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Blockade of CRF1 and CCK2 receptors attenuated the elevated anxiety-like behavior induced by immobilization stress

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ABSTRACT

Two highly co-localized neurotransmitters: corticotropin-releasing factor (CRF) and cholecystokinin (CCK), have been implicated in the development of stress-related anxiety disorders. This study was designed to examine the role of CRF1 and CCK2 receptors on the anxiety-like behavior induced by immobilization stress. Our results showed that 30-min immobilization enhanced the anxiety-like behavior in C57BL/6J mice examined in the elevated plus maze (EPM). The combined pretreatment of CR2945 (a CCK2 receptor antagonist) and antalarmin (a CRF1 receptor antagonist) fully blocked this elevated anxiety-like behavior, while the application of CR2945 or antalarmin alone showed only partial effects. The increased expression of CRF1 and CCK2 receptors at protein levels in three anxiety-related brain regions: cortex, hippocampus and hypothalamus, was detected by Western blot. The increased mRNA expression of CCK, CRF, CCK2 and CRF1 receptors was also examined by real-time RT-PCR. Our study demonstrated that the blockade of CRF1 and CCK2 receptors attenuated the elevated anxiety-like behavior induced by immobilization stress, suggestive of the CRF and CCK systems contributing to the development of stress-related anxiety behavior.

1. Introduction

Corticotropin-releasing factor (CRF) and cholecystokinin (CCK), which are two highly colocalized neuropeptides in the brain, have been implicated in the etiology of stress-related anxiety disorders (Bradwejn, 1993; Smoller et al., 2005; Sherrin et al., 2009). CCK was first identified and characterized in the gastrointestinal tract as a hormone and later was found to be one of the most abundant neuropeptides in the brain with high concentrations in the cortex and limbic brain regions (Beinfeld et al., 1981; Beinfeld and Palkovits, 1982). CCK has been linked to anxiety and panic disorders, but also has a role in satiety, learning and memory, thermoregulation, dependence and withdrawal processes (Harro, 2006; Rotzinger and Vaccarino, 2003; Wang et al., 2005). Thus far, the actions of CCK have been attributed to two types of receptor, CCK_1 and CCK_2 (formerly known as CCK-A and CCK-B, respectively). CCK_1 is mainly found in the digestive system, whereas CCK_2 receptors are the predominant subtype found in the central nervous system (CNS) and have been implicated in the experimentally induced anxiety and fear in rodents (Farook et al., 2001, 2004; Wang et al., 2003a,b) and panic attacks in humans (Bradwejn and Koszycki, 1994).

The effects of stress on CCK neuronal system have been studied in the last three decades. Siegel et al. (1984) demonstrated that CCK concentrations in the prefrontal cortex and the medial and lateral septum were increased after 30 min of foot-shock stress exposure. A study on marathon runners suggested that CCK is an important regulation factor in response to anticipatory stress, indicated by the higher level under pre-run conditions than control conditions and the highest after run (Philipp et al., 1992). Later, the effect of 30-min immobilization on the marked increase in cortical CCK-like material release in rats was also demonstrated (Nevo et al., 1996). So far, extensive studies have been carried out on the involvement of the CCKergic system in anxiety-, panic- and stress-related behaviors. Moreover, the functional relationship between CCK and the hypothalamic–pituitary–adrenal (HPA) axis, which is believed to be sensitized after exposure to certain stressors, was also investigated (Bhatnagar et al., 2000; Cournil et al., 2000).

CRF, as a major hormone and neurotransmitter in HPA axis, has been accepted as the main neuropeptide involved in both physical and emotional stress (Spiess et al., 1981; Vale et al., 1981). CRF functions as the prime mediator in stress-induced HPA axis activation by triggering the immediate release of ACTH from the anterior pituitary. Subsequently, ACTH stimulates corticosterone release from the zona fasciculate of the adrenal cortex. Two CRF receptors, CRF1

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and CRF2, have been identified in the mouse. CRF1 receptor has been hypothesized to be widely expressed in mammalian brain and pituitary and well involved in anxiety behavior (Sanchez et al., 1999). Centrally administered CRF was shown to produce several signs of increased anxiety and transgenic mice that over-express CRF exhibit increased anxiogenic behavior (Stenzel-Poore et al., 1994). Conversely, central administration of either a CRF antisense oligodeoxynucleotide or a CRF receptor antagonist produced anxiolytic effects in the rat (Skutella et al., 1994). These data indicated an increase in anxiety-like behavior after the activation of CRF1 receptor.

