fig. 3F).

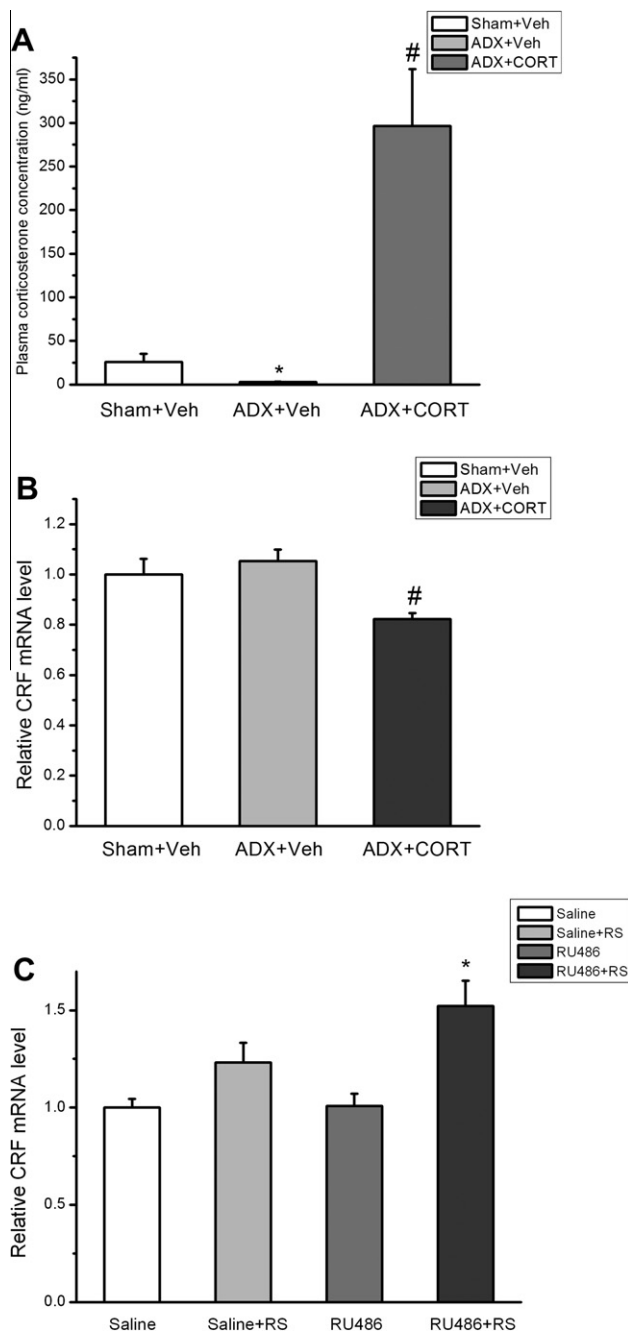


**Fig. 1.** Effect of acute restraint stress on plasma corticosterone level, PFC CRF and CRFR1 mRNA expression in the Sprague-Dawley rats. Rats were placed in Plexiglas restrainers and killed by decapitation at the times indicated. (A) Analysis of plasma corticosterone level by using ELISA. Analysis of CRF (B) and CRFR1 (C) mRNA level in rat PFC by real-time quantitative PCR. Data presented are the mean  $\pm$  SEM. A significant difference from controls is denoted by an asterisk (\*) for  $P < 0.05$  ( $n = 5$ –6 per group).

#### 3.4. Characterization of specificity of the GR antibody

Specificity of the antibody used in this experiment was tested in the rat PFC tissues by Western blot. Western blotting with anti-GR antibody revealed a single band of the expected molecular weight approximately 85 kD (Fig. 4A). In addition, staining Western blot with omission of primary anti-GR antibody did not show any bands (Fig. 4B), which supported the specificity of the anti-GR antibody in the rat PFC.





**Fig. 2.** Effect of corticosterone and RU486 on PFC CRF mRNA expression. (A, B) The plasma corticosterone (A) and PFC CRF mRNA level (B) in the ADX rats. The ADX rats were treated with corticosterone or vehicle twice daily and the sham rats treated with vehicle were used as the control. (C) Administration of RU486 took place 20 and 2 h before the acute restraint stress and the unstressed rats pretreated with saline or RU486 were used as the control. Rats were then killed by decapitation following 1 h restraint stress. Analysis of CRF mRNA level in rat PFC was performed by real-time quantitative PCR. Data presented are the mean  $\pm$  SEM. For (A, B) the significant difference from the sham-operated controls is denoted by the asterisk (\*) for  $P < 0.05$ . A significant difference from the vehicle treated ADX group is denoted by a pound (#) for  $P < 0.05$ . Sham+Veh group,  $n = 6$ ; ADX+Veh group,  $n = 7$ ; ADX+CORT group,  $n = 7$ . For (C) the significant difference from all the other groups is denoted by an asterisk (\*) for  $P < 0.05$ . Saline group,  $n = 7$ ; Saline + RS group,  $n = 10$ ; RU486 group,  $n = 6$ ; RU486 + RS group,  $n = 10$ .

### 3.5. GR is recruited by CRF promoter in the rat PFC

To determine whether GR can be recruited by the CRF promoter *in vivo*, we performed a ChIP assay with the rat PFC tissue. The occupancy of rat CRF promoter by GR was observed when

immunoprecipitated by GR antibody, which was located between –470 and –147 relative to the transcription initiation site (Fig. 4C). In both negative controls, no PCR bands were present.

### 3.6. Effect of dexamethasone on CRF mRNA expression in PFC primary neurons

To confirm whether the primary cells are neurons, triple labeling of NeuN/GFAP/DAPI (Fig. 5A–D) or MAP2/GFAP/DAPI (Fig. 5E–H) were performed on the 10 day cultured cells. In the primary cells, the neurons were immunolabeled by NeuN (Fig. 5A) or MAP2 (Fig. 5E) antibody, the astrocytes were immunolabeled by GFAP (Fig. 5B,F) antibody, and the nucleus were stained by DAPI (Fig. 5C,G). Over 90% DAPI stained cells were colocalized with NeuN (Fig. 5D) or MAP2 (Fig. 5H), and the GFAP staining cells are rare, which all depict the high purity of the neuronal culture.

