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Research article

New antidepressant mechanism of Yueju Pill: Increasing ghrelin level by inhibiting gastric mTOR/S6K signaling pathway and sensitizing hippocampal GHS-R

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ABSTRACT

Background and aim: Yueju Pill (YJ) not only has good antidepressant effect but also can effectively treat digestive system diseases. However,it remains unclear whether the mechanism of antidepressant action of YJ is related to the peripheral digestive system. The purpose of this study was to elucidate the antidepressant mechanism of YJ on ghrelin level based on gastric mTOR/S6K signal pathway and sensitized hippocampal Ghrelin/GHS-R system in CUMS mice.

Experimental procedure: The depression model was induced by chronic unpredictable mild stress (CUMS) and social isolation. The antidepressant effect of YJ was observed by behavioral experiment and hemodynamic experiments. Ghrelin levels in in hippocampus and blood were measured by Elisa kit, and the mRNA of ghrelin in mice stomach was measured by Real-time Quantitative PCR (RT-qPCR). The activation level of gastric mTOR/S6K signal pathway was detected by Western Blot (WB). Rapamycin (Rapa) and L-Leucine (L-Leu) were used to verify the effects of YJ on the synthesis and release of ghrelin. The activity of GHS-R in hippocampus was observed by immunofluorescence. Hippocampal neuronal damage was evaluated by HE staining and Nissl staining. The level of central neurotransmitter was measured by liquid chromatograph mass spectrometer (LC-MS).

Results and conclusion: YJ ameliorates CUMS-induced depressive-like behavior by inhibiting the gastric mTOR/S6K signaling pathway and increasing GHR expression in the mouse stomach. However, these effects of YJ could be resisted by L-Leu (a mTOR receptor agonist). Further studies have shown that YJ can sensitize the Ghrelin/GHS-R system in the hippocampus, with significant neuroprotective effects, and is also involved in regulating the levels of key neurotransmitters (5-hydroxytryptamine, Dopamine and γ-aminobutyric acid) in depressive-like states.

Abbreviations: YJ, Yueju Pill; Rapa, Rapamycin; L-Leu, L-Leucine; GHS-R, Growth hormone secretagogue receptor; CUMS, Chronic Unpredictable Mild Stress and Social isolation; 5-HT, 5-hydroxytryptamine; DA, Dopamine; GABA, γ-aminobutyric acid; Glu, Glutamine.

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1. Introduction

Depression, being a prevalent mental disorder, imposes significant psychological and economic burdens on patients and their families, thereby disrupting social stability and harmony [1]. Currently, classical antidepressants such as selective serotonin reuptake inhibitors (SSRIs), represented by sertraline, have side effects such as low drug resistance and many adverse effects [1]. People have questioned the potential addictive properties of newer antidepressants like ketamine, despite their rapid effectiveness [2]. In view of this, Chinese medicine's treatment of depression with a strong holistic concept, low side effects, comprehensive regulation, high safety, and other advantages is gradually attracting people's attention.

Yueju Pill (YJ) is a long-standing traditional Chinese medicine prescription that has been utilized for over 800 years to address disorders stemming from Yu (stagnation), boasting a commendable safety profile. In recent years, there has been consistent documentation of its antidepressant properties [3] and underlying mechanisms, including its ability to enhance the expression of BDNF in the hippocampus [4], rectify Akt signaling deficiencies, decrease the expression of NR1 [5], and activate the PKA-CREB signaling pathway [6], among others. Clinical settings have observed the use of YJ for the treatment of various digestive ailments, including esophageal diseases, indigestion, stomach diseases, intestinal diseases, peptic ulcers, liver diseases, gallbladder diseases, and pancreatic diseases [5,6]. The notable impact of YJ on the digestive system implies a potential correlation between its antidepressant properties and its peripheral effects.

Ghrelin (GHR) is a major metabolic hormone produced and released mainly by the stomach, but also in lower amounts in the small intestine and gastric sinuses, especially in rodents [7]. Research has demonstrated its ability to stimulate the release of growth hormone and appetite [8,9]. The synthesis and secretion of ghrelin are intricately linked to the mTOR/S6K signaling pathway in the stomach. Previous research has shown that the mTOR/S6K signal pathway in the stomach and the production of ghrelin by X/A-like cells are linked in a bad way [10–12]. Current research has confirmed that ghrelin is exclusively produced in the periphery [13]. It can enter the brain through the blood-brain barrier and the blood-cerebrospinal fluid barrier, exerting its physiological role [14]. Animal experiments have demonstrated that peripheral administration of ghrelin exhibits anti-anxiety and antidepressant effects [15,16]. Additionally, ghrelin in the central nervous system has been found to reduce neuroinflammation [17], regulate neurotransmitters [18], and protect neurogenesis [19]. Recent studies have also suggested a potential role for ghrelin in sleep and memory [20], indicating its possible association with various neuropsychiatric diseases.

As the sole receptor protein of ghrelin, the growth hormone secretagogue receptor (GHS-R) plays a crucial role in the physiological function of ghrelin. It is widely distributed in brain regions associated with emotions, such as the hippocampus [21,22]. When Ghrelin binds to GHS-R, it fulfills its physiological role, including the protection of hippocampal neurogenesis and enhancement of the 5-HT system [23]. However, the absence of the GHS-R receptor protein can result in depression-like behavior [24]. More importantly, a study found that long-term stress can significantly reduce the binding sites between ghrelin and GHS-R in the basal lateral amygdala (BLA) of mice. This is known as ghrelin resistance [25].

Therefore, in this study, the link between the regulation of ghrelin by YJ and its antidepressant mechanism of action through the gastric mTOR/S6K signal pathway was investigated by behavioral, hemodynamic, molecular biology, and pathological methods using CUMS depression model mice as the research subjects. We used L-Leucine (L-Leu is an mTOR receptor agonist) and Rapamycin (Rapa is an mTOR receptor blocker) in this study to learn more about how YJ works as an antidepressant. Our study may promote the clinical application of YJ as an antidepressant and provide new ideas for investigating the mechanisms of antidepressant action in YJ.

2. Material and methods

2.1. Materials

YJ is composed of Xiang Fu (*Cyperus rotundus* L., Cyperaceae), Chuan Xiong (*Ligusticum striatum* DC., Apiaceae), Zhi Zi (*Gardenia jasminoides* J. Ellis, Rubiaceae), Cang Zu (*Atractylodes lancea* (Thunb.) DC., Compositae.) and Shen Qu (Massa Medicata Fermentata). These traditional Chinese medicines were all purchased from Jiangxi JiangZhong Prepared Slices of Chinese Crude Drugs Co., Ltd. and verified for quality by Wu Shuyao, the pharmacist-in-charge of Jiangxi University of Chinese Medicine. The voucher specimen (No. 22-06-15) was deposited in the Institute of Chinese Medicine and Brain Science of Jiangxi University of Chinese Medicine. Fluoxetine (FLU, Sigma, USA, CAT. No. 56296-78-7) is the positive drug in the behavior test experiments.