The co-localization of CRF and CCK has been investigated in extensive studies. Sutin and Jacobowitz (1988) examined the immunocytochemical localization of peptides and neurochemicals in the rat laterodorsal tegmental nucleus and found the existence of CRF, atrial natriuretic factor (ANF), neurotensin (NT), vasoactive intestinal polypeptide (VIP) and dynorphin B (Dyn B) cell bodies in addition to CCK, neuropeptide Y (NPY), serotonin (5HT), glutamic acid decarboxylase (GAD), and tyrosine hydroxylase (TH). Evidence from the neurons in the cat raphe nucleus also showed the immunoreaction for 5-HT, CRF, gamma-aminobutyric acid (GABA), CCK, NPY, thyrotropin-releasing hormone (TRH), and vasoactive intestinal polypeptide (VIP) (Batten, 1995). The coexistence of CCK and CRF peptides was even observed in the entire CNS in the marine worm Nereis by immunochemistrical and electron microscopical studies (Dhainaut-Courtois et al., 1985a,b, 1986).

The similar functions of CCK and CRF systems and their close localization raise a question: does the interaction between CCK and CRF systems exist and contribute to the modulation of anxiety-like behavior in animal models? Current knowledge in this field is quite limited. It was found that the chronic treatment of antalarmin, a CRF1 receptor antagonist, significantly increased CCK2 receptor-binding density and the expression of preproCCK mRNA (Lodge and Lawrence, 2003). Another direct example is the experiment from Biro et al. (1993), who indicated that the pretreatment with CRF antiserum and a CRF receptor antagonist, alpha-helical CRF (ahCRF) prevented the anxiogenic response to CCK8 in rats in a dose-dependent manner. Our preliminary data showed that the pre-treatment of CRF1 antagonist [$Glu^{11,16}$]Astressin blocked the anxiogenic effect of CCK4 in mice (Wang et al., 2005). Recently, we demonstrated that chronic i.c.v. administration (5 days) of CRF1 agonist cortagine resulted in the increased sensitivity of the central CCK system as indicated by the effectiveness of sub-threshold doses of CCK4 during elevated plus maze paradigm and fear conditioning (Sherrin et al., 2009). These data point to the possibility that some of the anxiogenic effects of the central CCK system take place via interacting with the CRF system.

Thus, in the present study, we examined the immobilization-induced anxiety-like behavior in C57BL/6J mice tested by the elevated plus maze, screened the mRNA and protein expression of CCK and CRF systems in key brain regions and further explored the role of CRF1 and CCK2 receptors in the enhanced anxiety-like behavior. Although it has been demonstrated in rats that CRF and CRF1 receptor mRNAs were increased in several brain regions, e.g. hypothalamic paraventricular nucleus (PVN) and amygdala, after exposure to immobilization stress (Bonaz and Rivest, 1998; Imaki et al., 1996), the expression of CRF and CRF1 receptors in mice after immobilization has been poorly studied.

2. Materials and methods

2.1. Animals

Male, healthy, 8-week-old C57BL/6J mice (Laboratory Animal Center, National University of Singapore), were used in the experiments. The mice were housed under conventional conditions (room temperature 22 ± 1 °C; 12 h light/dark cycle with lights on 07:00 am–19:00 pm). Food and water were available ad libitum. Animals were allowed one week of habituation to the handling and the animal facility before being used in experiments.

The animals were brought into the experimental room 1 h before the experiment. All behavioral experiments were performed between 10:00 am and 17:00 pm. Each animal was exposed to the behavioral apparatus only once. All housing and behavioral procedures were approved by the animal ethics committee, National University of Singapore, and conformed to the principles of laboratory animal care issued by the National Institutes of Health (NIH).

2.2. Drug treatment

CCK_2 antagonist CR2945 (β-[2-([2-(8-azaspiro[4,5]dec-8-ylcarbonyl)-4,6-dimethylphenyl]amino)-2-oxoethyl]-(R)-1 naphthalenepropanoic acid; Sigma-Aldrich, USA) was freshly prepared and dissolved in 10% dimethyl-sulphoxide (DMSO; Merck). CRF1 receptor antagonist antalarmin (N-butyl-N-ethyl-2,5,6-trimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolol[2,3-d]pyrimidin-4-amine hydrochloride; Sigma-Aldrich, USA) was dissolved in 5% Cremephor (Sigma-Aldrich, USA), 5% DMSO and 90% sterile saline. Animals received i.p. injection of CR2945 (5 mg/kg bw) or antalarmin (10 mg/kg bw). The selection of drug doses and the time of drug administration were based on the previous literature (Marinelli et al., 2007; Revel et al., 1998; Specio et al., 2008) and our previous experiments (Farook et al., 2001, 2004).

