

Original Article

miR-335 over-expression in ventrolateral orbital cortex specifically down-regulates GRM4 to improve depressive behaviors in mice

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Abstract: Objective: To observe the effect of miR-335 over-expression on the depressive behaviors of mice, as well as the potential regulatory mechanism. Methods: A chronic unpredictable mild stress (CUMS)-induced depression model was constructed in C57BL/6 mice to detect the changes in the expression of miR-335 and glutamate metabotropic receptor 4 (GRM4) in the ventrolateral orbital cortex (VLOC). miR-335 over-expressing lentiviral vector LV-miR-335 was injected into the VLOC of CUMS mice; then, the changes in depressive behaviors of mice were detected through sucrose preference test (SPT), open field test (OFT), forced swimming test (FST), and tail suspension test (TST). Changes in GRM4 expression in mouse VLOC tissues were also examined through Western blot. Additionally, GRM4 silencing lentiviral vector LV-shRNA-GRM4 was injected in the VLOC of CUMS mice to detect the depressive behaviors of mice. Then, dual-luciferase reporter gene assay was conducted to verify the specific regulation of miR-335 on GRM4. Results: CUMS mice exhibited obvious depression tendency, with reduced SPT value, markedly decreased horizontal and vertical motion scores, and clearly extended immobility time in FST and TST. In addition, miR-335 expression in VLOC was down-regulated, while GRM4 expression was up-regulated. When up-regulating miR-335 expression in VLOC, the SPT value in CUMS mice elevated, the horizontal and vertical motion scores clearly increased, the immobility time in FST and TST was remarkably shortened, and GRM4 expression was down-regulated. When down-regulating GRM4 expression in VLOC, the SPT value in CUMS mice elevated, the horizontal and vertical motion scores notably increased, and the immobility time in FST and TST was dramatically shortened. Further, results of dual-luciferase reporter gene assay demonstrated that, miR-335 directly regulated GRM4. Conclusions: CUMS-induced depressive behaviors are related to the down-regulation of miR-335 in VLOC, while over-expressing miR-335 in VLOC specifically down-regulates GRM4 to improve the depressive symptoms in mice.

Keywords: Depression, miR-335, ventrolateral orbit cortex (VLOC), glutamate metabotropic receptor 4 (GRM4), mice

Introduction

Depression is a severe mental disease, which mainly manifests as long-term and persistent sad moods of patients [1]. According to reports, about 19% of people in the world develop depression; among them, nearly 0.3 billion people have major depressive disorder (MDD) [2]. As predicted by the World Health Organization (WHO), depression will become a leading cause of the loss of labor capacity among human beings by 2030 [3]. At present, many methods and substances have been applied to treat depression in the clinic, but most of them have

definite adverse reactions and have no obvious improvements in 30% of patients [4]. The ventrolateral orbital cortex (VLOC) is a component of the brain's limbic system-hypothalamus-cortex loop; in recent years, the important role of the VLOC in affective disorder-related mental disease has aroused extensive attention [5, 6]. For instance, Xing et al. [7] discovered that the VLOC participated in regulating the efficacy of antidepressants in the forced swimming test (FST). In the social isolation depression mouse model, the depressive behaviors were related to elevated VLOC neuronal activity [8]. The relationship of VLOC in stressful environments and

depressive behavior, together with the precise molecular mechanisms involved still presently remains unclear.

microRNA (miRNA) is a type of single-stranded small nucleotide sequence molecule, which regulates the expression of nearly 60% of the genes in mammalian cells through post-transcriptional regulatory mechanisms. miRNA mainly binds with the 3' untranslated region (3'UTR) of specific target mRNA, thus suppressing further translation of the target mRNA into protein. Recent research suggests that, miRNA possesses extensive and important biological regulatory functions, including regulation of neuronal development, differentiation and apoptosis [9]. In addition, changes in miRNA expression are also considered to be related to the occurrence and development of mental diseases, such as depression, schizophrenia and bipolar disorder [10]. Some research finds that, miR-335 expression is down-regulated in the cerebral cortex of people who committed suicide due to depression [11], and it is markedly down-regulated in the blood of MDD patients [12]. However, the mechanism of action of miR-335 in the occurrence and development of depression remains unclear so far. In this study, a mouse model of depression was constructed through chronic unpredictable mild stress (CUMS), so as to observe the changes in miR-335 expression in the VLOC of depressed C57BL/6 mice, and to further explore the influences of changes in miR-335 expression in the VLOC on the mouse depressive behaviors, as well as the underlying regulatory mechanisms.