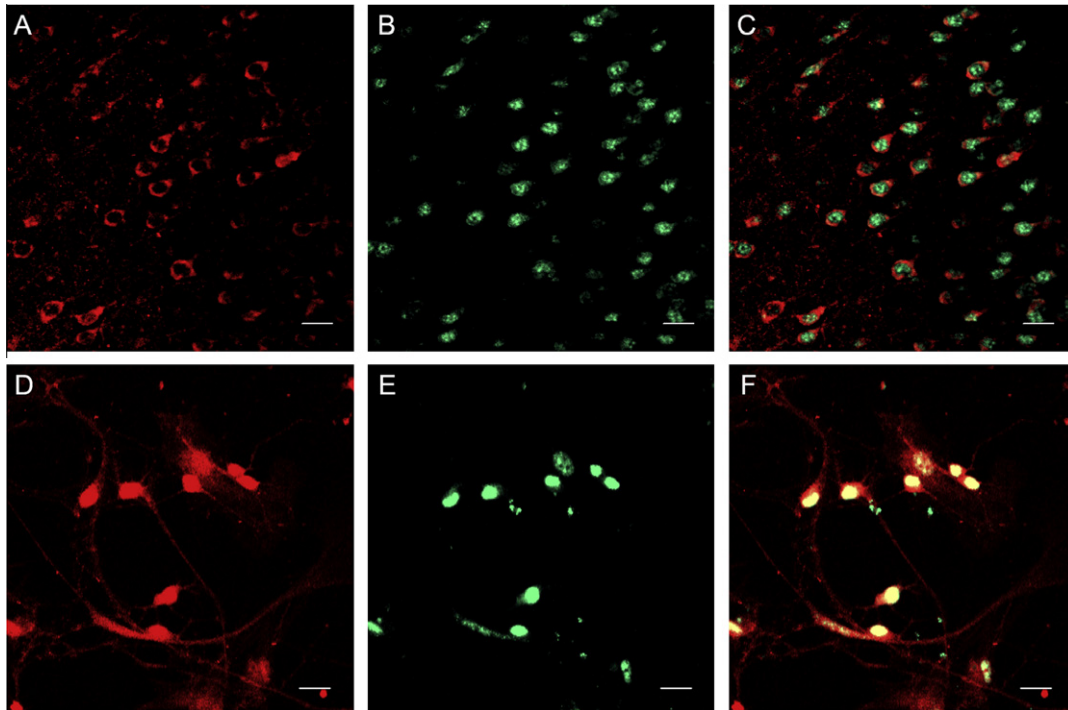
To further confirm the effect of glucocorticoid on CRF expression, PFC primary cell cultures was used by treating the cells with dexamethasone, a synthetic glucocorticoid. Twenty-four hour dexamethasone treatment induced a dose-dependent decrease in CRF mRNA level, with a maximal reductive effect occurring at a concentration of  $10^{-7}$  M (Fig. 6A). When incubation time was fixed for 24 h, the CRF mRNA levels in  $10^{-7}$  and  $10^{-6}$  M dexamethasone-treated cells were significantly decrease by about 25% compared to the control cells [ $F(4,35) = 2.095$ ,  $P < 0.05$ , Fig. 6A]. On the other hand, when the dexamethasone concentration was fixed at  $10^{-6}$  M, the influence of incubation time on the CRF mRNA level in PFC primary cell cultures was also investigated. The administration of  $10^{-6}$  M dexamethasone resulted in a time dependent decrease in CRF mRNA level, in which 12 and 24 h incubation caused a significantly decrease in CRF mRNA levels by about 25% relative to the control [ $F(3,26) = 3.606$ ,  $P < 0.05$ , Fig. 6B]. This maximal inhibitory effect was observed at 24 h (Fig. 6B).

To test the selectivity of the dexamethasone-induced decrease in CRF mRNA level, glucocorticoid receptor antagonists were performed. As shown in Fig. 6C,  $10^{-6}$  M RU486 significantly diminished the effect of  $10^{-6}$  M dexamethasone on CRF mRNA level [ $F(3,32) = 2.587$ ,  $P < 0.05$ ]. A 24 h treatment with RU486 alone did not alter CRF mRNA level.

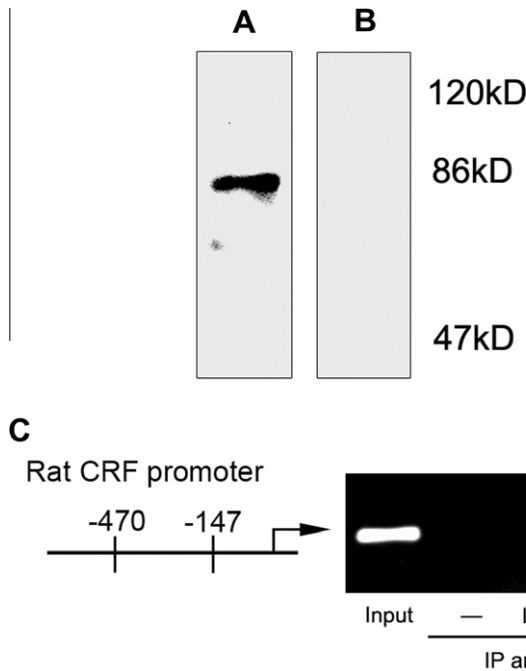
Forskolin is a well known factor that stimulates CRF expression. To further confirm the effect of dexamethasone, we examined the combined effect of forskolin and dexamethasone on CRF expression in rat PFC primary neurons. Consistent with previous studies, 12 h incubation with  $3 \times 10^{-7}$  M forskolin caused a significantly increase in CRF mRNA level by about 70% compared to the control [ $F(4,25) = 5.248$ ,  $P < 0.05$ , Fig. 6D]. In this experiment,  $10^{-6}$  or  $10^{-5}$  M dexamethasone decreased forskolin induced CRF mRNA level to about 60% of forskolin alone [ $F(4,25) = 5.248$ ,  $P < 0.05$ , Fig. 6D]. The minimum effective inhibitory dose of dexamethasone on forskolin induced CRF mRNA level was found to be  $10^{-6}$  M.

### 3.7. Effect of CRF on CRFR1 mRNA expression in PFC primary neurons

To characterize the possible function of CRF in the PFC, we investigated the effect of CRF on CRFR1 mRNA expression in PFC primary neurons. As shown in Fig. 7A, 12 h incubation with  $10^{-7}$  M CRF caused a significantly increase in CRFR1 mRNA level by about 70% compared to the control [ $F(3,20) = 5.026$ ,  $P < 0.05$ ]. ERK phosphorylation was then detected and compared with total ERK. We observed that  $10^{-7}$  M CRF cause a time dependent increase in ERK activity, with a maximal effect occurring at 12 h (Fig. 7B). 6 and 12 h incubation with  $10^{-7}$  M CRF caused a significantly increase in ERK activity by about 50% relative to the control [ $F(3,20) = 4.386$ ,  $P < 0.05$ , Fig. 7B]. In addition, we examined whether CRF induce CRFR1 mRNA expression through the MEK-ERK1/2 pathway. Considering ERK is the only known substrate for the upstream dual



**Fig. 3.** Colocalization of CRF and GR in both the rat PFC brain slices and the primary PFC neurons. (A,D) Single CRF fluorescence (red) in the rat PFC brain slices and the primary PFC neurons, respectively. (B,E) Single GR fluorescence (green) in the rat PFC brain slices and the primary PFC neurons, respectively. (C,F) Double fluorescence of GR and CRF (green, red, and yellow) in the rat PFC brain slices and the primary PFC neurons, respectively. Scale bar = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