2.3. Immobilization stress

Acute immobilization of the mice consisted of taping each mouse's limbs to a Plexiglas surface with each mouse kept in a prone position for 30 min.

2.4. Elevated plus maze (EPM)

The experimental apparatus consisted of a central part (5×5 cm), two opposing open arms (30×5 cm) and two opposing closed arms (30×5 cm) with 15 cm high, non-transparent walls. The maze was elevated 55 cm above the floor. The test room was maintained with controlled light levels and temperature. The maze platforms and walls were thoroughly cleaned with 70% ethanol between sessions and allowed to dry. The animals were individually placed in the center of the maze facing an open arm and allowed 5 min of free exploration. All sessions were videotaped and the total distance moved, the number of entries into the open and closed arms, and the total time spent in the open and closed arms were measured by Ethovision software (Noldus). The percentage of time spent on and the number of entries to open arms were calculated as the standard anxiety indices. The total distance moved was considered as the index of locomotor activity.

2.5. Behavioral procedures

Stress0 and stress30 group mice were exposed to the EPM immediately or 30 min after the cessation of a 30-min immobilization. The antagonists CR2945, antalarmin or CR2945+ antalarmin were applied 15 min before the beginning of immobilization or 45 min before EPM without exposure to the immobilization stress. The control group mice were tested in EPM without any immobilization stress exposure. Each group contained 8–11 mice.

2.6. Sample collection, RNA preparation and protein isolation

Mice were decapitated 3 h after EPM exposure. In a dorsal view of a mouse's skull, we cut along the coronal suture and sagittal suture, and then pull off both sides of the frontal and the parietal bone. The brain was removed from the cranial cavity. In a ventral view of the brain, we cut the brain into left and right hemispheres. Whole cerebral cortex, hippocampus and hypothalamus were dissected bilaterally

and immersed immediately in liquid nitrogen and stored at −80 °C for RNA and protein isolation.

For RNA isolation, the brain tissue was briefly homogenized in a 10-fold volume (wt/vol) of Trizol reagent (Gibco/BRL, Singapore) with a Polytron homogenizer (Janke and Kunkel, Selangor, Malaysia). After phenol–chloroform extraction, total RNA was precipitated by adding isopropanol and centrifuged at 15,000 *g* for 12 min. The RNA pellets were suspended in diethylpyrocarbonate-treated (DEPC) water. Finally, the quality and quantity of total RNA were determined by a spectrophotometer at 260/280 nm wavelengths.

For protein isolation, the brain tissue was homogenized in a ratio of 1:10 (1 g tissue/10 ml reagent) of CelLytic™ MT reagent (Sigma) supplemented with 1% protease inhibitor cocktail (Sigma). The lysed sample was centrifuged at 12,000–20,000 *g* for 10 min, and then the protein-containing supernatant was removed. Protein concentration was measured by the Bradford method using Quick Start Bradford dye reagent (Bio-Rad Laboratories, Hercules, CA, USA).

2.7. Western blot

Brain samples containing equal amounts of total proteins were mixed with XT sample buffer 4× (Bio-Rad Laboratories, Hercules, CA, USA). After being heated at 99 °C for 5 min, the denatured samples were electrophoresed on 10% sodium dodecyl sulfate-polyacryamide (SDS-PAGE) gel and then transferred onto a PVDF membrane using an electroblotting apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked with 5% BSA in PBS containing 0.1% Tween-20 for 1 h. The membrane was washed three times, incubated overnight at 4 °C with a primary antibody (mouse anti-β-tubulin monoclonal antibody (Sigma-Aldrich, USA), 1:1000 dilution; or goat anti-CRF1 receptor polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:500 dilution; or goat anti-CCK2 receptor polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:500 dilution), washed again and incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (rabbit anti-mouse IgG for β-tubulin, 1:10,000 dilution; or donkey anti-goat IgG for CRF1 and CCK2, 1:10,000 dilution). After washing three times, the membrane was incubated in SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) for 5 min at room temperature. Protein bands were visualized and analyzed by Quantity One analysis software using Gel Doc XRS (Bio-Rad Laboratories, Hercules, CA, USA), and expressed as the ratio of band intensity relative to the β-tubulin (the internal standard).