2.2. Preparation of YJ extract

In light of the findings from the existing literature [5] and the preliminary foundation of our team, an ethanol extraction method was employed to obtain YJ extract. The preparation method is as follows: Take 100 g of each of the above five Chinese herbal medicines and break them into small particles. Next, they were soaked in 75 % ethanol at a material-liquid ratio of 1:8 for 1 h and refluxed and heated for 1 h. The solution was filtered and collected, and the process was repeated twice. Next, we mixed the twice extracted solution and volatilized the solvent using a rotary evaporator. After using the freeze-dryer, we got 17.3 g of freeze-dried powder. Finally, we dissolved it into an appropriate concentration for intragastric administration using ultra-pure water. This concentration means that 3.6 g of mixed crude drug was extracted according to the above method and then dissolved in 1 mL of pure water.

Table 1
The chromatographic conditions of YJ extract.

Total time (min)	Flow Rate (μl/min)	A (%)	B (%)
0.00	300	95	5
45.00	300	15	85
55.00	300	5	95
57.00	300	5	95
57.10	300	95	5
60.00	300	95	5

2.3. Analysis of the chemical composition of these YJ extracts

As shown in Table S1, the HPLC-MS was used for component analysis of the YJ extract. 87 speculative substances were obtained through gradient elution separation, using 0.1 % formic acid water and acetonitrile as mobile phases. The chromatographic conditions are listed in Table 1.

2.4. Animals and treatments

Male C57BL/6 J mice (7 weeks), purchased from Jiangsu Jicuiyaokang Biological Co., Ltd. (certificate number: NO: 202317760) Before experiments, the animals were exposed to a SPF-grade room with a temperature of 22 ± 2 °C, humidity of 55 ± 5 %, and 12 h of lighting and darkness for at least one week. They were free to get water and food. The animal experiment protocol was approved by the Animal Ethics Committee of the Animal Experimentation of Jiangxi University of Chinese Medicine (Nanchang, China) on November 9, 2021 (Approval No. JZLLSC20210061).

Let the mice adapt to the environment for 5 days. According to the result of the sucrose preference test, the mice with abnormal sugar preference were excluded, and the remaining mice were divided into a control group and a CUMS group. The mice in the CUMS group were stimulated with CUMS for 5 weeks, according to our previous research [26]. Rapa (Cat# AB1641) and L-Leu (Cat# AB2454-1000) were purchased from Alfa Biotechnology Co., Ltd., and the administration methods and doses of these two drugs were determined according to previous studies [10]. The study was divided into two experiments.

In the first experiment, mice were randomly divided into 3 groups (n = 10). 1) control group; 2) CUMS group; 3) CUMS + YJ group (3.6 g/kg/day, intragastric gavage); 4) CUMS + Flu group (10 mg/kg/day, intragastric gavage). The dosage selection of YJ is based on the conversion of the clinical dosage in the Chinese Pharmacopoeia (edition 2020). The CUMS group and the control group used ultra-pure water to eliminate the influence of the solvent. The experimental timetable is shown in Fig. 1A, and tissue samples were collected after the behavioral test.

In the second experiment, mice were randomly divided into 6 groups (n = 10). 1) control group; 2) CUMS group; 3) CUMS + YJ group; 4) CUMS + YJ + Rapa group (1 mg/kg/day, intraperitoneal injection); 5) CUMS + YJ + L-Leu (450 mg/kg/day, intraperitoneal injection) group; 6) CUMS + Rapa (1 mg/kg/day, intraperitoneal injection) group, Rapa and L-Leu were administered 2 h before YJ. The experimental timetable is shown in Fig. 3A, and tissue samples were collected after the behavioral test.

2.5. Behavioral tests

2.5.1. Sucrose preference test (SPT)

The sucrose preference test is a common experiment to evaluate the depressive state of rodents. It measures mice's lack of pleasure based on their preference for sugar water. Two bottles of sucrose solution (1 % w/v) were placed in mice cages 72 h before the experiment, and one of them was replaced with ultra-pure water after 24 h (the positions of the two bottles were exchanged after 12 h). After the adaptation, mice were deprived of water and food for 24 h. Then animals were allowed free access to two bottles containing 80 mL of sucrose solution (1 % w/v) and 80 mL of water to measure their sugar water preference coefficient. After the test, the following formula was used to calculate sucrose consumption: sucrose preference (%) = (sucrose intake/total intake) × 100.

2.5.2. Open field test (OFT)

Open field test (OFT) was used to evaluate the autonomous activities and anxiety-depression-like behaviors of the mice. Each mouse was placed in the center of an arena (50 cm × 50 cm) for 6 min. The area in the middle 1/4 of the bottom of the box, is defined as the central area. A camera placed overhead is used to record the whole process. Its movement trajectory was analyzed by behavioral analysis software (Smart 3.0, USA). Between each trial, the open field was cleaned to avoid interference between individuals.

2.5.3. Elevated plus maze (EPM)

Elevated plus maze (EPM) was used to evaluate anxiety-related behaviors in mice. The EPM apparatus consisted of a central platform (7 cm × 7 cm) with four branching arms (30 cm × 7 cm), where one pair of opposite arms was walled while the other was open. The equipment was about 70 cm above the floor. Each mouse was placed on the central platform for 6 min. A camera placed overhead is used to record the whole process. The time spent in the open and closed arms was analyzed by behavioral analysis software (Smart 3.0, USA). Between each test, the equipment was cleaned to avoid interference between individuals.

2.5.4. Forced swim test (FST)

Forced swim test (FST) was used to evaluate the desperate behavior of rodents. Each mouse was put into a cylinder (height, 60 cm; diameter, 15 cm) filled with 20 cm of deep water (24 ± 1 °C). The duration of the experiment was 6 min. The immobile time was analyzed by behavioral analysis software (Smart 3.0, USA). In order to avoid the influence of smell on the test, the water will be changed for each test.

2.5.5. Tail suspension test (TST)

Tail suspension test (TST) was used to evaluate the desperate behavior of rodents. A shelf with hooks serves as the test instrument. Before the measurement began, the back 1/3 of the mouse's tail was taped to the hook, and then the mouse was hung upside down. The mouse's head was 25 cm above the ground for 6 min. Behavioral analysis software (Smart 3.0, USA) was used to analyze the immobile time of the test.

2.6. Doppler ultrasound imaging

The hair on the mouse's neck was removed by a mild hair removal cream. And the changes in blood flow and diameter of the left carotid artery in mice were detected by the Doppler ultrasound imaging system (Visual Sonics Vevo 2100, Toronto, Canada).