**Fig. 4.** GR is recruited by CRF promoter in the rat PFC. (A) Western blot of rat PFC tissue showing a band near 85 kD confirming the specificity of the GR antibody. (B) No staining was detected when incubation with the primary antibody was omitted. (C) Chromatin immunoprecipitation assay (ChIP) showing the recruitment of GR by the rat CRF promoter. Chromatin solutions from the rat PFC were immunoprecipitated with anti-GR antibody, and final DNA extraction were amplified with primers that cover the regions of rat CRF gene promoter as indicated. Similar results were observed in two independent experiments.

kinase MEK, the effect of the specific MEK inhibitor U0126 on CRF induced CRFR1 mRNA expression was investigated. As shown in

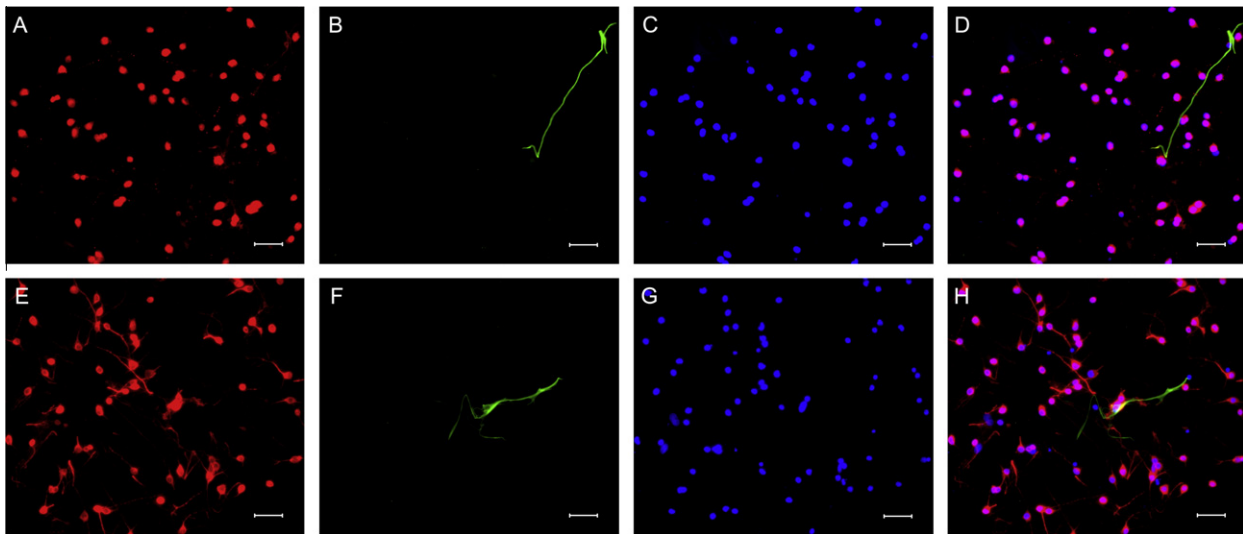
Fig. 7C,  $10^{-5}$  M of the MEK inhibitor U0126 significantly inhibited the effect of CRF on CRFR1 mRNA level [ $F(3,27) = 4.540$ ,  $P < 0.05$ ]. A 12 h treatment with U0126 alone did not alter CRFR1 mRNA level.

#### 4. Discussion

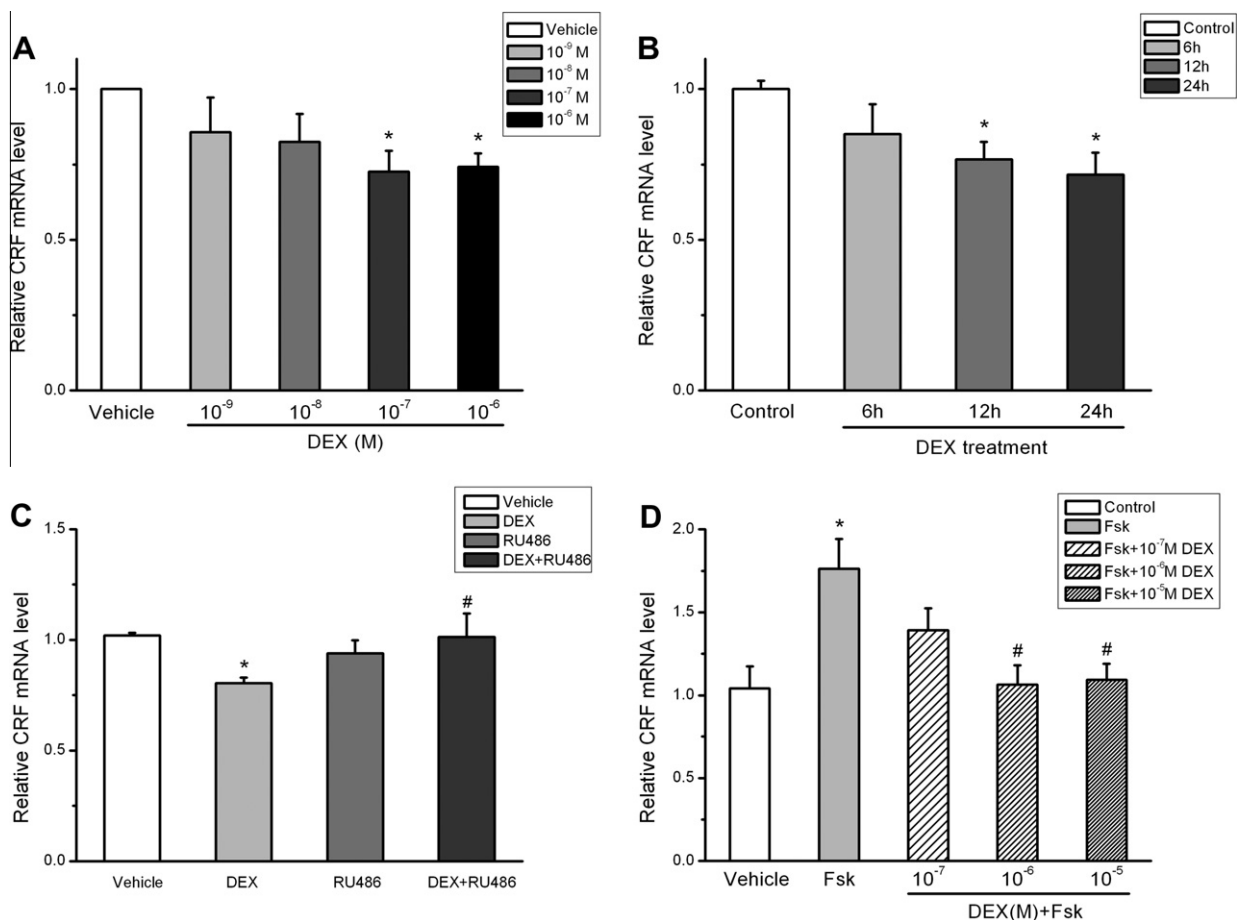
In this study, we find that acute stress resulted in an increase of the CRF mRNA expression in the PFC. Glucocorticoid could inhibit the expression of CRF mRNA in PFC. In PFC primary cell culture, CRF increased CRFR1 expression through the MEK-ERK1/2 pathway.