2.8. Quantitative real-time RT-PCR

Real-time RT-PCR was carried out according to the manufacturer's instruction (Applied Biosystems, USA). To synthesize cDNA, 400 ng total RNA was mixed with TaqMan reverse transcription reagents in a total volume of 20 μl and incubated at 25 °C for 10 min, 37 °C for 60 min, 95 °C for 5 min and a hold cycle at 25 °C. 2 μl cDNA was added into a 20 μl reaction buffer containing 10 μl 2× Taqman universal PCR master mix and 1 μl specific primers from TaqMan gene expression array. The cycling conditions were holding at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. ABI PRISM 7000 SDS Sequence Detection System equipment and ABI PRISM 7000 SDS Software were used. For No Template Control (NTC) reactions, 2 μl of cDNA was replaced with 2 μl of RNase-free water. 18s rRNA was used as the endogenous reference gene. Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected. All reactions were carried out in triplicate.

The amount of target genes in three brain regions was obtained by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) after being normalized against an internal control (18S ribosomal RNA) and a calibrator, in this case, the corresponding brain region in the control group (the relative gene expression was defined as 1). The relative expression of mRNA amount of target genes is shown as the fold change in Table 1.

2.9. Statistical analysis

Data are analyzed using SPSS 11.5 software and presented as mean ± SEM. The level of significance for all statistical tests was set to 0.05. Statistical evaluation in animal behavior study was performed by one-way analysis of variance (ANOVA) followed by the Bonferroni's post hoc test for individual between-group comparisons. The correlation among the percentage of time spent on open arms and the locomotor activity (total distance traveled (cm)) was assessed with Pearson's correlation coefficient.

Independent samples *t* test was applied in the Western blot study. The gene expression detected by real-time RT-PCR was shown as the fold change with an error term, which was determined by evaluating the expression: $2^{-\Delta\Delta Ct}$ with ΔΔCt ± the standard deviation using the ABI PRISM 7000 SDS Software.

3. Results

3.1. Effects of 30-min immobilization stress on the anxiety-like behavior in C57BL/6J mice tested in EPM

We initiated this study to examine the effects of 30-min immobilization stress on the anxiety-like behavior in C57BL/6J mice tested in EPM. Mice from the stress0 group (exposed to the EPM test immediately after 30-min immobilization) showed remarkable enhancement of anxiety-like behavior (Fig. 1) as indicated by the decreased time spent on ($F_{(8, 85)} = 9.257$; $p < 0.001$; Bonferroni test, $p < 0.05$ stress0 vs control) and the number of entries into the open arms of EPM ($F_{(8, 85)} = 15.637$; $p < 0.001$; Bonferroni test, $p < 0.05$ stress0 vs control). This effect disappeared in stress30 mice which were tested in EPM 30 min after the cessation of immobilization stress (Bonferroni test, $p > 0.05$ stress30 vs control; $p < 0.05$ stress30 vs stress0).

In order to examine the effect of CRF1 and CCK2 receptors in immobilization-induced anxiety, we employed antalarmin (a CRF1

Table 1
mRNA expression of CRF, CRF1, CCK and CCK2 genes in three brain regions in mice after immobilization stress.

Gene name	Group	Cortex	Hippocampus	Hypothalamus
CRF	Control	1.00 (0.92–1.09)	1.00 (0.86–1.16)	1.00 (0.91–1.09)
	Stress0	3.67 (3.34–4.03)	2.62 (2.45–2.82)	2.32 (2.18–2.46)
	Stress30	0.95 (0.81–1.12)	1.63 (1.56–1.71)	1.95 (1.89–2.02)
CRF1	Control	1.00 (0.89–1.12)	1.00 (0.89–1.12)	1.00 (0.96–1.04)
	Stress0	1.27 (1.14–1.42)	1.59 (1.51–1.68)	1.51 (1.46–1.57)
	Stress30	0.86 (0.81–0.91)	1.15 (1.08–1.22)	0.92 (0.88–0.97)
CCK	Control	1.00 (0.90–1.11)	1.00 (0.87–1.14)	1.00 (0.92–1.08)
	Stress0	2.41 (2.30–2.54)	2.88 (2.52–3.29)	1.10 (1.06–1.15)
	Stress30	1.21 (1.19–1.23)	1.33 (1.24–1.42)	1.10 (1.04–1.16)
CCK2	Control	1.00 (0.88–1.13)	1.00 (0.91–1.10)	1.00 (0.92–1.09)
	Stress0	4.82 (4.31–5.4)	2.33 (2.16–2.50)	1.64 (1.43–1.89)
	Stress30	1.98 (1.83–2.15)	0.96 (0.85–1.09)	1.11 (1.02–1.22)

Value ($2^{-\Delta\Delta Ct}$) means the fold change in gene expression relative to the respective control group (the gene expression was defined as 1). Values >1 are indicative of an increase in gene expression, and <1 a decrease. The error term was determined by evaluating the expression $2^{-\Delta\Delta Ct \pm \text{standard deviation}}$. The asymmetric distribution is a consequence of converting the results of an exponential process into a linear comparison of amounts.

