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Original Article

Hypothalamic gastrin-releasing peptide receptor mediates an antidepressant-like effect in a mouse model of stress

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Abstract: Evidence has shown that gastrin-releasing peptide receptor (GRPR) is involved in responses to stress and anxiety. The primary role of GRPR is to stimulate corticotrophin-releasing hormone (CRH) or adrenocorticotropic hormone (ACTH) secretion. Thus, the mechanisms of GRPR signaling should be elucidated to discover novel therapeutic targets for treating depression. This study aimed to investigate GRPR alterations in the C57 mouse hypothalamus after the animals were subjected to stress and fluoxetine treatments. Specifically, we subjected the mice to isolation and chronic unpredictable mild stress (CUMS) for three weeks to establish an experimental model of depression. These mice were subsequently treated with fluoxetine for three weeks. Then, we performed the sucrose preference test and the open field test and measured food intake and body weight to explore the effects of stress and fluoxetine on activity and anhedonia. After fluoxetine treatment, we also assessed changes in the levels of GRPR expression in the hypothalamus using immunohistochemistry, western blotting, and real-time quantitative PCR (RT-PCR). We found that stressed mice showed significant reductions in locomotion, food intake/body weight, and sucrose preference; these reduced parameters indicated a state of anhedonia. Marked increases in mRNA and protein expression of GRPR in the hypothalamus of CUMS-exposed mice were also observed, although treatment with fluoxetine reversed these stress-induced changes. Our results also demonstrated the feasibility and effectiveness of the C57 mouse model of depression established by CUMS and isolation. After fluoxetine treatment was administered, the animals' depression symptoms were alleviated, and these behavioral alterations were accompanied by specific changes in mRNA and protein expression of GRPR in the hypothalamus. These results suggest that GRPR may be implicated in depression; therefore, new therapeutic targets of depression focused on GRPR signaling should be explored.

Keywords: Depression, GRPR, hypothalamus, antidepressant

Introduction

Depression, which is one of the most prevalent mental disorders with high prevalence and mortality, is expected to become the second most common disease after coronary heart disease by 2020 [1-3]. Depression is characterized by several clinical symptoms, such as chronic depressed mood, inability to experience pleasure, loss of interest in activities, fatigue, feelings of worthlessness, and suicidal tendencies [4, 5]. Although considerable improvements have been achieved in the treatment of depression using antidepressants, the etiology of depression remains unclear [6]. Many of these antidepressants often produce side effects, such as obesity, cognitive impairment, and sexual dysfunction. Therefore, novel antidepressants with enhanced efficacy and few adverse effects should be developed. Gastrin-releasing peptide receptor (GRPR) binds to gastrin-releasing peptide (GRP), which belongs to the family of bombesin (BB)-like peptides that are highly expressed in the hypothalamus [7]. This high expression corresponds to this peptide's function in regulating hypothalamus-pituitary-adrenal (HPA) axis hormone secretion. Indeed, evidence has implicated the

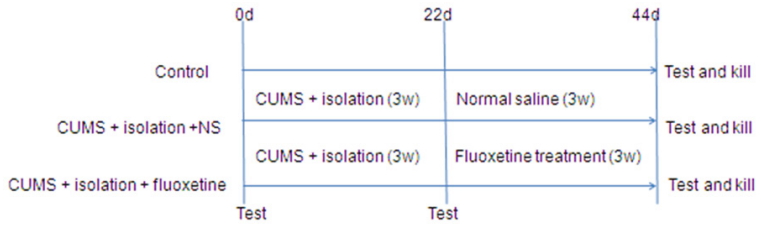

Figure 1. Experimental procedure. Mice in the ccontrol, CUMS + isolation + NS, and CUMS + isolation + fluoxetine were tested (sucrose preference test, food intake/body weight test, and open field test) at 0, 22, and 44 d and sacrificed within 12 h of the last tests.

GRPR signaling pathway in central nervous system diseases, and the anatomical distribution of GRPR is consistent with its proposed function in emotional and stress-related behaviors [8, 9]. GRPR also participates as a molecular key player in mental disorders [10]. The HPA axis plays an important role in the pathogenesis of depression because stress has been associated with the onset of depression [11]. Studies have shown that GRPR expression at the hypothalamus and pituitary levels is involved in the multifactorial regulation of adrenocorticotropic hormone (ACTH) secretion [12]. Although GRPR participates in several psychiatric diseases, including dementia, schizophrenia, autism, and anxiety disorders [10], the potential role for this receptor in the pathophysiology of depression remains unknown.