2.7. Laser Doppler imaging of brain

The images of the brain were obtained by a laser Doppler imaging system (moorLDI2-HIR, Moor Instruments, DE, UK). The core temperature of the animals was monitored to ensure euthermia throughout the procedure.

2.8. ELISA measurement

The content of ghrelin in serum and hippocampus was measured by ELISA kits based on the manufacturer's protocols. The mouse ELISA kits were purchased from Jiangsu Meimian Industrial Co., Ltd. (Cat# MM-0621M1).

2.9. Real-time quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA in stomach tissue was collected from frozen tissues by a Trizol (TRANS, Cat# ER502-01) reagent based on the manufacturer's recommended protocol. Transcription Kit (TRANS, Cat# AUQ-01) was used to reverse RNA to cDNA. Table 2 lists the primer sequences for RT-qPCR.

2.10. Western blotting analysis

Proteins in the stomach were collected in RIPA buffer containing protease and phosphatase inhibitors. The BCA Assay Kit (Solarbio, Cat# PC0020) was used to measure and balance the protein concentration between samples. SDS-PAGE electrophoresis was used to isolate the protein. And the protein was then transferred onto a PVDF membrane. The below primary antibodies were applied: (a) mouse anti-mTOR (1:10000, Proteinetch); (b) mouse anti-p-mTOR (1:4000, Proteinetch); (c) rabbit anti-S6K (1:4000, Proteinetch); (d) rabbit anti-p-S6K (1:8000, Proteinetch); (e) mouse anti-GAPDH (1:50000, Proteinetch). Blots were enhanced with chemiluminescence. And GAPDH expression was considered to normalize the relative expression of targeted protein. Target protein bands were analyzed by AnalytikJena Vision Works systems.

2.11. Immunofluorescence

The brain was dissected and immersed in a 4 % paraformaldehyde solution for 24 h to prepare 10 μm frozen sections. Shake the slides gently with PBS for 3 min each time. The slices were permeated with citric acid buffer (10 mm pH6.0) and 0.3 % TX-100 (RT for 15 min). After that, they were blocked by an endogenous biotin blocking kit (Sangon Biotech, Cat# E674001). Then acyl ghrelin (QYAOBIO, Cat# 04010077024) labeled with biotin was incubated overnight at 4 °C. The next day, after being washed 3 times with PBS, the slices were incubated with streptavidin-binding fluorescent dye (AAT Bioquest, Cat# 16955) at room temperature for an hour, and then PBS was used to wash the slices 3 times. Finally, the tablets were sealed with an anti-fluorescence quenching agent containing DAPI. The images were analyzed by an inverted fluorescence microscope and ImageJ software.

Table 2
The RT-qPCR primer sequences.

Primer Name	Sequence (5′-3′)
Ghrelin-S	AGAAAGGAATCCAAGAAGCCACC
Ghrelin-A	GCCAACATCGAAGGGAGCATT
GAPDH-S	CCTCGTCCCGTAGACAAAATG
GAPDH-A	TGAGGTCAATGAAGGGGTCGT

2.12. Hematoxylin and eosin (HE) staining

The brain was dissected and immersed in a 4 % paraformaldehyde solution for 24 h. After ethanol gradient dehydration, the tissues of the brain were embedded in transparent paraffin and then cut into 5 μm-thick coronal sections. After dewaxing with xylene, xylene was removed from tissue slides by reducing the concentration of ethanol and washed thoroughly with water. The hippocampal tissue was stained with HE according to the standard HE staining procedure. The slide is sealed with neutral resin. Use a microscope to observe and take pictures (Leica, Multiview).

2.13. Nissl staining

Brain slices of the hippocampal region of 10um were prepared using cryosectioning. The sections were stained with a Nissl staining solution at 37 °C for 5 min and then washed in a 95 % ethanol solution for 5 min. After drying, the sections were washed twice in xylene for 5 min each. After sealing the sections with neutral resin, the slides were observed and recorded under a light microscope.

2.14. LC-MS

The reference substance and samples were analyzed by the Shimadzu Controlle CBM20Alite system from the AB Sciex Instruments company. The mobile phase consisted of 0.01 % formic acid-water (A) and acetonitrile (B), followed by a linear gradient elution procedure. The total flow rate was 0.2000 ml/min.

The mass spectrometer is a Triple Quad 5500 in positive-ion mode. The ionization parameters are as follows: air curtain gas (CUR) 35.00 psi; ion source gas 1 (GS1) 45.00; ion source gas 2 (GS2) 40.005; ion source temperature (TEM) 550.00 °C; inlet potential (EP) 10.00 V; collision-chamber output voltage (CXP) 14 V; collision induced pyrolysis voltage (CAD) 7 psi. The mass spectrometer and UPLC system are controlled by Analyst 1.6.2 software. The details of rustic conditions and total ion flow diagrams can be seen in supplementary materials.

2.15. Statistical analyses

The data are displayed as mean ± SEM. The statistical analyses were implemented by GraphPad Prism Software 7 (GraphPad Software, San Diego, CA, USA). The comparisons of multiple groups were implemented by one-way ANOVA with Dunnett's test, P values < 0.05 were regarded as statistically significant.

3. Results

3.1. YJ has a therapeutic effect on CUMS-induced depressive behavior in mice

Fig. 1A displays the experimental design. After administration of pure water or YJ for 2 weeks, the depressive behavior induced by CUMS could be recovered by YJ. It is characterized by an increase in sugar water consumption in SPT (Fig. 1B), and a decrease in immobility time in TST (Fig. 1E) and FST (Fig. 1F). At the same time, under the treatment of YJ, the residence time in the center of OFT (Fig. 1C) and the duration time in the open arms of EPM (Fig. 1D) were also increased, and showed a trend of regression to the control group.

Clinical studies have shown that cerebral blood flow is significantly reduced in patients with depression [27]. In addition, our previous study also found reduced cerebral blood flow in CUMS model mice [28]. This suggests that hemodynamics is one of the important indicators for evaluating depression. Moreover, 80 % of the blood flow supply to the brain comes from the bilateral carotid arteries in the neck. As showed in Fig. 1G and H that CUMS caused a decrease in left carotid and cerebral blood flow. On the 14th day after administration of YJ, the left carotid artery and the degree of cerebral ischemia were significantly improved, and the blood flow increased.

Based on the results of behavioral tests and hemodynamics, it indicates that YJ has a therapeutic effect on depressive mice caused by CUMS.

3.2. YJ inhibits the activation of the gastric mTOR/S6K signal pathway and increases the expression and secretion of ghrelin and AG

The content of ghrelin in the serum of mice in each group was measured by the Elisa double sandwich method. As shown in Fig. 2A and B, CUMS caused a decrease in serum ghrelin, while the content of serum ghrelin in mice was significantly reversed after the intervention of YJ for 14 days. At the same time, the results of RT-qPCR showed that CUMS significantly decreased the mRNA of ghrelin in the stomachs of mice, while YJ alleviated this phenomenon. These results suggest that YJ can affect the synthesis and release of ghrelin.