C_t means the threshold cycle. $\Delta\Delta Ct = \Delta C_{t,\ sample} - \Delta C_{t,\ calibrator}$; $\Delta C_{t,\ sample}$ is the C_t value for any sample normalized to the endogenous housekeeping gene (18S ribosomal RNA) and $\Delta C_{t,\ calibrator}$ is the C_t value for the calibrator also normalized to the endogenous housekeeping gene. The calibrator, in this case, is the corresponding brain region in the control group.

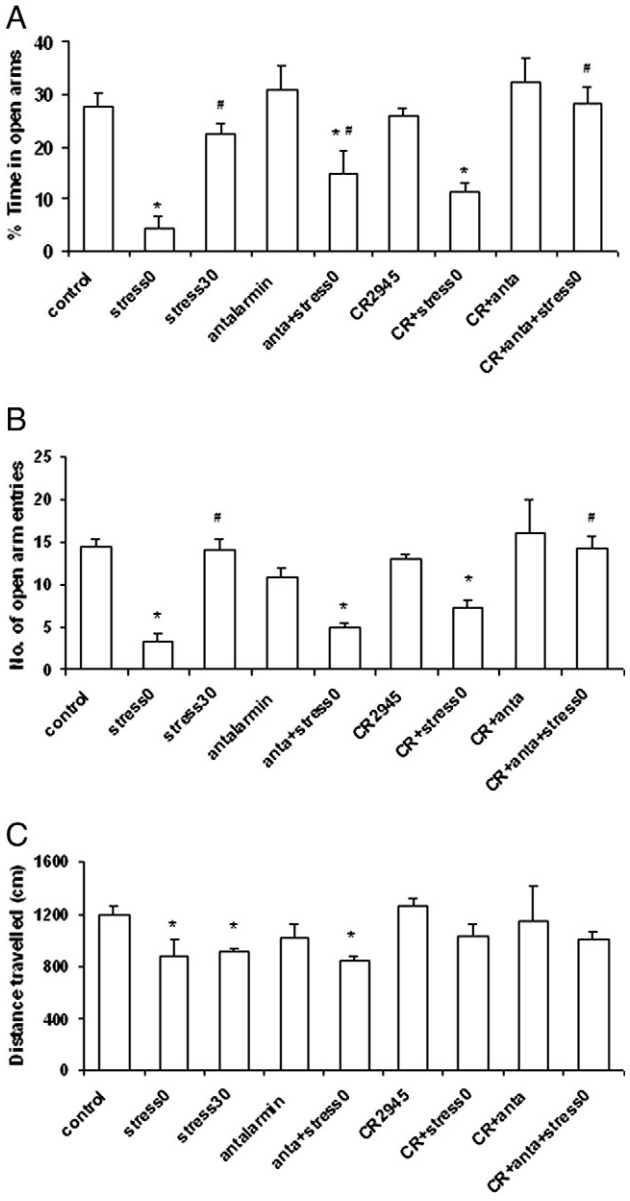

Fig. 1. The anxiety-like behavior in C57BL/6J mice was tested by the elevated plus maze. Panel A: the percentage of time spent on the open arms; Panel B: the number of open arm entries; Panel C: the total distance travelled. Stress0 and stress30 group mice were exposed to the EPM immediately or 30 min after cessation of 30-min immobilization. Control group mice were exposed to EPM without immobilization stress. The antagonists CR2945 (5 mg/kg bw) and antalarmin (10 mg/kg bw) were applied by *i.p.* 15 min before the beginning of immobilization or were injected alone 45 min before EPM. Each group contains 8-11 mice. Data are presented as mean ± SEM. One-way ANOVA followed by the Bonferroni's post hoc test for individual between-group comparisons was used for statistical analysis. Significant level was set at 0.05. * $p<0.05$ versus control. # $p<0.05$ versus stress0.

receptor antagonist) and CR2945 (a CCK2 receptor antagonist) to block the CRF1 and CCK2 receptors. The administration of antalarmin or CR2945 alone showed no effect on the percentage of time spent on open arms, number of open arm entries and distance traveled (Fig. 1). Nevertheless, the pretreatment with antalarmin or CR2945 15 min before immobilization stress attenuated the enhanced anxiety-like behavior as indicated by the increase of the time spent on the open arms. These increases did not reach the control group level (Bonferroni test, $p<0.05$ CR + stress0 vs control; Bonferroni test, $p<0.05$ anta + stress0 vs control), suggesting that the blockade of CRF1 or CCK2 receptor alone was unable to fully reverse the enhancement of anxiety-like behavior induced by immobilization stress. Thus, we further pretreated the animals with combined CR2945 and antalarmin and found that the co-treatment of CR2945 and antalarmin fully blocked this enhancement (Bonferroni test, $p>0.05$ CR + anta + stress0 vs control; Bonferroni test, $p<0.05$ CR + anta + stress0 vs stress0), indicating that both CRF1 and CCK2 receptors are necessary for the immobilization-induced increase in anxiety-like behaviors.