Previous studies have showed that the stressor-dependent CRF gene activation in the central nervous system is evolutionarily conserved (Yao and Denver, 2007). In the PVN, increases in CRF mRNA and in some cases hnRNA levels have been demonstrated after exposure of rats to different physical, physiologic, and psychologic stressors (e.g., restraint stress, foot shock, forced swimming, ether stress and endotoxin stimulation) (Bartanusz et al., 1993; Chen et al., 2001; Herman et al., 1989; Hsu et al., 1998; Imaki et al., 1991; Kovacs and Sawchenko, 1996a,b; Liu et al., 2001; Ma et al., 1997a,b; Rivest et al., 1995; Yao and Denver, 2007). Furthermore, Kalin et al. determined that restraint stress increased CRF mRNA in the amygdala (Kalin et al., 1994) and Pich et al. revealed via microdialysis that restraint stress led to amygdala CRF release (Pich et al., 1993). To our knowledge, our study is the first to find that acute restraint stress significantly elevated the level of CRF mRNA expression in the PFC (Fig. 1B).

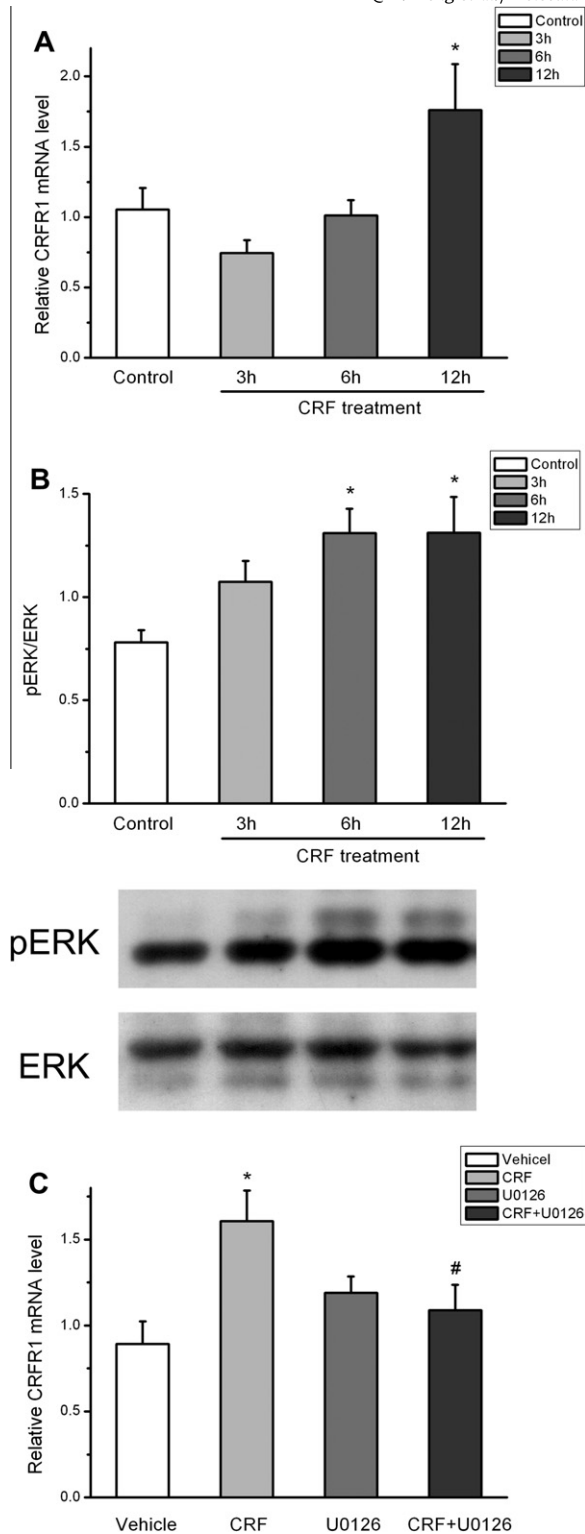
Within about past three decades, since the discovery of CRF, investigations of the CRF regulation by glucocorticoids have focus mostly on hypothalamus and amygdala (Pisarska et al., 2001; Yao and Denver, 2007). In our study, the ADX rats received high-dose corticosterone treatment showed significantly lower PFC CRF mRNA level compared to the ADX group (Fig. 2B), suggesting an inhibitory effect of glucocorticoids on PFC CRF expression.



**Fig. 5.** Composition of PFC primary culture cells. (A–C) The rat primary PFC cells were triple labeled by NeuN (red, A), GFAP (green, B) and DAPI (blue, C). D is the overlay of A–C. (E–G) The rat primary PFC cells were triple labeled by MAP2 (red, E), GFAP (green, F) and DAPI (blue, G). H is the overlay of E–G. Scale bar = 40  $\mu$ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



**Fig. 6.** Effect of dexamethasone (DEX) on CRF mRNA expression in PFC primary neurons. CRF mRNA level were determined by Real-time quantitative RT-PCR analysis using the beta-actin as internal control. (A) Effects of increasing concentrations of dexamethasone on CRF mRNA expression of PFC cells in culture. PFC cells were treated after 10 days in culture with dexamethasone ( $10^{-9}$ – $10^{-6}$  M) or vehicle for 24 h. (B) Effects of increasing incubation time of dexamethasone on CRF mRNA expression of PFC cells in culture. PFC cells were treated after 10 day in culture with  $10^{-6}$  M dexamethasone for the indicated time periods. (C) Diminishment by GR antagonist RU486 of dexamethasone-decreased CRF mRNA level of PFC cell cultures. PFC primary cells were treated with  $10^{-6}$  M dexamethasone,  $10^{-6}$  M RU486, or both for 24 h. (D) Effect of combined treatment with dexamethasone and forskolin (Fsk) on the CRF mRNA expression of PFC cell cultures. PFC primary cells were treated with  $3 \times 10^{-7}$  M forskolin or combined with dexamethasone at the indicated concentration ( $10^{-7}$ – $10^{-5}$  M). Each value represents the mean  $\pm$  SEM of 6–9 culture wells. For (A–D) the significant difference from the controls is denoted by the asterisk (\*) for  $P < 0.05$ . For (D) a significant difference from the forskolin treated group is denoted by a pound (#) for  $P < 0.05$ .