Material and methods

Animal grouping

SPF-grade male C57BL/6 mice with a body weight of 18-22 g were purchased from SiKeBeiSi Biotechnology Co., Ltd (Shandong, China), and raised in an SPF barrier system under the following environmental conditions, temperature of $(24\pm 1)^{\circ}\text{C}$, light/dark cycle of 12-h/12-h, and relative humidity of $(50\pm 5)\%$. During the entire experimental process, all animals had free access to food and water. Mice in the first part of experiment were randomized into two groups, with 10 in each group, including control group (normal feeding without any treatment) and the CUMS group (in which CUMS was applied to construct a mouse model of depression). Mice in the second part of the

experiment were randomly divided into 2 groups, with 10 in each group, including; (1) CUMS+LV-miR-NC group, in which CUMS was used to construct the mouse model of depression, and then 1 μl LV-miR-NC (Gene Pharma, China) was injected bilaterally into the VLOC of mice using the Stereotaxic device via the micro-injection pump. (2) CUMS+LV-miR-335 group, in which CUMS was used to construct a mouse model of depression, and then 1 μl LV-miR-335 (Gene Pharma, China) was injected bilaterally into the VLOC of mice using the Stereotaxic device. Mice in the third part of experiment were randomized into two groups, with 10 in each group, including; (1) CUMS+LV-shRNA-NC group, in which CUMS was used to construct a mouse model of depression, and then 1 μl LV-shRNA-NC (Gene Pharma, China) was injected bilaterally into the VLOC of mice using a Stereotaxic device via a micro-injection pump. (2) CUMS+LV-shRNA-GRM4 group, in which CUMS was used to construct a mouse model of depression, and then 1 μl LV-shRNA-GRM4 (Gene Pharma, China) was bilaterally injected into the VLOC of mice using a Stereotaxic device.

Construction of the CUMS-induced depression mouse model

CUMS was adopted to construct the mouse model of depression in this experiment [13, 14]. The CUMS model is a classical model used to simulate unpredictable adverse changes in the body under a long-term and increasingly stressful environment, thus inducing abnormal behaviors. To avoid the tolerance of mice to single or regular stress stimulation, this experiment adopted multiple unpredictable stimulation methods for alternation. The specific stress methods were as follows, tail clamping for 10 min, food deprivation for 24 h, low temperature (8°C) swimming for 20 min, and food deprivation for 24 h, five mice were set in a group for shaking at the frequency of 240 Hz for 1 h, limitation of motion (LOM) for 60 min, warm water (40°C) swimming for 20 min, and restriction to a high-density space for 24 h (5 mice were in a cage). CUMS was applied to each mouse for once a day for 42 consecutive days in total.

Sucrose preference test (SPT)

Prior to experiment, the mice were trained to adapt to 1% sucrose, to be specific; 2 bottles of 1% sucrose solution (200 ml) were placed in

miR-335 and GRM4 in depression

Table 1. Primer sequences for qRT-PCR

Name	Forward primer (5'-3')	Reverse primer (5'-3')
miR-335	GATACGACTCAAGAGCAATAACGAA	CCAGTGCGTGTGCTGGAGTC
GRM4	GGAACCCGGTGACCTTCAAC	CTGTCCATGAGCCGATGACCTTGTA
U6	TGGAACGATACAGAGAAGATTAGCA	ACGAATTTGCGTGCATCCTTG
GAPDH	AAGGTGGTGAAGCAGGCATCTGAG	CGGCATCGAAGGTGGAAGAGTG