However, exposure to immobilization significantly decreased the total distance traveled in mice ($F_{(8,85)}=3.266$; $p<0.005$; Bonferroni test, $p<0.05$ stress0 vs control; $p<0.05$ stress30 vs control). The correlation analyses between locomotor activity (total distance traveled (cm)) and anxiety (percentage of time on the open arms) indicated that there was no significant correlation between locomotion and anxiety-like behavior ($r=0.076$; $p=0.489$) in the EPM test. These data indicated that the locomotion change did not influence the percentage of time spent on the open arms, which is a main indicator for measuring the anxiety-like behavior in mice. Thus, we concluded that the immobilization stress increased the anxiety behavior level of C57BL/6J mice in EPM.

3.2. Protein expression of CRF1 and CCK2 receptors in three brain regions after 30-min immobilization detected by Western blot

Three brain regions: cortex, hippocampus and hypothalamus, were isolated and subjected to the protein expression test for CRF1 and CCK2 receptors in control, stress0 and stress30 mice. Compared to the control group, mice from group stress0 showed the increased expression of CRF1 receptors in cortex ($p<0.05$ stress0 cortex vs control cortex), hippocampus ($p<0.05$ stress0 hippocampus vs control hippocampus) and hypothalamus ($p<0.05$ stress0 hypothalamus vs control hypothalamus), and this enhancement was also observed in the hypothalamus in the stress30 group ($p<0.05$ Stress30 hypothalamus vs control hypothalamus) (Fig. 2B). The increased expression of CCK2 receptor was only detected in the cortex in mice from group stress0 ($p<0.05$ stress0 cortex vs control cortex). The other two areas: hippocampus and hypothalamus, did not differ significantly in CCK2 protein levels (Fig. 2C).

3.3. mRNA expression of CRF, CRF1, CCK and CCK2 genes in three brain regions after 30-min immobilization detected by real-time RT-PCR

Quantitative real-time RT-PCR was employed to check the mRNA expression of CRF, CRF1, CCK and CCK2 genes in three brain regions: cortex, hippocampus and hypothalamus. Compared to the control group, mice from group stress0 showed the increased expression of CRF1 receptors in the cortex, hippocampus and hypothalamus (Table 1), which was corresponding to the protein expression by Western blot (Fig. 2B). The gene expression of the CCK2 receptor was found to be up-regulated markedly in the cortex and moderately in the hippocampus and the hypothalamus in group stress0 (Table 1), though only the expression in the cortex was confirmed by the Western blot test at the protein level (Fig. 2C). The enhancement of CRF and CCK gene expression was also observed in these anxiety-related brain regions in mice after 30-min immobilization stress.

4. Discussion

Our study showed that 30-min immobilization enhanced the anxiety-like behavior in C57BL/6J mice examined in the EPM as indicated by the decreased time spent on open arms and the number of open arm entries. The combined pretreatment of CR2945 (a CCK2 receptor antagonist) and antalarmin (a CRF1 receptor antagonist) fully blocked this elevated anxiety-like behavior, while the application of CR2945 or antalarmin alone showed only partial effects. The increased mRNA and protein expression of CRF1 and CCK2 receptors in three anxiety-related brain regions: cortex, hippocampus and hypothalamus, was detected by Western blot and real-time RT-PCR.