**Fig. 7.** Effect of CRF on CRFR1 mRNA expression in primary PFC cultures. CRFR1 mRNA level was determined by Real-time quantitative RT-PCR analysis using the beta-actin as internal control. (A) Effects of increasing incubation time of CRF on CRFR1 mRNA expression of PFC cells in culture. PFC cells were treated after 10 day in culture with  $10^{-7}$  M CRF for the indicated time periods. (B) Effects of increasing incubation time of CRF on ERK activity of PFC cells in culture. PFC cells were treated after 10 day in culture with  $10^{-7}$  M CRF for the indicated time periods. ERK phosphorylation was then detected by Western blot and normalized to total ERK. (C) The effect of MEK inhibitor U0126 on CRF-stimulated CRFR1 mRNA level of PFC cell cultures. PFC primary cells were treated with  $10^{-7}$  M CRF,  $10^{-5}$  M U0126, or both for 12 h. For (A–C) Each value represents the mean  $\pm$  SEM of 6–8 culture wells. The significant difference from the controls is denoted by the asterisk (\*) for  $P < 0.05$ . For (C) a significant difference from the CRF treated group is denoted by a pound (#) for  $P < 0.05$ .

Notably, in contrast with increased PVN CRF expression by ADX (Tanimura and Watts, 1998), we did not find significant increase of PFC CRF expression in ADX rats compared with sham-operated rats. This could be explained by different regulatory effect of ADX on the expression of glucocorticoid receptor (GR). In the hypothalamus, the level of GR expression was not affected by ADX (Peiffer et al., 1991), while GR expression in the cortex is up-regulated by ADX (Herman and Spencer, 1998), which may compensate the reduced inhibitory effect by decreased corticosterone level. It has also reported that corticosterone replacement could normalize the decreased GR expression in the cortex of ADX rats (Herman and Spencer, 1998), which further supports our observation of the inhibitory effect of corticosterone on PFC CRF expression.

In addition, our results also demonstrated that the stressed animals pretreated with RU486 showed significantly higher PFC CRF mRNA expression compared to the stressed ones pretreated with saline (Fig. 2C), which confirmed our hypothesis that glucocorticoids may exhibit inhibitory effects on CRF expression in the PFC. Recently, another study reported that corticosterone facilitates CRF release in response to airpuff within the medial prefrontal cortex (Merali et al., 2008). The differences in the experimental methods may lead to this discrepancy. First, the brain regions studied were different. According to the previous studies, the rat prefrontal cortex consists of cytoarchitecturally and functionally distinct areas located over the medial, orbital, and insular surfaces of the rostral cerebral hemispheres (Gabbott et al., 2005; Neafsey, 1990). The PFC block we dissected in our study including the medial, orbital and insular PFC, because a previous study has shown that CRF immunoreactive cells presented not only in the medial prefrontal cortex but also in the orbital and insular area of prefrontal cortex (Merchenthaler, 1984). Second, we examined the mRNA level of CRF, while they examined the interstitial levels of CRF. Finally, airpuff was used as the stressor in their study, while the acute restraint stress was used in our study. Considering the report that the expression of CRF mRNA was dependent on the stressors and brain regions measured (Funk et al., 2006), the use of different stressors might be another explanation for the discrepancy.

To further prove our hypothesis, double labeling of GR and CRF was performed on the rat PFC. According to the results of immunofluorescence, a high proportion of PFC CRF cells were colocalized with GR in both the rat PFC brain slices and the primary PFC cells (Fig. 3A–F). In addition, our observation that GR can be recruited to the CRF promoter in the rat PFC provided another *in vivo* demonstration for glucocorticoid to regulate CRF expression (Fig. 4C). We demonstrated relative larger amount of CRF-ir cells in the rat PFC compared with previous studies and their data showed that there was only a limited number of CRF-ir cells in neocortex of the adult rats (Merchenthaler, 1984; Olschowka et al., 1982; Sakanaka et al., 1987). Several reasons may account for this discrepancy. For the rat brain slices, the immunofluorescence was performed using the paraffin sections. Microwave treatment was used in our experiment for antigen retrieval, which has been proved by our previous study (Zhou et al., 1996). In addition, the rats in this study were kept free of disturbance and handling stress before perfusion to avoid diminishing the intensity of the CRF-ir signals, because it has been reported that secretion of CRF from CRF-ir neurons in the hypothalamus occurs within minutes after the onset of a stressful stimulus (Herman and Cullinan, 1997; Lightman and Harbuz, 1993; Rivier et al., 1983; Yi and Baram, 1994).

In our study, primary PFC cultures were also used to analyze the effect of glucocorticoid on CRF mRNA expression. We found that dexamethasone can decrease the expression of CRF mRNA (Fig. 6A,B) and the GR antagonist RU486 significantly blocked the effect of dexamethasone on CRF mRNA level (Fig. 6C). Therefore, the inhibitory effect of glucocorticoid on PFC CRF expression was demonstrated by not only *in vivo* studies but also *in vitro* models.



Previous studies have shown that glucocorticoids decrease CRF expression *in vitro* hypothalamic dissociated culture systems (Hu et al., 1992). However, in primary dissociated amygdala cells, dexamethasone did not alter CRF gene or peptide expression (Kasckow et al., 1997). Furthermore, in our experiments, we also found that forskolin (a PKA activator) caused a significantly increase in CRF mRNA expression and dexamethasone diminished this inducible effect (Fig. 6D). These results were consistent with some of the previous studies, for example, dexamethasone also significantly reduced PKA pathway dependent activation of the human CRF promoter in AtT20 cell (Guardiola-Diaz et al., 1996; Rosen et al., 1992; van der Laan et al., 2008). By contrast, some other studies showed that dexamethasone enhanced PKA pathway dependent induction of CRF mRNA in PC-12 cells (Guardiola-Diaz et al., 1996) and had no effect on forskolin induced CRF expression in primary amygdala cells (Kasckow et al., 1997).

It is well known that in coping to stress the HPA axis requires input from cortical and limbic regions involved in the processing of cognitive information (Herman and Cullinan, 1997; Herman et al., 2005) and PFC is a key brain region controlling cognition and emotion (Miller, 1999). Jaferi et al. have reported an excitatory influence of CRF in the mPFC on stress induced HPA activity and anxiety related behavior (Jaferi and Bhatnagar, 2007). They found that blockade of CRF receptor in the mPFC with the non-selective receptor antagonist significantly inhibited HPA responses and intra-mPFC injections of CRF significantly increased anxiety related behavior in the elevated plus maze. In addition, previous studies have shown that implants of corticosterone in this region attenuate HPA axis responses to restraint (Akana et al., 2001; Diorio et al., 1993). Considering glucocorticoid could down regulated CRF expression in the PFC found in our study, we speculate that PFC CRF may be a new site for glucocorticoid negative feedback regulation of response to stress. Interestingly, another previous study showed that CRF and acute stress prolongs serotonergic regulation of GABA transmission in PFC pyramidal neurons and the stress induced prolongation of the effects of serotonin on sIPSCs was diminished by CRF antagonist (Tan et al., 2004). We thus also speculated that increases in CRF and CRFR1 of the prefrontal cortex might control HPA axis and stress response through pyramidal cells. Further studies are required to test these speculations.