Chronic unpredictable mild stress (CUMS) has been successfully used to establish a rat depression model, and isolated male mice subjected to CUMS also show anxiety and depressive-like behaviors [13]. Therefore, we isolated animals and subjected them to CUMS to produce a mouse model of depression. Furthermore, we explored the likely mechanisms for the involvement of GRPR in depression by analyzing its expression levels in the hypothalamus of mice subjected to CUMS.

Materials and methods

Animals

A total of 30 male C57 mice, weighing 14 g to 18 g, were used in this experiment. These C57 mice were obtained from the Center of Experimental Animals of Wuhan University. All of the mice were housed in plastic cages and maintained under standard laboratory conditions (12 h/12 h light/dark cycle; 22 ± 2°C; food and water ad libitum) for one week before the start of the experiment. The experimental procedures were conducted in accordance with the guidelines for the care and use of laboratory animals issued by the Ministry of Science and Technology of the People's Republic of China in 2006.

Fluoxetine treatment

Fluoxetine (Shanghai Jinhuan Chemical Co., Ltd., China) was immediately dissolved in normal sodium before each use. Daily injections of fluoxetine (10 mg/kg) [14-16] or saline vehicle were intraperitoneally administered to the C57 mice for three weeks.

Experimental design

A total of 30 male C57 mice were randomly divided into three groups: the CUMS + isolation + NS (normal saline) group (CNS group; n = 10); the CUMS + isolation + fluoxetine group (CF group; n = 10); and the control group (Ctrl group; n = 10). We recorded the results of the behavioral experiments, including the sucrose preference test and open field test, and measured food intake and body weight. This experiment was performed before stress was induced (0 d), after stress was induced (22 d), and after fluoxetine was administered (44 d). The details of the experimental procedures are shown in **Figure 1**. These mice were individually subjected to stress induction, treatment injections, and behavioral assessments.

CUMS

The majority of the stressors used to induce CUMS were slightly modified from the procedures described in previous studies [13, 17-19]. The mice were subjected to the following mild stressors for three weeks: housed in a cage tilted at 45° for 24 h; exposed to damp sawdust for 24 h; maintained in an empty cage for 24 h; subjected to food deprivation for 24 h; subjected to water deprivation for 24 h; tail clamped for 3 min; immobilized in a 30-ml syringe for 1 h; and exposed to alterations of the light/dark cycle. Each mouse was individually exposed to these stressors and these stressors were never presented simultaneously.

Sucrose preference test

All of the mice were trained to consume 1% sucrose solution. The training consisted of an initial sucrose solution exposure for 24 h without another water source, followed by a consecutive 12 h with a bottle of 1% sucrose solution and a bottle of water [20]. Before each test was performed, the mice were deprived of water for 12 h (9 p.m. to 9 a.m.). The mice were subsequently presented with a bottle of 1% sucrose solution and a bottle of water for 24 h (the positions of these bottles were exchanged after 12 h). Sucrose intake and water intake were measured by comparing the weights of the bottle before and after the test window, as described by Willner et al. (1987). Sucrose preference was calculated using the following equation: sucrose preference = sucrose intake/total fluid intake.

Food intake/body weight test

The mice were deprived of food for 12 h (9 p.m. to 9 a.m.) before each test. Then, these mice were provided with food and water ad libitum for 24 h. We compared the weights of food before and after the test window and divided the result by the animals' corresponding body weights.

Open field test

An open field test was performed, as previously described, to determine the spontaneous activity of the mice [21]. In this test, each experimental mouse was placed at the center of a rectangular cage (80 cm × 40 cm × 40 cm). The mice were observed directly and continuously using a video tracking system for 10 min (Ethovision 3.0, Noldus, The Netherlands). The locomotor activity (distance traveled) of the mice was quantified using the video tracking system. The apparatus was thoroughly cleaned before the next animal test was performed.