The synthesis and release of ghrelin are closely related to the activation of the gastric mTOR/S6K signal pathway. The expression of mTOR and S6K proteins and their phosphorylated proteins were detected by Western blot. The results showed that the expression of p-mTOR protein (Fig. 2C) and p-S6K protein (Fig. 2D) in the gastric tissue of CUMS mice increased significantly. However, YJ could effectively reduce the expression in both of them. These results suggested that YJ can increase the expression and secretion of ghrelin by inhibiting the gastric mTOR/S6K signal pathway.

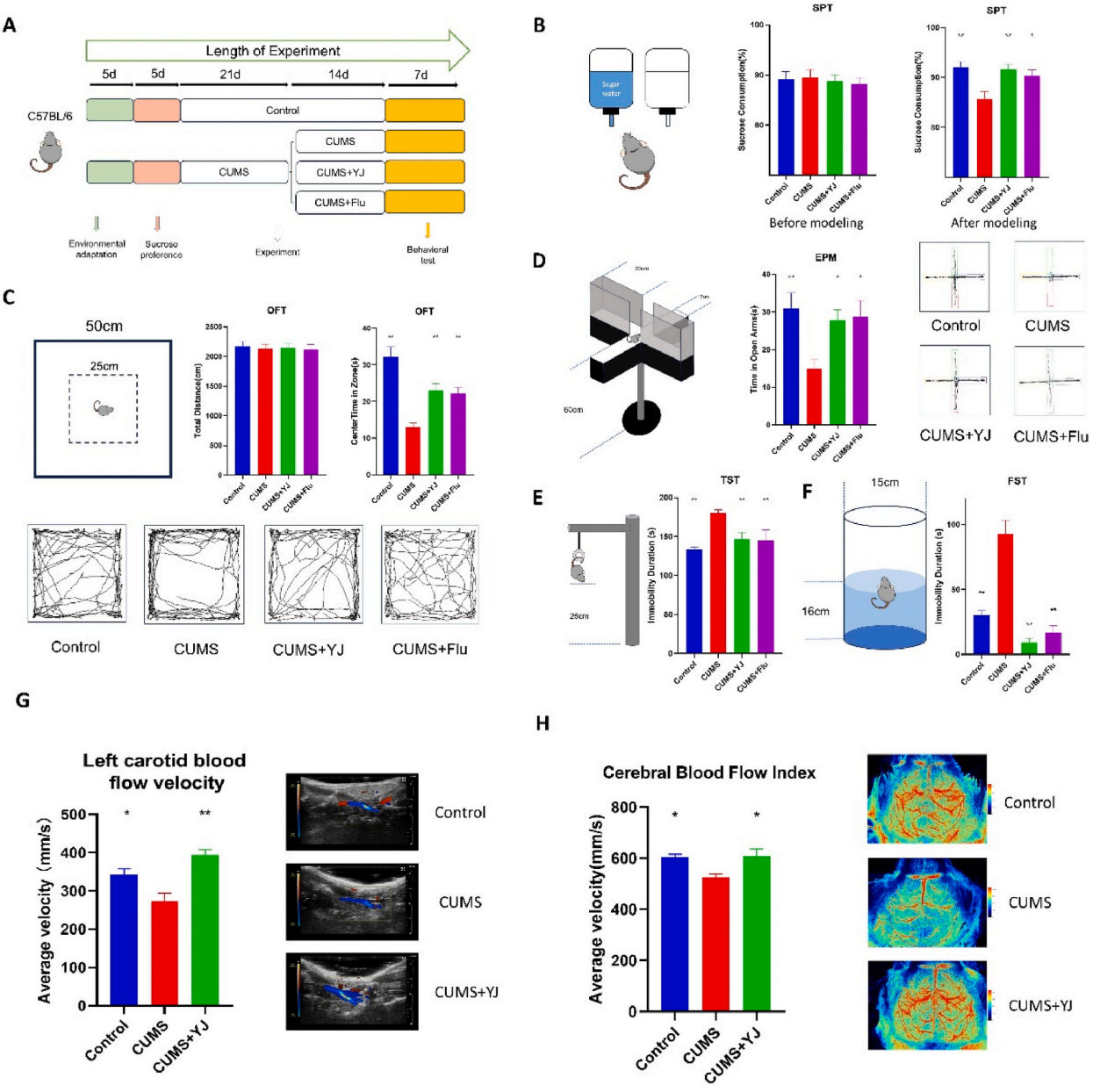

Fig. 1. Effects of YJ on depression-like behavior and cerebral blood flow changes in CUMS model mice. (A) Schematic diagram of experimental procedure. (B–F) The behavioural tests: (B) SPT, (C)OFT, (D) EPM, (E) TST, (F)FST. (G) Left carotid artery blood flow. (H) Cerebral blood flow. $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant differences vs. the CUMS group. (mean ± SEM, n ≥ 8).

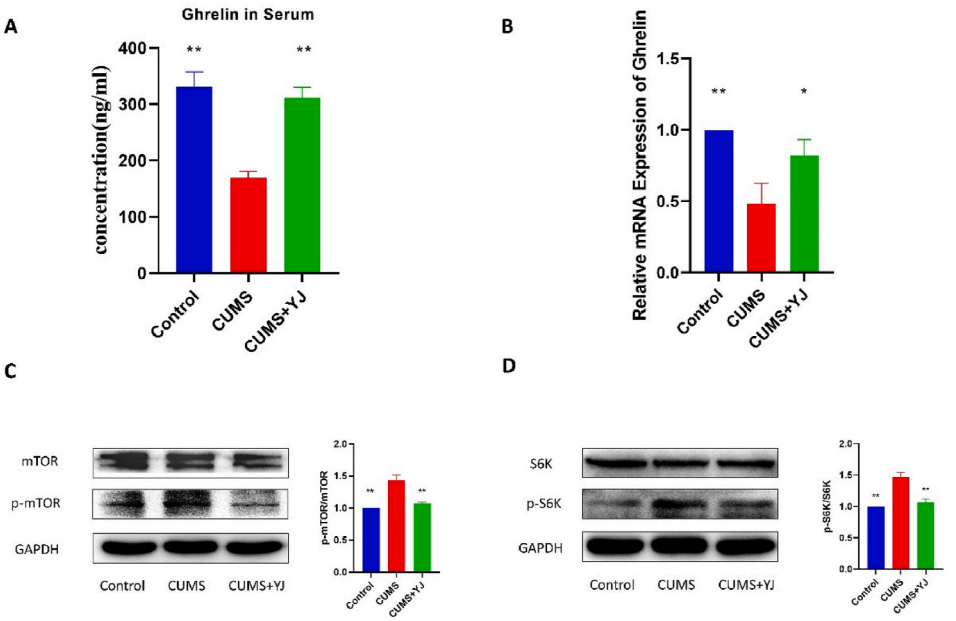

Fig. 2. Influence of YJ on the expression of ghrelin based on mTOR/S6K signal pathway (A). The content of ghrelin in serum. (B). Expression of ghrelin mRNA in gastric tissue. (C). Relative abundance of mTOR and p-mTOR protein. (D). Relative abundance of S6K and p-S6K protein. $^{*}P < 0.05$, $^{**}P < 0.01$, indicate significant differences vs. the CUMS group (mean ± SEM, n = 3). Comparisons were performed by One-way ANOVA.