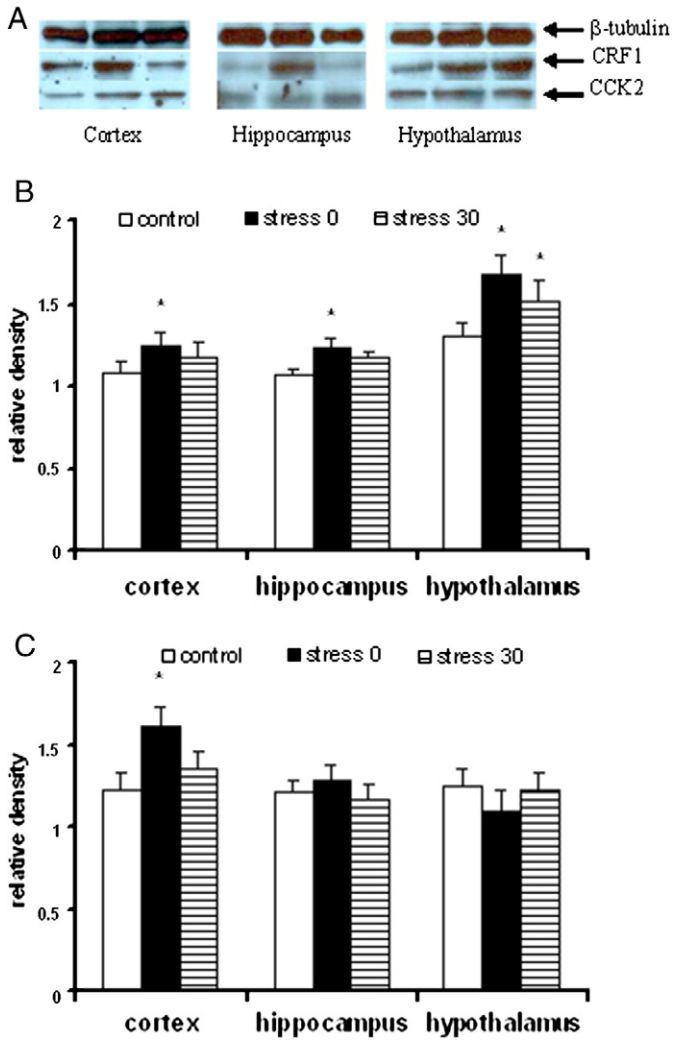

Fig. 2. The protein expression of CRF1 and CCK2 receptors detected by western blot in cortex, hippocampus and hypothalamus in three groups: control, stress0 and stress30. Panel A shows representative bands of β-tubulin, CRF1 and CCK2 receptors. Panel B is the statistical result of CRF1 receptor expression; Panel C is the result of CCK2 receptor expression. Band density of target receptor is expressed as the ratio relative to the corresponding β-tubulin (the internal standard) band. Data are presented as mean ± SEM. Independent samples t test, * $p<0.05$ versus control.

The results suggested that both CRF and CCK systems contribute to the development of stress-related anxiety behavior.

Previous observations showed that the maximal responses of the stress effector systems are usually seen within the first 30 min after the beginning of immobilization stress (Pacák and Palkovits, 2001). The magnitude of central stress responses usually gradually diminishes after the end of acute immobilization stress and returns to basal levels (Pacák and Palkovits, 2001). This is consistent with our observation that the increased anxiety induced by 30-min immobilization disappeared in stress30 mice, which were tested in EPM 30 min after the cessation of immobilization stress.

Todorovic et al. (2007) showed that mice subjected to 1-h immobilization exhibited the enhancement of anxiety-like behavior 15 and 30 min after the end of immobilization and this enhancement disappeared 1 h afterwards, which is in agreement with the observation from Radulovic et al. (1999). In their experiments, 1-h immobilization did not significantly alter the locomotor activity. In contrast to their findings, our results showed that 30-min immobilization reduced the total distance traveled in the stress group and meanwhile increased the anxiety-like behavior. This discrepancy might be related to the experimental design or time-dependent changes in immobilization stress-induced behavior. 30-min immobilization was chosen as the acute stressor in this experiment because preliminary work had indicated that a 30 min of immobilization was sufficient to increase anxiety-like behavior in the behavioral tests (Henry et al., 2006). The correlation analysis among the percentage of time spent on open arms and the total distance traveled showed no significant correlation between the percentage of time spent on open arms and the total distance traveled. These data suggested that the effects of the immobilization stress on anxiety-related behavior were not confounded by alterations in locomotor activity, which is in agreement with the observation of Henry et al. (2006).

Stress is usually comprehended as an event affecting mainly the HPA axis and initiating the alarm reaction represented by activation of the adrenal medulla. This means that the levels of related hormones and neurotransmitters are significantly elevated during and after the stress. CRF, following its discovery 30 years ago, has been postulated to mediate both hormonal and behavioral responses to stressors. It has been well documented that CRF mRNA was increased after immobilization stress in PVN (Bartanusz et al., 1993; Harbuz and Lightman, 1989; Harbuz et al., 1991, 1993; Kiss et al., 1996; Pacak et al., 1996) and amygdala in rats (Kalin et al., 1994; Mamalaki et al., 1992). CRF receptor mRNA expression in rats was also significantly increased after exposure to acute immobilization stress. 120 min restraint significantly increased CRF1 receptor and CRF mRNA signals in the PVN examined by in situ hybridization (Imaki et al., 1996). CRF receptor mRNA hybridization in rats became evident 2 h after the initiation of acute immobilization, with levels substantially increasing from 2 to 4 h, decreasing at 8 h and disappearing by 24 h (Luo et al., 1994). This is in line with an observation from Bonaz and Rivest (1998) that exposure to a 90-min immobilization stress induced a robust increase in CRF1 receptor mRNA expression in rats, which reached a peak level at 3 h after the cessation of immobilization and decreased gradually from 3 to 12 h.