Detecting GRPR in the paraventricular nucleus of the hypothalamus (PVN) by immunohistochemical (IHC) staining

The mice were decapitated, and their hypothalamus was dissected. The tissue samples were preserved in a 4% paraformaldehyde solution, dehydrated, embedded in paraffin, and then cut systematically into a series of 7-μm coronal sections. Specifically, we chose the paraventricular nucleus of the hypothalamus (PVN) as the targeting nucleus for our study because of our preliminary supportive results and its easily recognized anatomical feature (adjacent to the third ventricle). The paraffin-embedded sections were removed and subsequently immersed in distilled water. These sections were washed in 0.1 M PBS, treated with 1% H_2O_2 for 20 min, and incubated using a blocking buffer (5% normal goat serum) at room temperature for 10 min. The sections with goat serum were discarded and were incubated with an anti-GRPR antibody (MBL, MC-830, 1:200 dilution) at 4°C overnight. Then, the sections were incubated with a secondary antibody at room temperature for 15 min, washed three times with 0.1 M PBS, and stained with 3,3'-diaminobenzidine for 15 min without any light. These specimens were dehydrated, cleared, and mounted with neutral gums. Positively stained images of the PVN were analyzed using a light microscope. The reagents used for the entire process were obtained from UltraSensitive TMS-Pk (Fuzhou Maixin Company, China).

Western blot analysis of GRPR

Western blot analysis was performed as previously reported [22, 23] with slight modifications. The frozen tissue samples were thawed and homogenized in 50 μl of RIPA lysate containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, and 0.5% sodium deoxycholate (Beyotime, P0013B). The homogenates were cleared by centrifugation (12,000×*g*, 30 min, 4°C), and the supernatants were collected and stored at -80°C. The protein concentration was subsequently determined using a colorimetric assay (BCA protein assay kit, Beyotime, P0010) in accordance with the manufacturer's protocol. The protein samples were separated at 100 V on 10% polyacrylamide gel electrophoresis for GRPR analyses. After electrophoresis was performed, the proteins were transferred onto polyvinylidene membranes (Millipore, IPVH00010) using a transfer unit that was run at 200 mA for 130 min. The membranes were blocked using 5% non-fat dry milk with Tris-buffered saline containing Tween 20 (TBST) at room temperature for 2 h. Then, these membranes were incubated with anti-GRPR antibody (MBL, MC-830, 1:200 dilution) at 4°C and gently shaken overnight. These membranes were washed in TBST buffer five to six times for 5 min each and then blocked with secondary antibodies at room temperature for 2 h. GAPDH was used as a loading control to analyze relative protein quality.