It is reported that CRF could regulate the expression of CRF receptor in some brain regions, such as hypothalamus, anterior pituitary, etc. (Konishi et al., 2003; Moriyama et al., 2005; Pozzoli et al., 1996). Given that the expression of CRF mRNA expression in the PFC was elevated by acute stress (Fig. 1B), we further studied whether the expression of CRF receptor could be regulated by CRF in the PFC. We focus on the regulation of CRFR1 in this study because it has been reported to be the main subtype in the PFC (Chalmers et al., 1995; Hillhouse and Grammatopoulos, 2006; Steckler and Holsboer, 1999). Our results showed that CRF increased CRFR1 mRNA expression through the MEK-ERK1/2 pathway (Fig. 7A–C). In addition, we found that acute restraint stress significantly increase the expression of CRFR1 mRNA (Fig. 1C). The up regulation of receptor expression may represent a positive regulatory loop between CRF and cells expressing CRFR1.

Together, we demonstrated for the first time the regulatory effect of acute restraint stress and glucocorticoid on PFC CRF expression. These findings contribute to a better understanding of the function of CRF in PFC in stress response.

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## References

- Akana, S.F., Chu, A., Soriano, L., Dallman, M.F., 2001. Corticosterone exerts site-specific and state-dependent effects in prefrontal cortex and amygdala on regulation of adrenocorticotrophic hormone, insulin and fat depots. *J. Neuroendocrinol.* 13, 625–637.
- Bartanusz, V., Jezova, D., Bertini, L.T., Tilders, F.J., Aubry, J.M., Kiss, J.Z., 1993. Stress-induced increase in vasopressin and corticotropin-releasing factor expression in hypophysiotrophic paraventricular neurons. *Endocrinology* 132, 895–902.
- Bissette, G., 1990. Central nervous system CRF in stress: radioimmunoassay studies. In: De Souza, E., Nemeroff, C. (eds.), *Corticotropin-releasing Factor: Basic and Clinical Studies of a Neuropeptide*, CRC-Press, Boca Raton, FL, USA, pp. 21–28.
- Chalmers, D.T., Lovenberg, T.W., De Souza, E.B., 1995. Localization of novel corticotropin-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF1 receptor mRNA expression. *J. Neurosci.* 15, 6340–6350.
- Chen, X.N., Meng, Q.Y., Bao, A.M., Swaab, D.F., Wang, G.H., Zhou, J.N., 2009. The involvement of retinoic acid receptor- $\alpha$  in corticotropin-releasing hormone gene expression and affective disorders. *Biol. Psychiatry* 66, 832–839.
- Chen, Y., Hatalski, C.G., Brunson, K.L., Baram, T.Z., 2001. Rapid phosphorylation of the CRE binding protein precedes stress-induced activation of the corticotropin releasing hormone gene in medial parvocellular hypothalamic neurons of the immature rat. *Brain Res. Mol. Brain Res.* 96, 39–49.
- Diorio, D., Viau, V., Meaney, M.J., 1993. The role of the medial prefrontal cortex (cingulate gyrus) in the regulation of hypothalamic-pituitary-adrenal responses to stress. *J. Neurosci.* 13, 3839–3847.
- Dunn, A.J., Berridge, C.W., 1990. Physiological and behavioral responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety or stress responses? *Brain Res. Brain Res. Rev.* 15, 71–100.
- Funk, D., Li, Z., Le, A.D., 2006. Effects of environmental and pharmacological stressors on c-fos and corticotropin-releasing factor mRNA in rat brain: relationship to the reinstatement of alcohol seeking. *Neuroscience* 138, 235–243.
- Gabbott, P.L., Warner, T.A., Jays, P.R., Salway, P., Busby, S.J., 2005. Prefrontal cortex in the rat: projections to subcortical autonomic, motor, and limbic centers. *J. Comp. Neurol.* 492, 145–177.
- Guardiola-Diaz, H.M., Kolinske, J.S., Gates, L.H., Seasholtz, A.F., 1996. Negative glucocorticoid regulation of cyclic adenosine 3', 5'-monophosphate-stimulated corticotropin-releasing hormone-reporter expression in AtT-20 cells. *Mol. Endocrinol.* 10, 317–329.
- Harbuz, M.S., Jessop, D.S., Lightman, S.L., Chowdrey, H.S., 1994. The effects of restraint or hypertonic saline stress on corticotropin-releasing factor, arginine vasopressin, and proenkephalin A mRNAs in the CFY, Sprague-Dawley and Wistar strains of rat. *Brain Res.* 667, 6–12.
- Herman, J.P., Cullinan, W.E., 1997. Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. *Trends Neurosci.* 20, 78–84.
- Herman, J.P., Dolgas, C.M., Carlson, S.L., 1998. Ventral subiculum regulates hypothalamo-pituitary-adrenocortical and behavioural responses to cognitive stressors. *Neuroscience* 86, 449–459.
- Herman, J.P., Ostrander, M.M., Mueller, N.K., Figueiredo, H., 2005. Limbic system mechanisms of stress regulation: hypothalamo-pituitary-adrenocortical axis. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 29, 1201–1213.
- Herman, J.P., Schafer, K.H., Sladek, C.D., Day, R., Young, E.A., Akil, H., Watson, S.J., 1989. Chronic electroconvulsive shock treatment elicits up-regulation of CRF and AVP mRNA in select populations of neuroendocrine neurons. *Brain Res.* 501, 235–246.
- Herman, J.P., Spencer, R., 1998. Regulation of hippocampal glucocorticoid receptor gene transcription and protein expression *in vivo*. *J. Neurosci.* 18, 7462–7473.
- Hillhouse, E.W., Grammatopoulos, D.K., 2006. The molecular mechanisms underlying the regulation of the biological activity of corticotropin-releasing hormone receptors: implications for physiology and pathophysiology. *Endocr. Rev.* 27, 260–286.
- Hsu, D.T., Chen, F.L., Takahashi, L.K., Kalin, N.H., 1998. Rapid stress-induced elevations in corticotropin-releasing hormone mRNA in rat central amygdala nucleus and hypothalamic paraventricular nucleus: an *in situ* hybridization analysis. *Brain Res.* 788, 305–310.
- Hu, S.B., Tannahill, L.A., Biswas, S., Lightman, S.L., 1992. Release of corticotropin-releasing factor-41, arginine vasopressin and oxytocin from rat fetal hypothalamic cells in culture: response to activation of intracellular second messengers and to corticosteroids. *J. Endocrinol.* 132, 57–65.
- Imaki, T., Nahan, J.L., Rivier, C., Sawchenko, P.E., Vale, W., 1991. Differential regulation of corticotropin-releasing factor mRNA in rat brain regions by glucocorticoids and stress. *J. Neurosci.* 11, 585–599.
- Imaki, T., Xiao-Quan, W., Shibasaki, T., Yamada, K., Harada, S., Chikada, N., Naruse, M., Demura, H., 1995. Stress-induced activation of neuronal activity and corticotropin-releasing factor gene expression in the paraventricular nucleus is modulated by glucocorticoids in rats. *J. Clin. Invest.* 96, 231–238.
- Jaferi, A., Bhatnagar, S., 2007. Corticotropin-releasing hormone receptors in the medial prefrontal cortex regulate hypothalamic-pituitary-adrenal activity and anxiety-related behavior regardless of prior stress experience. *Brain Res.* 1186, 212–223.
- Kalin, N.H., Takahashi, L.K., Chen, F.L., 1994. Restraint stress increases corticotropin-releasing hormone mRNA content in the amygdala and paraventricular nucleus. *Brain Res.* 656, 182–186.