each cage, 24 h later, one bottle of 1% sucrose solution was replaced with pure water for 24 h. After adaptation, the mice were deprived of water and food for 24 h to carry out SPT. Before initiating the experiment, the weight of each bottle was weighed (one bottle was 200 ml of 1% sucrose solution, while the other one was the 200 ml pure water), the mice were allowed to drink water in both bottles during the experiment; at 1 h later, the positions of these two bottles were replaced for 1 h, and then the remaining weights were measured to obtain the consumption (g) of sucrose solution and pure water. Moreover, the ratio of consumed sucrose solution to the overall consumed liquid (consumed amount of sucrose solution + consumed amount of pure water) denoted the SPT value.

Forced swimming test (FST)

The test was carried out in a transparent cylindrical swimming barrel with a height of 50 cm and a diameter of 20 cm. On the first day of experiment, water at the temperature of (23±3)°C was poured in the barrel to a height of 40 cm, so that the hind limbs of mice were unable to touch the bottom of the barrel to support their bodies, and the mice were allowed to swim freely for 6 min to adapt to the environment. On the second day, water at the same temperature and height was poured into the barrel, the mice were allowed to swim for 6 min, and the immobility time of mice within the later 5 min was recorded. The immobility time referred to the time at which the mice stayed still when floating on the water surface or only made some tiny limb motion to keep afloat.

Open field test (OFT)

The test was performed in an open box with the height of 40 cm, and the floor of the box was equally divided into 25, 50 cm × 50 cm squares, and all sides were black. The test was conducted in a quiet environment. At the beginning, the mice were placed in the center of the box for observation of their motion for 5 min; meanwhile, the horizontal motion score (number of

grids crossed by the mice) and the vertical motion score (frequency of standing up-right) of the mice were also recorded.

Tail suspension test (TST)

The distal 1/3 mouse's tail was suspended onto a self-made tail suspension device, so that the mouse's head was directly facing the lens and were about 5 cm away from the ground, the despair behaviors of mice within 6 min were recorded by a camera, and the accumulative immobility time (s) within the later 5 min was analyzed. The immobility time referred to the time during which the mice stopped struggling and stayed still, with an inverted hanging body.

qRT-PCR

Upon the completion of behavioral tests, the mice were immediately anesthetized and decapitated to dissect the brain. Then, the brain tissues were washed with cryohydrate, and coronal sections were made with a mouse brain mold. Later, the bilateral VLOC was rapidly separated according to mouse brain stereoscopic images, and then Trizol reagent (Tiangen, China) was used to extract the total RNA from tissues. cDNA was prepared using 2 µg of total RNA through reverse transcription, which was then used as the template for qPCR amplification (Takara, Japan). The PCR conditions were as follows: 95°C for 5 min; followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Later, U6 or GAPDH was used as the internal reference, and the relative expression levels of miR-335 and GRM4 mRNA were determined according to the $2^{-\Delta\Delta Ct}$ method. All primer sequences are shown in **Table 1**.

Dual-luciferase reporter gene assay

GRM4 was predicted by bioinformatic analysis to be the candidate target gene of miR-335. The wild type (Wt) and mutant (Mut) GRM4 3'UTR reporter gene plasmids were constructed, which were then co-transfected with miR-

335 mimic or miR-NC into HEK-293T cells (Shanghai Cell Bank, Chinese Academy of Sciences). Moreover, the luciferase reporter gene detection kit (Promega, USA) was used to detect the luciferase activity after cell transfection using the dual-luciferase detection system.

Western blotting

The total protein was extracted from the VLOC tissue. Then, the extracted total protein was degraded, isolated through 10% SDS-PAGE, transferred onto a PVDF membrane, blocked with 5% skim milk powder for 2 h and washed. Afterwards, primary antibody GRM4 (1:1000, Santa Cruz, USA) was added to incubate at 4°C overnight. On the following day, the membrane was washed with PBS once and with TBST twice; later, the horse radish peroxidase (HRP)-conjugated secondary antibody (1:5000, Santa Cruz, USA) was added to incubate at 37°C for 1 h on a horizontal shaking table. Subsequently, the membrane was washed, and ECL chemiluminescence was used to visualize the protein bands.