In contrast to the extensive research on the mRNA expression of CRF and CRF1 receptors after immobilization stress in rats, the expression of CRF and CRF1 receptors in mice has seldom been investigated. Two studies reported an increase in CRH mRNA, not CRF1 mRNA, in the PVN after 2 or 3 h restraint in mice by in situ hybridization (Imaki et al., 2003; Makino et al., 2005). By using quantitative real-time RT-PCR, we detected a pronounced increase in CRF mRNA expression in the cortex, hippocampus and hypothalamus after 30-min immobilization and the slight enhancement of the CRF1 mRNA expression in these brain regions, which was confirmed by the Western blot analysis for the CRF1 protein levels. The enhancement of the CRF1 receptor diminished in stress30 group mice. This disagreement of our results with previous reports could be explained by the different mRNA detection technique, stress time and type used. We chose 30-min immobilization as the acute stressor in this experiment instead of restraint. The restraint and immobilization stressors differ in the degree of movement restriction. In immobilization, all limbs are taped to a Plexiglas surface and the animals show increased exercise as they want to escape from that inconvenient position. In restraint, the animals are placed into a tube where their movement is impossible. Although there are clear similarities between these types of stressors, the effects on the animals sometimes are different.

CCK is another target neurotransmitter in our experiment. A large body of evidence, both in rodents and in human, supports the idea that central cholecystokininergic neurones play a role in the expression and control of anxiety-related behaviors (van Megen et al., 1996). Nevo et al. (1996) showed that a 30-min immobilization produced a marked but transient increase in cortical CCK-like material release in rats, thus providing the direct biochemical evidence in support of the activation of cortical CCK-ergic neurotransmission in relation with immobilization stress and anxiety-related behavior. Later, Giardino et al. (1999) demonstrated that restraint stress alone increased CCK mRNA levels in rats in the hippocampus, whereas no changes were found in the cerebral cortex, amygdaloid complex and thalamus. A study conducted

in jerboas by Barakat et al. (2006) indicated that the number of CCK-immunoreactive neurones within the PVN was significantly increased (138%) in stressed animals compared to controls. Similarly, the number of CRH-containing neurones was higher in stressed jerboas (128%) compared to controls. This evidence suggested that in addition to CRF, CCK is another neuropeptide involved in the response to stress, acting by controlling HPA axis activity.

In our study, the gene expression of CCK2 receptor was found to be up-regulated dramatically in cortex and moderately in the hippocampus and hypothalamus in group stress0 mice, though only the expression in the cortex was confirmed at protein level by Western blot. Moreover, the CCK gene expression was found to be increased in the cortex and hippocampus in mice from immobilization stress groups, but did not differ significantly in the hypothalamus.

We further examined our hypothesis that the activation of CRF and CCK systems is responsible for the enhanced anxiety-like behavior induced by immobilization stress using two antagonists to block the expression of CRF1 and CCK2 receptors. It was found that CCK2 receptor antagonist CR2945 or CRF1 antagonist antalarmin partially reversed the enhanced anxiety-like behavior induced by 30-min immobilization, which is in agreement with a study from Henry et al. (2006), who demonstrated that the administration of the CRF1 antagonist antalarmin into the central amygdala reduced the anxiogenic effect of the 30-min immobilization stress. Although the blockade of CRF1 or CCK2 receptor alone only partially reversed the enhancement of anxiety-like behavior induced by immobilization stress, the combined treatment of CR2945 and antalarmin could fully block this enhancement, suggesting the synergetic effects of CRF and CCK systems in the immobilization-induced anxiety in mice.

In summary, our data suggest that 30-min immobilization enhanced the anxiety-like behavior in EPM in mice, accompanied by the activation of CRF and CCK systems. The co-blockade of CRF1 and CCK2 receptors could fully reverse the enhancement of anxiety-like behavior induced by immobilization stress, while the pretreatment of CRF1 or CCK2 receptor antagonist alone only showed partial effect, indicating that both CRF and CCK systems participate in the immobilization-induced increase in anxiety-like behaviors.

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