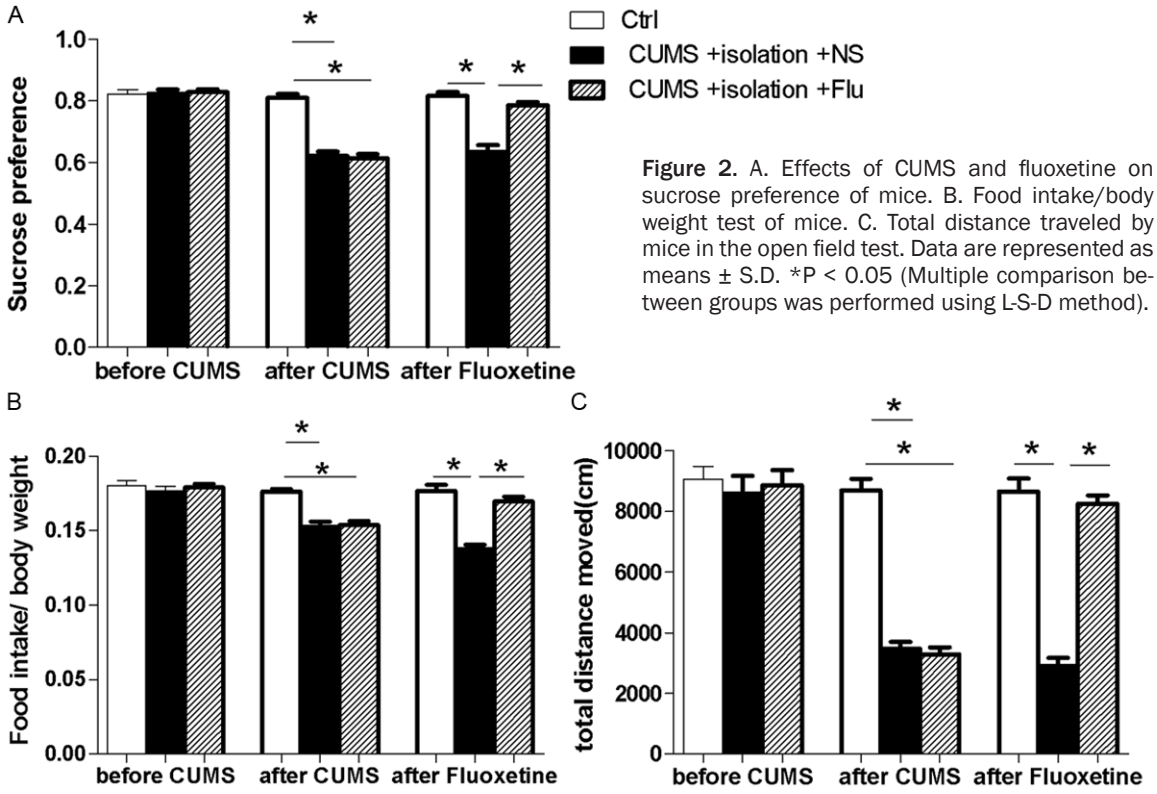

Figure 2. A. Effects of CUMS and fluoxetine on sucrose preference of mice. B. Food intake/body weight test of mice. C. Total distance traveled by mice in the open field test. Data are represented as means ± S.D. *P < 0.05 (Multiple comparison between groups was performed using L-S-D method).

Measuring mRNA levels by real-time PCR

The C57 mice were euthanized by cervical dislocation. Their brains were rapidly removed, snap-frozen, and stored at -80°C until use. Total RNA was extracted from the hypothalamus and collected using TRIzol reagent (Invitrogen, 15596-018) containing DNase to remove potential genomic DNA contamination in accordance with the manufacturer's instructions. mRNA was reverse transcribed to cDNA using a first-strand synthesis kit (Fermentas, #K1622). A total of 20 μl of the RT reaction was subjected to PCR amplification using the SYBR Green/Fluorescein qPCR Master Mix (2×; Fermentas, #K0242) according to the supplier's manual. The primer pair sequences for β-actin (5'-CACGATGGAGGGGCCGGACTCATC-3' and 5'-TAAAGACCTCTATGCCAACACAGT-3') and GRPR (5'-AGCTGACAGGTACAAAGCCATT-3' and 5'-AGGGTGTAGCTCATTGGAGTGT-3') were subjected to the following amplification conditions: 1 cycle of 50°C for 2 min, 95°C for 10 min and then 40 cycles of 95°C for 30 s and 60°C for 30 s. β-actin was quantified and used as an internal control for normalization. Fold differences in mRNA levels from the control values were calculated using the cycle threshold values. PCRs were run in triplicate for each brain sample; at least three independent sample pairs were used for each statistical analysis.

Statistical analysis

All the analyses were carried out in individual animals by using different statistical tests according to the effect examined. Specifically, for behavior experiment, the data were analyzed by 1-way analysis of variance. Conversely, the effects of prolonged CUMS and pharmacological treatment were evaluated by 2-way analysis of variance, with stress (CUMS vs Ctrl) and treatment (CF vs CNS) as independent factors. Between-group comparisons were conducted by analyzing the data using a post hoc method. For graphic clarity, data are presented as means ± standard error of the mean (SEM). The analyses were performed using SPSS (Statistical Package for the Social Sciences) 18.0 software. Statistical significance was set at $P < 0.05$.

Results

Sucrose preference tests, food intake/body weight test and open field tests

Figure 2 shows the results for sucrose preference (**Figure 2A**), food intake/body weight