Statistical methods

SPSS 20.0 software was used to analyze data, the measurement data were expressed as ($\bar{x} \pm s$) and compared by *t*-test, and $P < 0.05$ indicated a statistical significance.

Results

miR-335 expression decreased in the VLOC tissue of CUMS mice, while GRM4 expression increased

Compared with control group, the SPT value, horizontal motion and vertical motion scores in the CUMS group decreased ($P < 0.05$, **Figure 1A-C**). Meanwhile, the immobility time in FST and TST increased ($P < 0.05$, **Figure 1D, 1E**). The miR-335 expression in the VLOC tissue was down-regulated ($P < 0.05$, **Figure 1F**), while GRM4 mRNA and protein expression was up-regulated ($P < 0.05$, **Figure 1G, 1H**).

Up-regulating miR-335 expression in the VLOC markedly improved the depressive behaviors in CUMS mice

Compared with the CUMS+LV-miR-NC group, miR-335 expression in the VLOC tissue of the

CUMS+LV-miR-335 group was remarkably up-regulated ($P < 0.05$, **Figure 2A**). Whereas the SPT value, horizontal motion and vertical motion scores increased ($P < 0.05$, **Figure 2B-D**), and the immobility time in FST and TST was reduced ($P < 0.05$, **Figure 2E, 2F**).

Up-regulating miR-335 expression dramatically suppressed GRM4 expression in the VLOC tissue of CUMS mice

Compared with the CUMS+LV-miR-NC group, the GRM4 mRNA and protein expression in the VLOC tissue of CUMS+LV-miR-335 mice was markedly down-regulated ($P < 0.05$, **Figure 3A, 3B**).

GRM4 might be a functional target gene of miR-335

The target gene of miR-335 was analyzed through bioinformatic analysis, and the results suggested that, GRM4 was a candidate target gene of miR-335. Additionally, results of the luciferase reporter gene assay (**Figure 4**) revealed that, miR-335 mimics remarkably decreased the luciferase activity of wild type Wt-GRM4 reporter gene plasmid ($P < 0.05$), but it had no obvious influence on the luciferase activity of mutant Mut-GRM4 reporter gene plasmid ($P > 0.05$).

Down-regulating GRM4 markedly improved the depressive behaviors of CUMS mice

Compared with the CUMS+LV-shRNA-NC group, the mRNA and protein expression of GRM4 in the VLOC tissue of CUMS+LV-shRNA-GRM4 mice was remarkably down-regulated ($P < 0.05$, **Figure 5A, 5B**). The SPT value, horizontal motion and vertical motion scores remarkably increased ($P < 0.05$, **Figure 5C-E**), and the immobility time in FST and TST markedly decreased ($P < 0.05$, **Figure 5F, 5G**).

Discussion

The current treatment for depression is unsatisfactory, and plenty of research suggests that, the drug therapy for depression is only effective in 30%-50% cases upon first receiving a 6-week anti-depressant treatment [15]. The pathogenesis and pathophysiological features of the onset of depression remain unclear. It is suggested that, depression is related to heredity and stress, but so far, the existing genetic