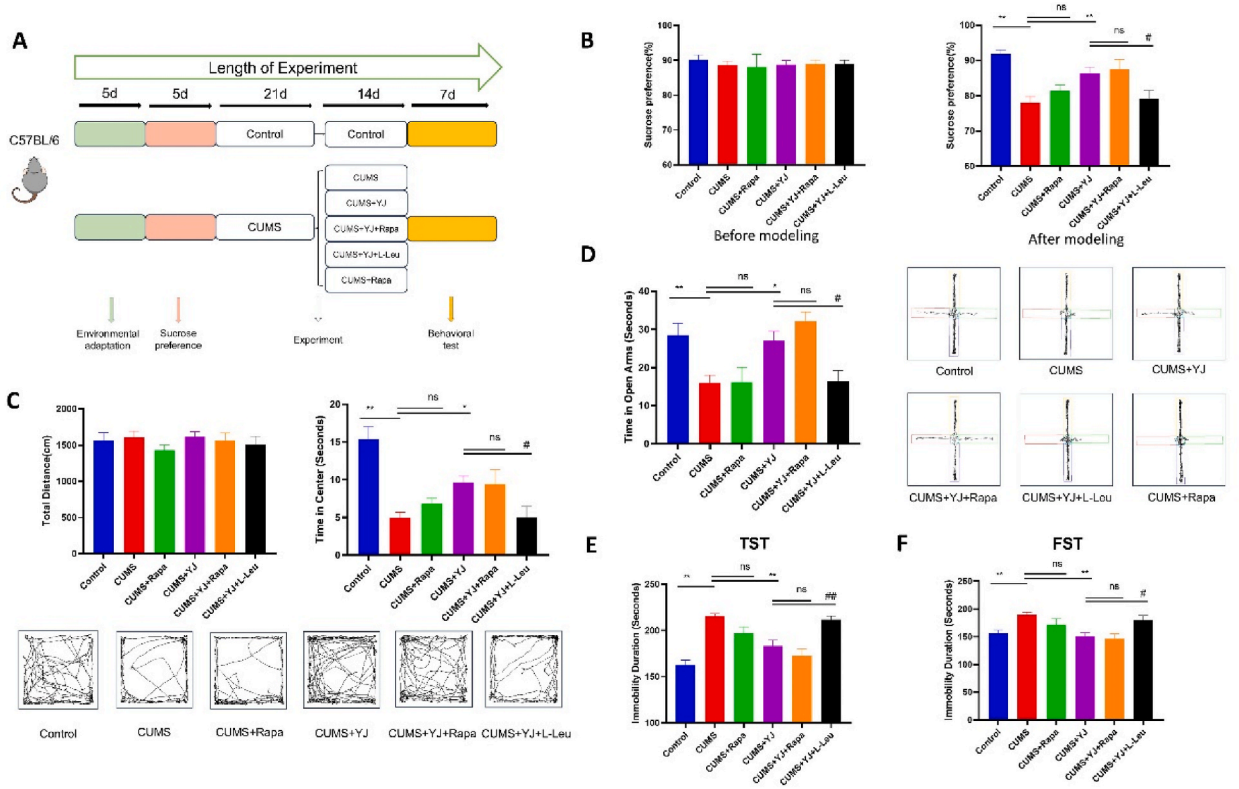

Fig. 3. The antidepressant effect of YJ was blocked by L-Leu, and Rapa alone had no antidepressant effect. (A) Schematic diagram of experimental procedure. (B–H) The behavioural tests: (B) SPT, (C) OFT, (D) EPM, (E) TST and (F) FST. $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant differences vs. the CUMS group (mean ± SEM, n ≥ 8). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ indicate significant differences vs. the CUMS + YJ group. (mean ± SEM, n ≥ 8) Comparisons were performed by One-way ANOVA.

3.3. L-Leu decreased total ghrelin and prevented the antidepressant effect of YJ

The experimental design is shown in Fig. 3A. Compared with the CUMS + YJ group, the CUMS + YJ + L-Leu group showed a significant decrease in sugar water preference in SPT (Fig. 3B), a significant decrease in open field center exercise time in OFT (Fig. 3C), a significant reduction in open arm exercise time in EMP (Fig. 3D), and significant increases in immobility time in TST (Fig. 3E) and FST (Fig. 3F). The behavioral experimental performance of the CUMS + YJ + L-Leu group exhibits a trend of regression toward the CUMS group.

L-Leu (a branched-chain amino acid) has been proven to activate mTOR signal. As shown in Fig. 4, compared with the CUMS + YJ group, the expression of p-mTOR protein and p-S6K protein in the CUMS + YJ + L-Leu group increased, and the expression of serum ghrelin and ghrelin mRNA decreased, displaying an inclination of returning toward the CUMS group.

These results suggested that L-Leu could increase the expression of ghrelin and block the antidepressant effect of YJ by activating the gastric mTOR/S6K signal pathway, which was inhibited by YJ. More importantly, it proved that the antidepressant effect of YJ was related to its inhibition of the gastric mTOR/S6K signal pathway and increase of ghrelin expression.

3.4. Rapa increased the expression and secretion of ghrelin, but Rapa alone had no antidepressant effect

From the characterization of behavioral tests (Fig. 3), the CUMS + YJ + Rapa group still had the same good antidepressant effect as the CUMS + YJ group. Rapa is a well-characterized mTOR inhibitor. As shown in Fig. 4, the CUMS + YJ + Rapa group exhibited a notable reduction in the expression of the gastric mTOR/S6K signal pathway, accompanied by a pronounced elevation in ghrelin mRNA levels in the stomach, in comparison to the CUMS + YJ group. When compared to the CUMS + YJ group, serum ghrelin