- Kasckow, J.W., Regmi, A., Gill, P.S., Parkes, D.G., Geraciotti, T.D., 1997. Regulation of corticotropin-releasing factor (CRF) messenger ribonucleic acid and CRF peptide in the amygdala: studies in primary amygdalar cultures. *Endocrinology* 138, 4774–4782.
- Keller-Wood, M.E., Dallman, M.F., 1984. Corticosteroid inhibition of ACTH secretion. *Endocr. Rev.* 5, 1–24.
- Konishi, S., Kasagi, Y., Katsumata, H., Minami, S., Imaki, T., 2003. Regulation of corticotropin-releasing factor (CRF) type-1 receptor gene expression by CRF in the hypothalamus. *Endocr. J.* 50, 21–36.
- Koob, G.F., Heinrichs, S.C., 1999. A role for corticotropin releasing factor and urocortin in behavioral responses to stressors. *Brain Res.* 848, 141–152.
- Kovacs, K.J., Sawchenko, P.E., 1996a. Regulation of stress-induced transcriptional changes in the hypothalamic neurosecretory neurons. *J. Mol. Neurosci.* 7, 125–133.
- Kovacs, K.J., Sawchenko, P.E., 1996b. Sequence of stress-induced alterations in indices of synaptic and transcriptional activation in parvocellular neurosecretory neurons. *J. Neurosci.* 16, 262–273.
- Lightman, S.L., Harbuz, M.S., 1993. Expression of corticotropin-releasing factor mRNA in response to stress. *Ciba Found Symp.* 172, 173–187, discussion 187–198.
- Liu, Y., Curtis, J.T., Fowler, C.D., Spencer, C., Houpt, T., Wang, Z.X., 2001. Differential expression of vasopressin, oxytocin and corticotrophin-releasing hormone messenger RNA in the paraventricular nucleus of the prairie vole brain following stress. *J. Neuroendocrinol.* 13, 1059–1065.
- Ma, X.M., Levy, A., Lightman, S.L., 1997a. Emergence of an isolated arginine vasopressin (AVP) response to stress after repeated restraint: a study of both AVP and corticotropin-releasing hormone messenger ribonucleic acid (RNA) and heteronuclear RNA. *Endocrinology* 138, 4351–4357.
- Ma, X.M., Levy, A., Lightman, S.L., 1997b. Rapid changes in heteronuclear RNA for corticotrophin-releasing hormone and arginine vasopressin in response to acute stress. *J. Endocrinol.* 152, 81–89.
- Makino, S., Gold, P.W., Schulkin, J., 1994a. Corticosterone effects on corticotropin-releasing hormone mRNA in the central nucleus of the amygdala and the parvocellular region of the paraventricular nucleus of the hypothalamus. *Brain Res.* 640, 105–112.
- Makino, S., Gold, P.W., Schulkin, J., 1994b. Effects of corticosterone on CRH mRNA and content in the bed nucleus of the stria terminalis; comparison with the effects in the central nucleus of the amygdala and the paraventricular nucleus of the hypothalamus. *Brain Res.* 657, 141–149.
- Merali, Z., Anisman, H., James, J.S., Kent, P., Schulkin, J., 2008. Effects of corticosterone on corticotrophin-releasing hormone and gastrin-releasing peptide release in response to an aversive stimulus in two regions of the forebrain (central nucleus of the amygdala and prefrontal cortex). *Eur. J. Neurosci.* 28, 165–172.
- Merchenthaler, I., 1984. Corticotropin releasing factor (CRF)-like immunoreactivity in the rat central nervous system. *Extrahypothalamic distribution. Peptides* 5 (Suppl. 1), 53–69.
- Miller, E.K., 1999. The prefrontal cortex: complex neural properties for complex behavior. *Neuron* 22, 15–17.
- Moriyama, T., Kageyama, K., Kasagi, Y., Iwasaki, Y., Nigawara, T., Sakihara, S., Suda, T., 2005. Differential regulation of corticotropin-releasing factor receptor type 1 (CRF1 receptor) mRNA via protein kinase A and mitogen-activated protein kinase pathways in rat anterior pituitary cells. *Mol. Cell Endocrinol.* 243, 74–79.
- Neafsey, E.J., 1990. Prefrontal cortical control of the autonomic nervous system: anatomical and physiological observations. *Prog. Brain Res.* 85, 147–165, discussion 165–166.
- Olschowka, J.A., O'Donohue, T.L., Mueller, G.P., Jacobowitz, D.M., 1982. Hypothalamic and extrahypothalamic distribution of CRF-like immunoreactive neurons in the rat brain. *Neuroendocrinology* 35, 305–308.
- Palkovits, M., Young 3rd, W.S., Kovacs, K., Toth, Z., Makara, G.B., 1998. Alterations in corticotropin-releasing hormone gene expression of central amygdaloid neurons following long-term paraventricular lesions and adrenalectomy. *Neuroscience* 85, 135–147.
- Paulmyer-Lacroix, O., Anglade, G., Grino, M., 1994. Insulin-induced hypoglycaemia increases colocalization of corticotrophin-releasing factor and arginine vasopressin mRNAs in the rat hypothalamic paraventricular nucleus. *J. Mol. Endocrinol.* 13, 313–320.
- Paxinos, G., Watson, C., 1998. *The Rat Brain in Stereotaxic Coordinates*, fourth ed. Academic Press, New York.
- Peiffer, A., Lapointe, B., Barden, N., 1991. Hormonal regulation of type II glucocorticoid receptor messenger ribonucleic acid in rat brain. *Endocrinology* 129, 2166–2174.
- Pich, E.M., Koob, G.F., Heilig, M., Menzaghi, F., Vale, W., Weiss, F., 1993. Corticotropin-releasing factor release from the mediobasal hypothalamus of the rat as measured by microdialysis. *Neuroscience* 55, 695–707.
- Pisarska, M., Mulchahey, J.J., Sheriff, S., Geraciotti, T.D., Kasckow, J.W., 2001. Regulation of corticotropin-releasing hormone in vitro. *Peptides* 22, 705–712.