miR-335 and GRM4 in depression

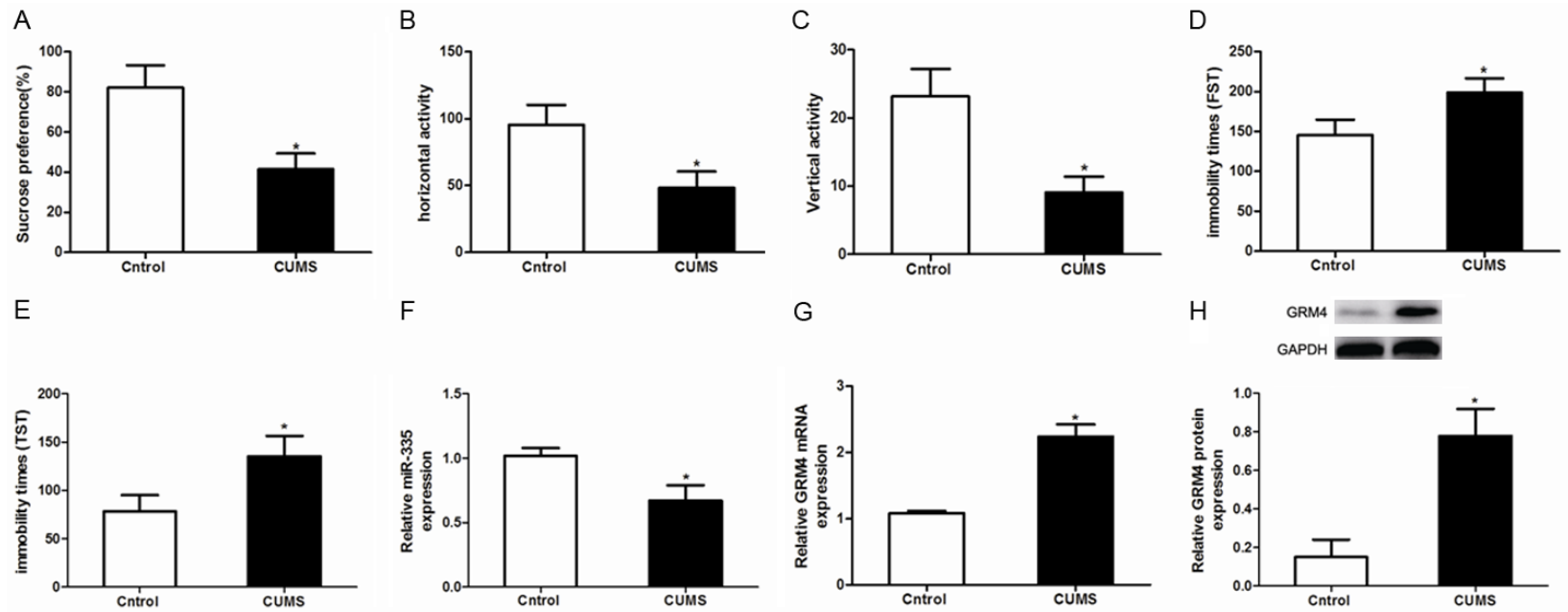


Figure 1. miR-335 expression decreased in the VLQC of CUMS mice, while GRM4 expression increased. Differences in the sucrose preference (A), horizontal activity (B), vertical activity (C), immobility times in FST (D) and in TST (E) were observed in control group and CUMS group. The miR-335 mRNA expression levels were examined with qRT-PCR (F). The mRNA and protein expression levels of GRM4 were detected by qRT-PCR (G) and Western blot (H). * $P < 0.05$.