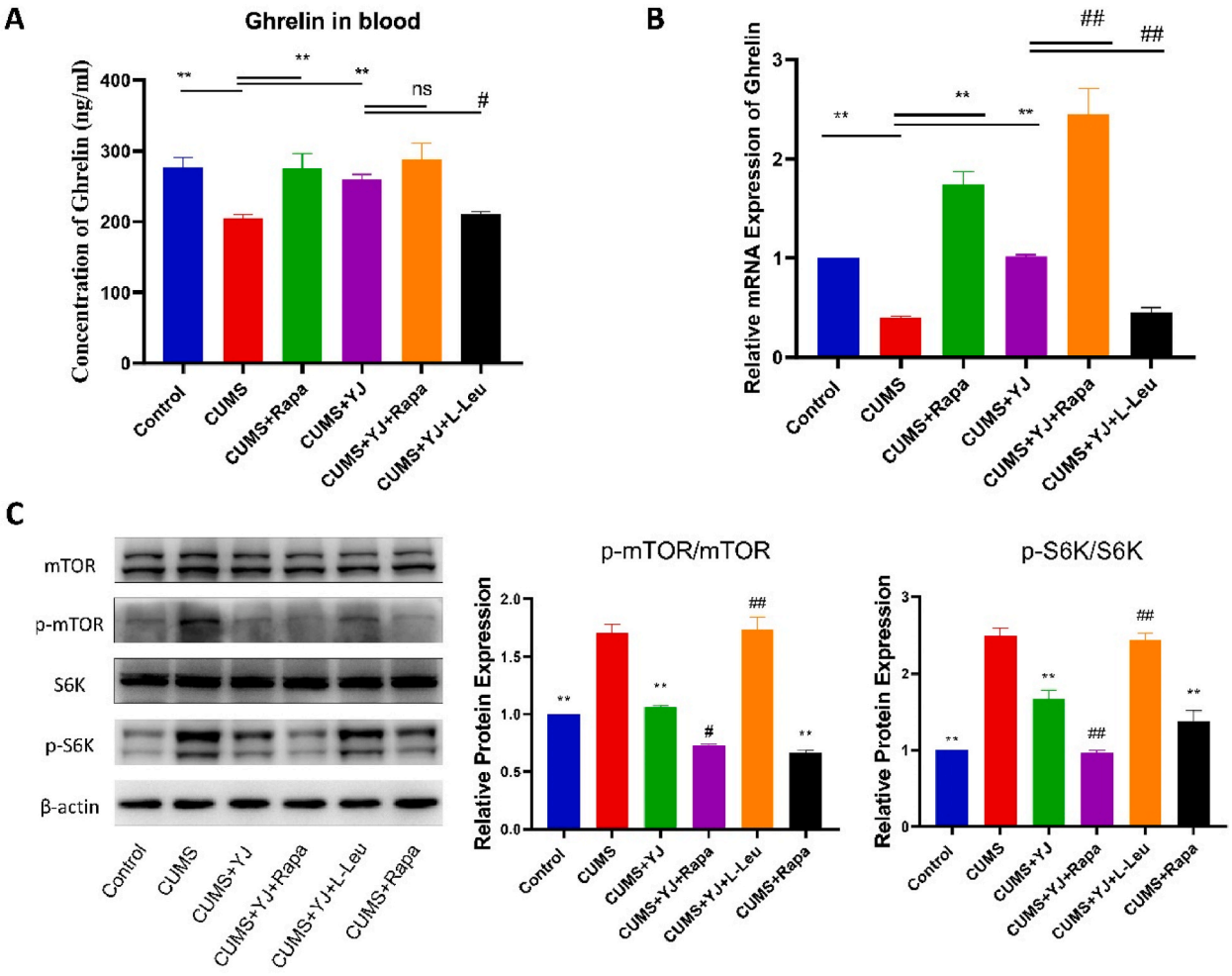

Fig. 4. The effect of YJ on the expression of GHR and the gastric mTOR/S6K signal pathway. (A). The content of ghrelin in serum. (B). Expression of ghrelin mRNA in gastric tissue. (C). Expression of the gastric mTOR/S6K signal pathway in each group of mice. $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant differences vs. the CUMS group. (mean ± SEM, n ≥ 8). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ indicate significant differences vs. the CUMS + YJ group. (mean ± SEM, n ≥ 8) Comparisons were performed by One-way ANOVA.

expression in the CUMS + YJ + Rapa group tended to increase. However, there was no significant difference between these two groups.

What's more, the CUMS + Rapa group showed the same depressive behavior as the CUMS group (Fig. 3). However, compared with the CUMS group, the CUMS + Rapa group had lower levels of gastric mTOR/S6K signal pathway activation, higher expression of serum ghrelin and ghrelin mRNA (Fig. 4), showing the same trend as the CUMS + YJ group.

These results meant that the antidepressant effect of YJ was not only to improve the level of ghrelin, but also played an equally important role in other ways.

3.5. YJ improved the binding ability of the GHS-R in hippocampus

The binding capacity of GHS-R and growth hormone-releasing peptide in the DG region of the hippocampus was measured by immunofluorescence. The results of IF in Fig. 5A showed that the binding ability of GHS-R and ghrelin in the CUMS group is significantly lower than that in the control group, while YJ could reverse this trend. Rapa and L-Leu did not affect YJ's sensitizing effect on GHS-R. Administration of Rapa alone could not reverse the decrease in binding ability of GHS-R and AG caused by CUMS. This suggests that YJ can sensitize GHS-R in the hippocampus of mice and activate more of the Ghrelin/GHS-R system.

At the same time, ghrelin was measured in the hippocampus (Fig. 5B). The results showed that the ghrelin content in the hippocampal region of mice in the CUMS group decreased compared to the control group. Compared to the CUMS group, YJ administration significantly increased the ghrelin content in mice's hippocampal region, whereas giving Rapa alone had no effect. The CUSM + YJ + Rapa group exhibited similar ghrelin concentrations in the hippocampal region compared to the CUMS + YJ group, while the CUSM + YJ + L-Leu group showed a decrease in ghrelin concentrations.

3.6. Effects of YJ on hippocampal pathological changes in mice with CUMS-induced depression

HE staining (Fig. 6A) showed that the hippocampal neurons of Control group mice had complete cellular morphology and were neatly arranged; the hippocampal neurons of the CUMS group showed partial nuclear fragmentation, cytoplasmic coalescence, disappearance of nucleoli, and disorganized arrangement; the hippocampal neuronal damage in the CUMS + YJ and CUMS + YJ + Rapa groups was alleviated as compared with that in the CUMS group. And the pathological manifestations in the hippocampus of mice in the CUMS + YJ + L-Leu group and the CUMS + Rapa group were the same as those in the CUMS group. Nissl staining observed