- Pozzoli, G., Bilezikjian, L.M., Perrin, M.H., Blount, A.L., Vale, W.W., 1996. Corticotropin-releasing factor (CRF) and glucocorticoids modulate the expression of type 1 CRF receptor messenger ribonucleic acid in rat anterior pituitary cell cultures. *Endocrinology* 137, 65–71.
- Rivest, S., Laflamme, N., Nappi, R.E., 1995. Immune challenge and immobilization stress induce transcription of the gene encoding the CRF receptor in selective nuclei of the rat hypothalamus. *J. Neurosci.* 15, 2680–2695.
- Rivier, J., Spiess, J., Vale, W., 1983. Characterization of rat hypothalamic corticotropin-releasing factor. *Proc. Natl. Acad. Sci. U S A.* 80, 4851–4855.
- Roosendaal, B., Brunson, K.L., Holloway, B.L., McGaugh, J.L., Baram, T.Z., 2002. Involvement of stress-released corticotropin-releasing hormone in the basolateral amygdala in regulating memory consolidation. *Proc. Natl. Acad. Sci. U S A.* 99, 13908–13913.
- Rosen, L.B., Majzoub, J.A., Adler, G.K., 1992. Effects of glucocorticoid on corticotropin-releasing hormone gene regulation by second messenger pathways in NPLC and AtT-20 cells. *Endocrinology* 130, 2237–2244.
- Sakanaka, M., Shibasaki, T., Lederis, K., 1987. Corticotropin releasing factor-like immunoreactivity in the rat brain as revealed by a modified cobalt-glucose oxidase-diaminobenzidine method. *J. Comp. Neurol.* 260, 256–288.
- Schulkin, J., Gold, P.W., McEwen, B.S., 1998. Induction of corticotropin-releasing hormone gene expression by glucocorticoids: implication for understanding the states of fear and anxiety and allostatic load. *Psychoneuroendocrinology* 23, 219–243.
- Schulkin, J., Morgan, M.A., Rosen, J.B., 2005. A neuroendocrine mechanism for sustaining fear. *Trends Neurosci.* 28, 629–635.
- Sherman, J.E., Kalin, N.H., 1987. The effects of ICV-CRH on novelty-induced behavior. *Pharmacol. Biochem. Behav.* 26, 699–703.
- Smagin, G.N., Heinrichs, S.C., Dunn, A.J., 2001. The role of CRH in behavioral responses to stress. *Peptides* 22, 713–724.
- Steckler, T., Holsboer, F., 1999. Corticotropin-releasing hormone receptor subtypes and emotion. *Biol. Psychiatry* 46, 1480–1508.
- Swaab, D.F., 2003. *The Human Hypothalamus: Basic and Clinical Aspects. (Part I: Nuclei of the Hypothalamus, Handbook of Clinical Neurology, vol. 79)*. Elsevier, Amsterdam, pp. 476.
- Swanson, L.W., Sawchenko, P.E., Rivier, J., Vale, W.W., 1983. Organization of ovine corticotropin-releasing factor immunoreactive cells and fibers in the rat brain: an immunohistochemical study. *Neuroendocrinology* 36, 165–186.
- Swanson, L.W., Simmons, D.M., 1989. Differential steroid hormone and neural influences on peptide mRNA levels in CRH cells of the paraventricular nucleus: a hybridization histochemical study in the rat. *J. Comp. Neurol.* 285, 413–435.
- Takahashi, L.K., Kalin, N.H., Vanden Burgt, J.A., Sherman, J.E., 1989. Corticotropin-releasing factor modulates defensive-withdrawal and exploratory behavior in rats. *Behav. Neurosci.* 103, 648–654.
- Tan, H., Zhong, P., Yan, Z., 2004. Corticotropin-releasing factor and acute stress prolongs serotonergic regulation of GABA transmission in prefrontal cortical pyramidal neurons. *J. Neurosci.* 24, 5000–5008.
- Tanimura, S.M., Sanchez-Watts, G., Watts, A.G., 1998. Peptide gene activation, secretion, and steroid feedback during stimulation of rat neuroendocrine corticotropin-releasing hormone neurons. *Endocrinology* 139, 3822–3829.
- Tanimura, S.M., Watts, A.G., 1998. Corticosterone can facilitate as well as inhibit corticotropin-releasing hormone gene expression in the rat hypothalamic paraventricular nucleus. *Endocrinology* 139, 3830–3836.
- van der Laan, S., Lachize, S.B., Vreugdenhil, E., de Kloet, E.R., Meijer, O.C., 2008. Nuclear receptor coregulators differentially modulate induction and glucocorticoid receptor-mediated repression of the corticotropin-releasing hormone gene. *Endocrinology* 149, 725–732.
- Viau, V., Soriano, L., Dallman, M.F., 2001. Androgens alter corticotropin releasing hormone and arginine vasopressin mRNA within forebrain sites known to regulate activity in the hypothalamic-pituitary-adrenal axis. *J. Neuroendocrinol.* 13, 442–452.
- Wang, X., Zhong, P., Gu, Z., Yan, Z., 2003. Regulation of NMDA receptors by dopamine D4 signaling in prefrontal cortex. *J. Neurosci.* 23, 9852–9861.
- Watts, A.G., Sanchez-Watts, G., 1995. Region-specific regulation of neuropeptide mRNAs in rat limbic forebrain neurones by aldosterone and corticosterone. *J. Physiol.* 484 (Pt 3), 721–736.
- Wu, L.M., Han, H., Wang, Q.N., Hou, H.L., Tong, H., Yan, X.B., Zhou, J.N., 2007. Mifepristone repairs region-dependent alteration of synapsin I in hippocampus in rat model of depression. *Neuropsychopharmacology* 32, 2500–2510.
- Yao, M., Denver, R.J., 2007. Regulation of vertebrate corticotropin-releasing factor genes. *Gen. Comp. Endocrinol.* 153, 200–216.
- Yi, S.J., Baram, T.Z., 1994. Corticotropin-releasing hormone mediates the response to cold stress in the neonatal rat without compensatory enhancement of the peptide's gene expression. *Endocrinology* 135, 2364–2368.
- Zhou, J.N., Hofman, M.A., Swaab, D.F., 1996. Morphometric analysis of vasopressin and vasoactive intestinal polypeptide neurons in the human suprachiasmatic nucleus: influence of microwave treatment. *Brain Res.* 742, 334–338.