miR-335 and GRM4 in depression

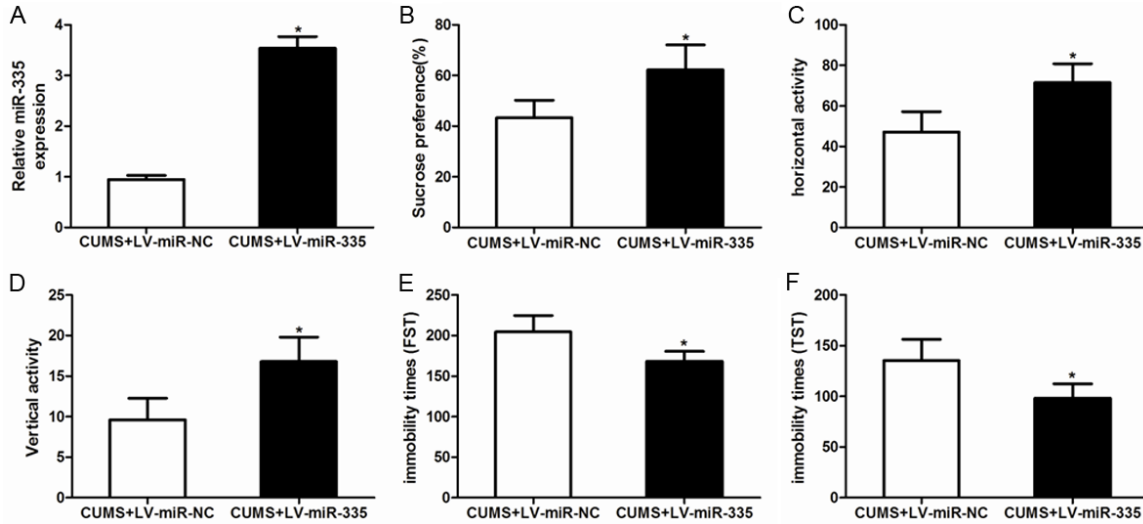


Figure 2. Effect of miR-335 on the depression-like behaviors in CUMS mice. (A) The miR-335 mRNA expression levels were detected by qRT-PCR. Differences in the sucrose preference (B), horizontal activity (C), vertical activity (D), immobility times in FST (E) and in TST (F) were observed in CUMS+LV-miR-NC group and CUMS+LV-miR-335 group. * $P < 0.05$.

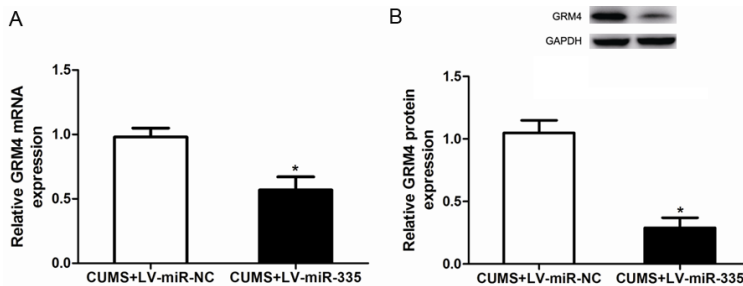


Figure 3. Effect of miR-335 on GRM4 mRNA and protein expression. A. The GRM4 mRNA expression levels were detected by qRT-PCR. B. The GRM4 protein expression levels were examined with Western blot. * $P < 0.05$.

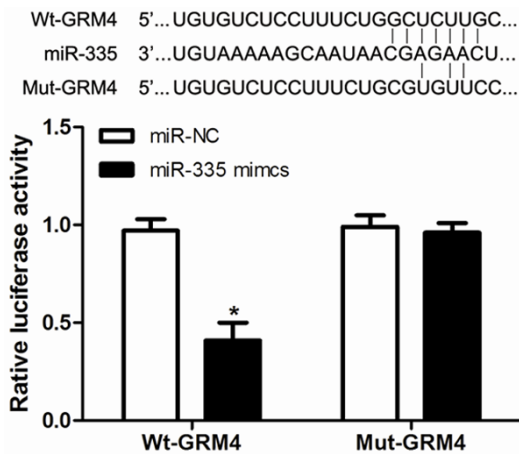


Figure 4. Binding activity between miR-335 and GRM4 shown by dual-luciferase reporter assay. * $P < 0.05$.

research has not determined the key sensitive gene related to depression. Therefore, searching for a depression-related key sensitive gene is of great significance to change the current status of treatment for depression.

miRNA is a type of non-coding single-stranded RNA molecule. Recent research suggests that miRNA plays an important role in the genesis and develop-

ment of multiple nervous system and mental disorders, such as depression and schizophrenia [16]. The VLOC is a component of the brain's limbic system-hypothalamus-cortical loop; in recent years, the important role of the VLOC in affective disorder-related mental diseases has aroused extensive attention [5, 6]. Zhao et al. [17] discovered that, miR-101 expression was markedly down-regulated in the VLOC of CUMS rats, while up-regulating miR-101 expression in the VLOC reversed the depressive behaviors in CUMS rats. miR-335 belongs to a newly discovered miRNA family, whose expression is found to be markedly down-regulated in the cerebral cortex of people who commit suicide due to depression [11]. Besides, Li et al. [12] found that, miR-335 expression in the blood was down-regulated in MDD patients, while the