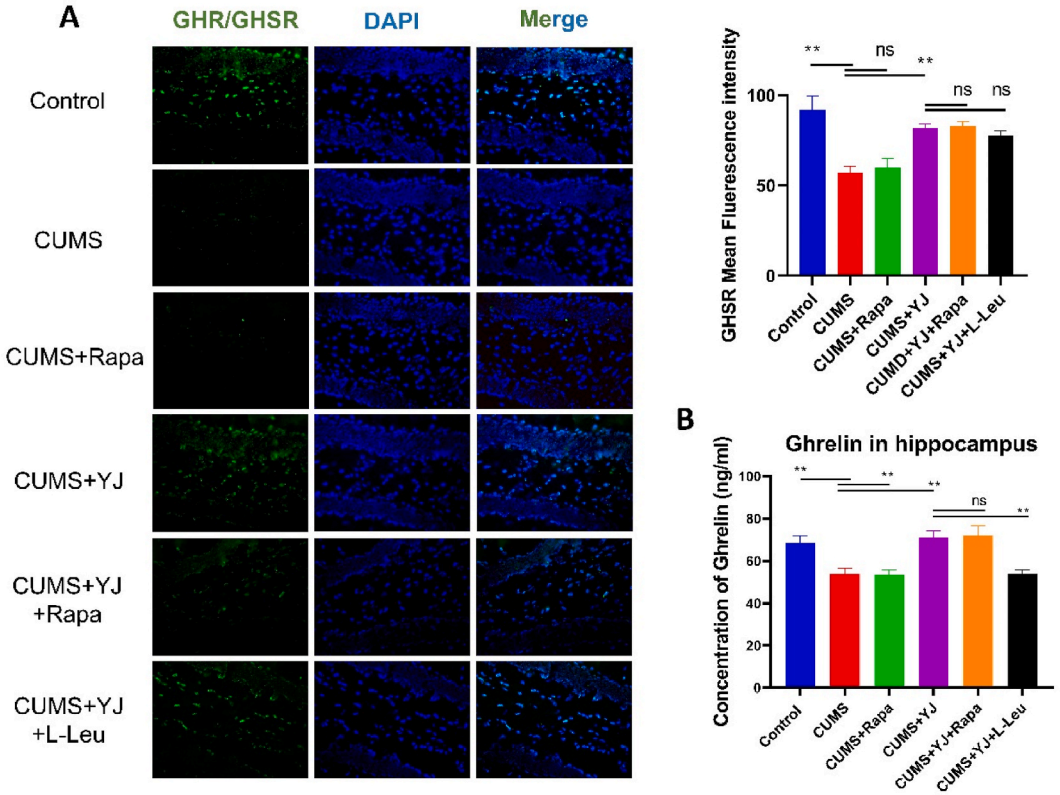

Fig. 5. Sensitization of GHS-R in the DG region of mouse hippocampus by YJ. (A) Representative immunofluorescence staining of ghrelin and GHS-R binding sites in the DG region of the hippocampus. (B) The content of ghrelin in hippocampus. $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant differences vs. the CUMS group (mean ± SEM, n = 3). Comparisons were performed by One-way ANOVA.

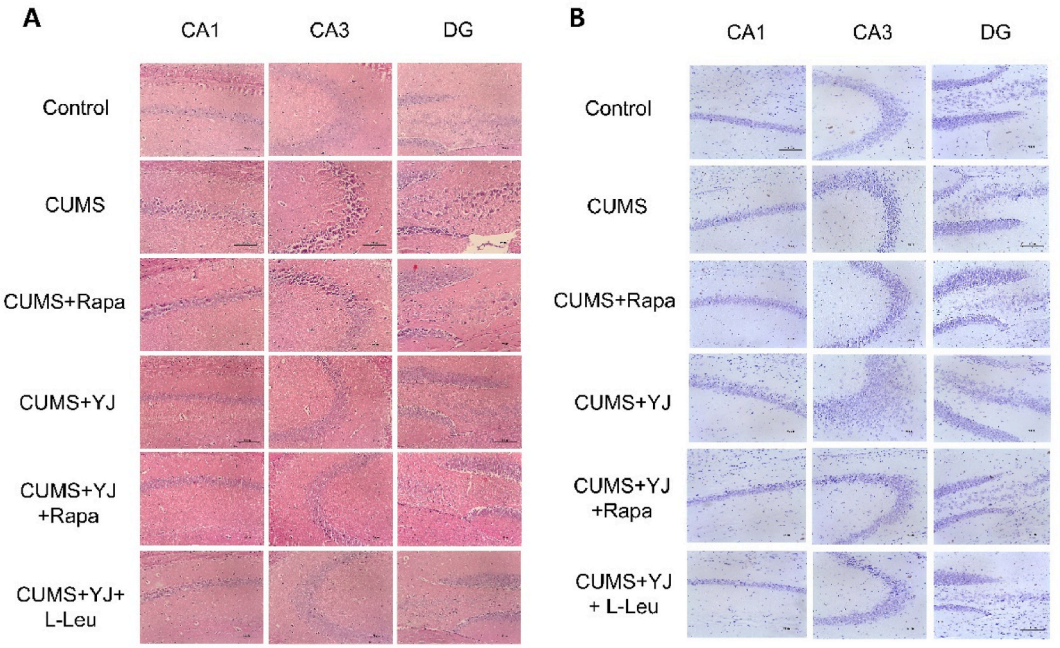

Fig. 6. Influence of YJ on histopathological damage in the hippocampus. (A) HE staining results of hippocampal tissue. (B) Nissl staining results of Hippocampal tissue.

atrophy of hippocampal neuronal cells in mice in the CUMS group, and neuronal degeneration was observed (Fig. 6B). Hippocampal neuronal damage was alleviated in the CUMS + YJ group and the CUMS + YJ + Rapa group. And the CUMS + YJ + L-Leu group and the CUMS + Rapa group showed the same pathological manifestations in the hippocampus of mice as the CUMS group.

These evidences suggest that YJ attenuated the damage to mouse hippocampal neurons, while L-Leu blocked the effect of YJ. Rapa alone did not ameliorate hippocampal neuronal injury in mice.

3.7. YJ changes the relative content of central neurotransmitters in mice

It has been shown that the activation of the central Ghrelin/GHS-R system can increase the level of central 5-HT. We used LC-MS to determine the relative content of central neurotransmitters in mice. As shown in Fig. 7, YJ could reverse the decrease in 5-HT level and