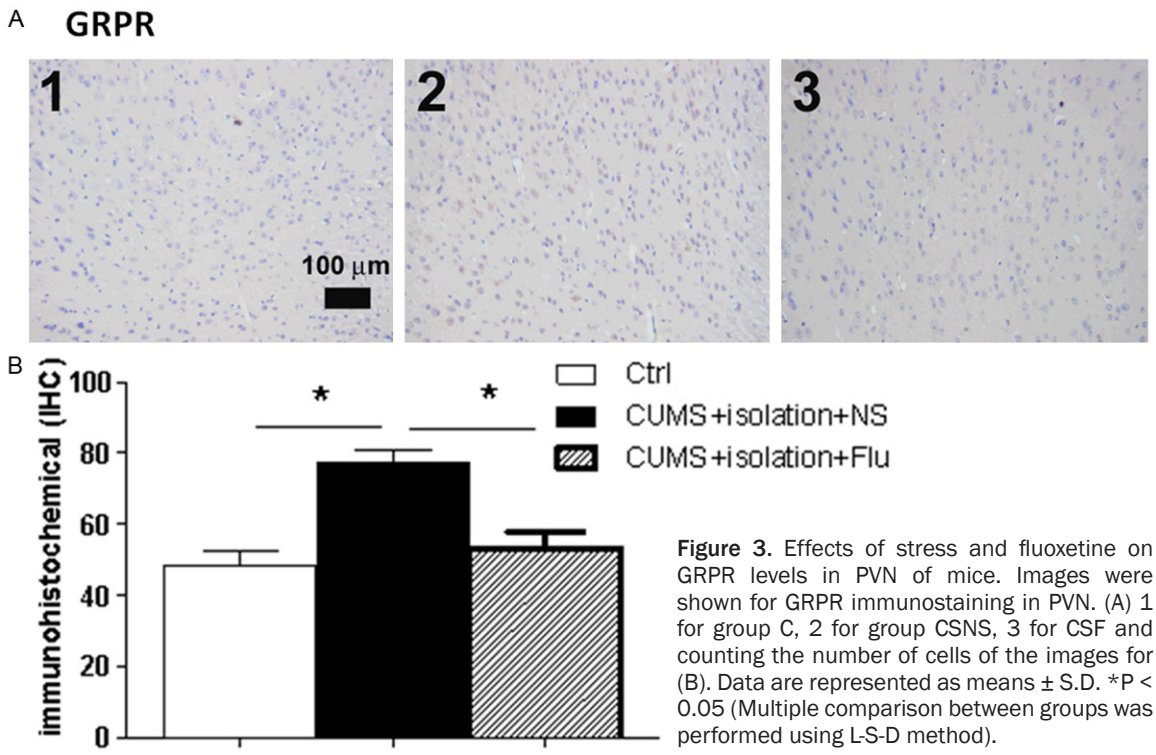

Figure 3. Effects of stress and fluoxetine on GRPR levels in PVN of mice. Images were shown for GRPR immunostaining in PVN. (A) 1 for group C, 2 for group CSNS, 3 for CSF and counting the number of cells of the images for (B). Data are represented as means ± S.D. *P < 0.05 (Multiple comparison between groups was performed using L-S-D method).