miR-335 and GRM4 in depression

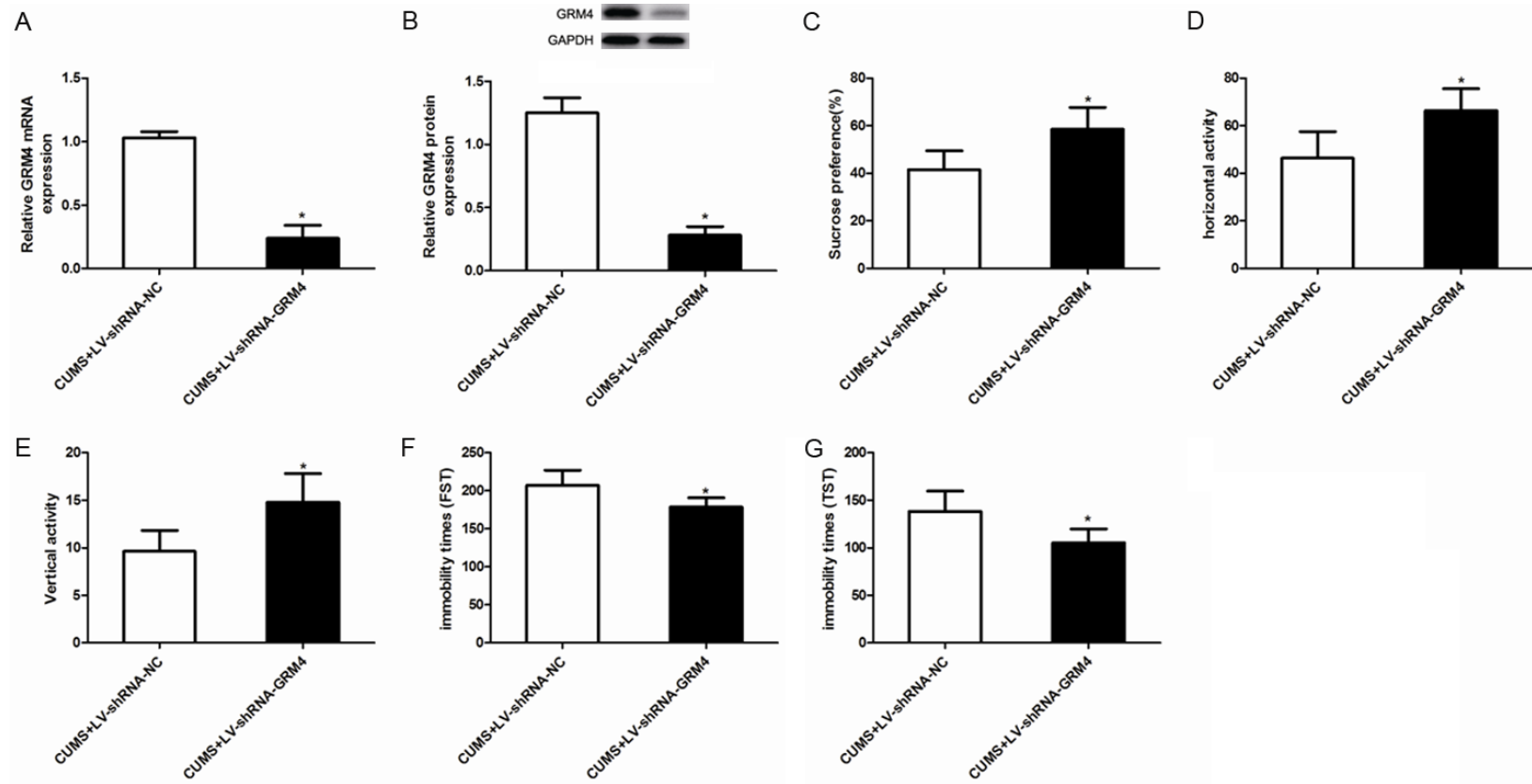


Figure 5. Effect of GRM4 on the depression-like behaviors in CUMS mice. (A) The GRM4 mRNA expression levels were detected by qRT-PCR. (B) The GRM4 protein expression levels were examined with Western blot. Differences in the sucrose preference (C), horizontal activity (D), vertical activity (E), immobility times in FST (F) and in TST (G) were observed in CUMS+LV-shRNA-NC group and CUMS+LV-shRNA-GRM4 group. * $P < 0.05$.

expression of glutamate metabotropic receptor 4 (GRM4) elevated. Moreover, after Citalopram treatment, miR-335 expression elevated in the blood, while GRM4 expression reduced, revealing that miR-335 might be related to the genesis and progression of depression, but the precise mechanisms of action remain unclear. This study adopted the classical CUMS model to construct a mouse model of depression, and we found that mice receiving CUMS modeling had dramatically decreased SPT value, horizontal motion and vertical motion scores, whereas increased immobility time in FST and TST. Typically, the formed depressive behaviors were extremely similar to the clinical manifestations of depression in humans induced by the long-term social, family or emotional pressures [18]. Further, we discovered that, miR-335 expression in the VLOC of CUMS mice was notably down-regulated, while GRM4 expression was apparently up-regulated, revealing that the abnormal expression of miR-335 and GRM4 in the VLOC might be related to the genesis and development of depression.

Subsequently, a miR-335 over-expressing lentiviral vector was injected into the VLOC of CUMS mice to up-regulate miR-335 expression in the mouse VLOC brain area. We discovered that, the SPT value, horizontal motion and vertical motion scores markedly elevated, while the immobility time in FST and TST notably decreased. Besides, the GRM4 expression in the VLOC tissue of mice was remarkably decreased with an increase in miR-335 expression, revealing that up-regulating miR-335 expression in the VLOC improved the depressive symptoms of CUMS mice, which might be related to the specific regulation of miR-335 on GRM4. GRM4 belongs to the metabotropic glutamate receptor III family, which is mainly expressed in the presynaptic membrane of neurons, and it mainly functions to regulate the metabolism of neurotransmitters (like dopamine, glutamic acid and tryptophan), thus affecting neurotransmitter release and mediating the transduction of neuronal signaling pathways [19]. Related research suggests that, GRM4 up-regulation is closely correlated with the genesis and development of depression [20]. Anti-depression treatment with ketamine is related to the down-regulation of GRM4 expression [21]. Lopez et al. [22] reported that, the anti-depression effect produced by the up-

regulation of miR-1202 was realized through specifically down-regulating GRM4. Further, a GRM4 silencing lentiviral vector was injected into the VLOC of CUMS mice to down-regulate GRM4 expression in the VLOC of mice. We discovered that, the SPT value, horizontal motion and vertical motion scores markedly increased, while the immobility time in FST and TST notably reduced, suggesting that down-regulating GRM4 expression in the VLOC also improved the depressive behaviors of CUMS mice. Moreover, the improvement by miR-335 on the depressive behaviors of CUMS mice was related to the specific regulation on GRM4. Afterwards, we discovered through luciferase reporter gene assay that, miR-335 bound with the 3'UTR of wild type Wt-GRM4 reporter gene plasmid, thus reducing the luciferase activity, and these findings verified that GRM4 was a direct target gene of miR-335.

To sum up, CUMS-induced depressive behaviors are related to the down-regulation of miR-335 in the VLOC, while over-expressing miR-335 expression in the VLOC specifically regulates GRM4 to improve depression symptoms, thus providing a new line of thinking for treating depression. However, there were certain deficiencies in this study. The downstream signaling of the effect of GRM4 on depressive symptoms in mice is still not clear yet. The results may not be representative. More extensive and in-depth research should be conducted in relation to humans, so as to provide effective methods for the clinical treatment of patients with depression.

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Disclosure of conflict of interest

None.

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