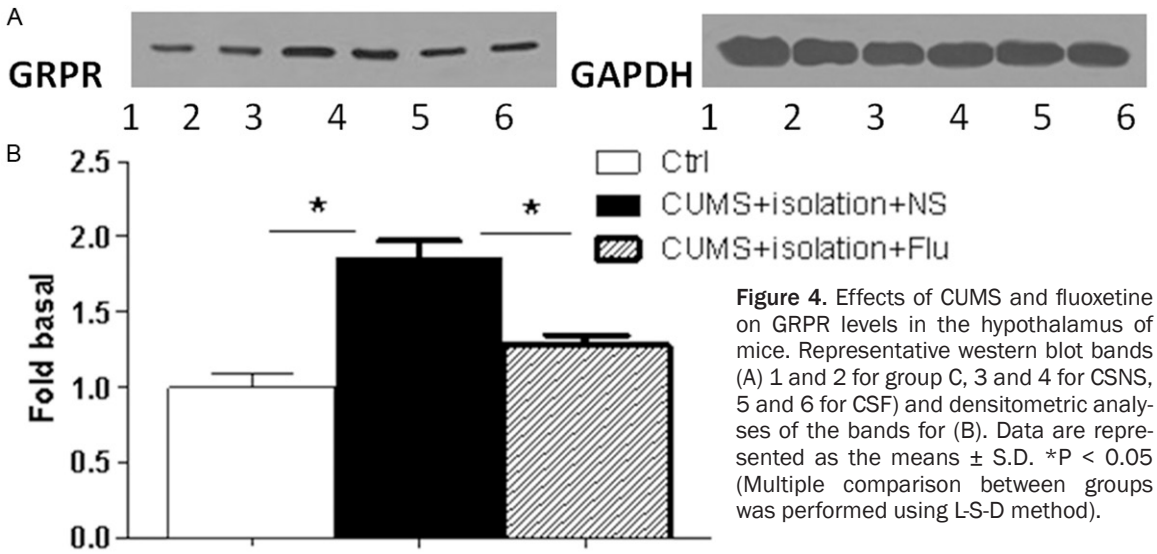

Figure 4. Effects of CUMS and fluoxetine on GRPR levels in the hypothalamus of mice. Representative western blot bands (A) 1 and 2 for group C, 3 and 4 for CSNS, 5 and 6 for CSF) and densitometric analyses of the bands for (B). Data are represented as the means ± S.D. *P < 0.05 (Multiple comparison between groups was performed using L-S-D method).

(**Figure 2B**), and total distance traveled (**Figure 2C**) among the three groups during the entire experimental period. At the beginning of the experiment, no significant difference was observed among the three groups.

At the end of the stress treatment, the CNS and CF groups displayed significant reductions in sucrose preference (mean ± SD; 0.81 ± 0.04 for the control group; 0.62 ± 0.05 for the CNS group; and 0.61 ± 0.04 for the CF group; F = 64.37, P < 0.05) (**Figure 2A**), food intake/body weight (0.18 ± 0.01 for the control group; 0.15 ± 0.01 for the CNS group; and 0.15 ± 0.01 for the CF group; F = 24.54, P < 0.05) (**Figure 2B**), and total distance traveled (8,695.64 ± 1,183.09 cm for control group; 3,482.55 ± 676.92 cm for the CNS group; and 3,288.82 ± 718.08 cm for the CF group; F = 118.91, P < 0.05) (**Figure 2C**). Compared with the control

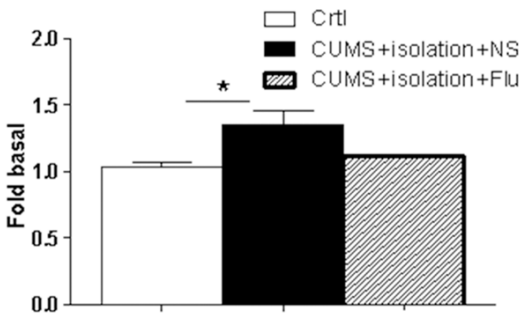

Figure 5. Effects of CUMS and fluoxetine on GRPR levels in the hypothalamus of mice. mRNA levels of GRPR determined by real-time PCR. Data are represented as means ± S.D. *P < 0.05 (Multiple comparison between groups was performed using L-S-D method).

mice, the CNS and CF groups showed no significant differences.

After fluoxetine was administered for three weeks, the CF group did not significantly differ from the control group but did show significant differences compared to the CNS group (sucrose preference: 0.82 ± 0.04 for the control group; 0.63 ± 0.07 for the CNS group; and 0.79 ± 0.03 for the CF group; F = 39.29, P < 0.05; food intake/body weight: 0.18 ± 0.01 for the control group; 0.13 ± 0.01 for the CNS group; and 0.17 ± 0.01 for the CF group; F = 35.74, P < 0.05; total distance traveled: 8,656.60 ± 1,363.05 cm for the control group; 2,900.21 ± 859.79 cm for the CNS group; and 8,253.92 ± 877.90 cm for the CF group; F = 91.99, P < 0.05).

The reductions in sucrose consumption and food intake indicated that the C57 mice exposed to stress decreased their responsiveness to rewarding stimuli. Moreover, the reduction in total distance traveled showed that the mice were less active. However, treatment with fluoxetine for three weeks could reverse these stress-induced changes.

IHC staining

We observed significant GRPR expression in the PVN, periventricular nucleus (PEV) and ventromedial hypothalamic nucleus (VMH). We selected the PVN as the targeting area because of its significantly up-regulated GRPR expression and easily recognizable anatomical features (adjacent to the third ventricle). **Figure 3** shows the GRPR expression in the PVN of the three groups after stress was induced and fluoxetine treatment was administered. The GRPR expression of the CNS group significantly increased compared with that of the control group and the CF group (GRPR: 48.60 ± 9.10 for the control group, F = 15.63; 77.60 ± 7.20 for the CNS group, F = 0.75; and 53.40 ± 9.86 for the CF group, F = 30.51, P < 0.01).

GRPR protein expression

Figure 4 shows GRPR protein expression in the three groups. The GRPR level of the CNS group significantly increased compared with that of the control group and the CF group (GRPR: 1.00 ± 0.13 for the control group, F = 42.11; 1.87 ± 0.15 for the CNS group, F = 4.72; and 1.28 ± 0.09 for the CF group, F = 23.41, P < 0.05).

GRPR mRNA expression

We also analyzed the mRNA expression of GRPR in the hypothalamus by RT-PCR after CUMS was induced and fluoxetine treatment was administered. The RT-PCR results revealed that the mRNA expression of GRPR in the hypothalamus of the control group did not significantly differ from that in the CF group but significantly differed from that in the CNS group (1.03 ± 0.04 for the control group, F = 12.53; 1.35 ± 0.15 for the CNS group, F = 0.76; and 1.11 ± 0.01 for the CF group, F = 6.65, P < 0.05) (**Figure 5**).

Discussion

In this study, three-week exposure to CUMS and isolation was used to establish a classical model of depression in mice. The results showed that fluoxetine treatment (10 mg/kg) could reverse the decreased activity of the mice in the open field test and increase sucrose preference and food intake/body weight. Chronic fluoxetine treatment could also restore the stress-induced increase in GRPR expression. These results provide additional insights into the potential value of targeting GRPR signaling in patients suffering from depression.

CUMS is one of the most validated rodent models used to study depression because of its etiological characteristics and predictive validity [18, 19, 24]. The locomotor activity in the open field test corresponds to certain aspects of explorative behavior in new environments [25]. This investigation demonstrated that stress significantly reduced the distance traveled by the mice in the open field test, which indicated that stressed mice were less active.

Moreover, the food intake/body weight of the mice in the CUMS group was lower than that in the control group. This decreased food intake/body weight in the stressed mice in our study suggested that stress induced depression. Studies on the CUMS paradigm have also used a reduction in sucrose intake as a measure of anhedonia and technique efficacy [26]. The index in our sucrose preference test represented anhedonia-like behavior, which is a major symptom of depression in humans [15].

Thus, behavioral defects, including stress-induced decreased activity and waned interest, indicate several depressive-like symptoms in humans. We found that stress-induced changes in mice could be normalized by chronically administering fluoxetine (10 mg/kg).

These behavioral changes associated with depression may be accompanied by alterations in GRPR expression in the hypothalamus. Our experiments showed a remarkable increase in the mRNA and protein expression of GRPR in the hypothalamus of stress-exposed mice. However, after these mice were treated with fluoxetine for three weeks, the mRNA and protein expression of GRPR in the hypothalamus was restored to values that were not significantly different from those in the control group.

GRP was first extracted from a European frog by Anastasi in 1971. GRP is a small regulatory peptide exhibiting homology to BB [27] . GRP is also widely distributed in the central nervous system [28], and the BB family of peptides has been recognized as stress peptides. Merali et al. [29] found that mice exhibiting depression show an increase in GRP, and this result is consistent with the outcome of our study. Furthermore, systemic administration or specific intraventricular injection of GRPR agonists was shown to produce endocrine and behavioral changes similar to stress-induced changes, and GRPR antagonists could reverse these effects [30].

Stress evokes a plethora of physiological and behavioral responses that can impair the prevention and restoration of homeostasis [31-33]. Physiological stress responses involve the activation of two interrelated systems: the sympathetic nervous system and the HPA axis [34, 35]. Studies have suggested that GRP induces corticosterone release by binding to GRPR; this release occurs by stimulating ACTH secretion [36]. Moreover, previous studies on molecular biology have suggested that GRP can regulate 5-serotonin and dopamine concentrations in the central nervous system [37, 38]. The results of our study further support GRPR-mediated chronic stress-induced depression. However, the role of the GRPR signaling pathway in the treatment of depression should be investigated in further studies.

The established C57 mouse model of depression induced by CUMS and isolation was reliable and stable, although additional research should be conducted using a larger sample size. In fact, our future studies will utilize GRPR knockout and conditional knockout mice to fully understand the precise role of GRPR in depression. The results of such studies will further facilitate the diagnosis and treatment of patients with depression.

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Disclosure of conflict of interest

None.

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