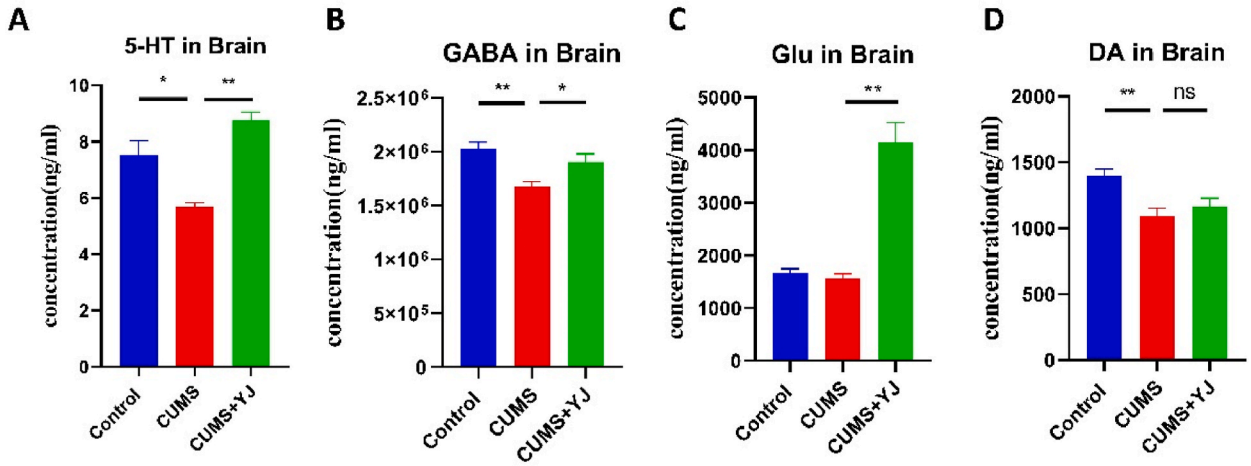

Fig. 7. Effect of YJ on neurotransmitters in mice (A) 5-HT. (B) GABA. (C) Glu. (D) DA. $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant differences vs. the CUMS group (mean ± SEM, n ≥ 6).

GABA level induced by CUMS. YJ also significantly increased the relative content of central Glu in mice. CUMS stimulation decreased the relative content of DA in the central nervous system of mice, and YJ showed a tendency to pullback, but there was no significant difference between the CUMS group and the CUMS + YJ group.

4. Discussion

YJ is a classic and famous prescription for treating depression in Chinese medicine. Not only that, in clinical practice, YJ is also widely used for treating digestive system diseases. This study demonstrated that YJ can exert antidepressant effects by inhibiting the gastric mTOR/S6K signal pathway to increase gastric hunger hormone levels, and on this basis, sensitizing the GHS-R in the hippocampus to ameliorate neurological damage in the hippocampus. Our work emphasizes the close link between the antidepressant effect of YJ and its effect on peripheral gastric hunger hormone and provides new ideas for the antidepressant mechanism of YJ.

The antidepressant pharmacodynamic study of YJ showed that, compared with the mice in the CUMS group, the mice treated with YJ exhibited a trend of regression in neurobehavior and hemodynamics consistent with that of the positive drug group, which is in line with the previous study that YJ has a better antidepressant efficacy [4,5]. Meanwhile, our results showed that CUMS induction led to a decrease in the mRNA expression level of GHR in the stomach and serum GHR in depressed mice, which was significantly increased by the intervention of YJ. This suggests a close relationship between YJ's antidepressant effect and its modulation of GHR. The antidepressant and antianxiety effects of ghrelin have been widely reported. A study reported that calorie-restricted mice had increased levels of ghrelin, and these mice exhibited more immobility time in the forced swim test (FST) and more exercise time in the open arm of the elevated plus maze (EPM) [29]. Another study revealed that ghrelin-injected subcutaneously C57BL6/J mice significantly reduced depression-like behavior in the EPM and FST [16]. Chronic peripheral or central administration of exogenous ghrelin therapy has shown significant antidepressant-like effects on rodents induced with CUMS in the FST [25]. These studies demonstrated that both increased endogenous ghrelin levels and peripheral or central administration of exogenous ghrelin can have an antidepressant effect.

The synthesis and release of ghrelin are regulated by the gastric mTOR/S6K signal pathway, and its level is inversely correlated with the activation of the gastric mTOR/S6K signal pathway. Li and Tian et al. discovered that inhibiting the gastric mTOR signal pathway could increase circulating ghrelin levels [30,31]. Interestingly, it was reported that the mTOR signal pathway is impaired in the PFC of individuals diagnosed with MDD [32]. Attenuation of the mTOR pathway was also found in the prefrontal cortex and hippocampus of rodents exposed to chronic stress [33,34]. Other studies have shown that activation of the mTOR signal pathway appears to underlie some classical antidepressants, such as NMDA and muscarinic receptors antagonists [35]. This suggests that expression of the mTOR signal pathway shows opposite trends in the brain and stomach of depressed rodents. Therefore L-Leu and Rapa were used to validate the previous results. Our experiments revealed that YJ reduced the activation of the gastric mTOR/S6K signal pathway in the stomachs of CUMS-induced mice, resulting in an increase in ghrelin expression levels. The co-administration of Rapa and YJ further inhibited the gastric mTOR/S6K signal pathway and slightly increased (but not significantly) GHR expression. However, it did not significantly enhance the antidepressant effect of YJ. Additionally, the administration of L-Leu counteracted the inhibitory effect of YJ on the gastric mTOR/S6K signal pathway and decreased ghrelin levels in mice. Furthermore, behavioral tests demonstrated that L-Leu also blocked YJ's antidepressant effect. These findings suggest a close relationship between the antidepressant effect of YJ and the ghrelin level, mediated by the gastric mTOR/S6K signal pathway. Interestingly, we found that Rapa alone did increase the expression of ghrelin but did not exhibit any antidepressant activity. This suggests that YJ's antidepressant effect may not rely solely on inhibiting the gastric mTOR/S6K signal pathway and increasing ghrelin levels. It is worth noting that the effectiveness of a ligand depends not only on itself, but also on the receptor it can bind to. To further understand this phenomenon, we directed our attention towards the receptor for ghrelin.

Importantly, as a hormone primarily produced in the periphery [13], ghrelin can enter the central nervous system through various pathways. Our experimental results showed that the GHR content in mice's hippocampal region in all groups was consistent with the mRNA trend of GHR in blood and GHR in the stomach. One study demonstrated that injections of isotope-labeled ghrelin appeared in the brains of mice [36]. Another study discovered that RBE4 brain microvascular endothelial cells completely absorbed and released isotope-labeled ghrelin [37]. Ghrelin can also cross the blood-cerebrospinal fluid barrier (BCSFB) to reach the cerebrospinal fluid, allowing hormones to enter the periventricular area [38,39]. These studies show that peripheral ghrelin can reach the brain and provide strong evidence to establish a link between YJ's effect on peripheral ghrelin and its central antidepressant mechanism.

GHS-R is currently the sole known receptor that binds to ghrelin, and it is expressed in various regions of the brain, including the hippocampus [21]. The Ghrelin/GHS-R system in the hippocampus plays a protective role in hippocampal neurogenesis [19]. However, impaired neuroplasticity is a significant contributor to severe depression [40]. Recent studies have showed that loss of GHS-R increases susceptibility to depression-like behavior [18]. More importantly, the mice under long-term chronic stress showed a decrease in ghrelin binding sites in their amygdala, which they explained as the desensitization of GHS-R [15]. Our results showed that CUMS and social isolation stimulation can lead to desensitization of GHS-R in mice's hippocampus, whereas YJ rather than Rapa can sensitize GHS-R. The HE staining and Nysted staining experiments also showed that YJ had a better neuroprotective effect.

An abnormal function of the central 5-HT system is a significant mechanism of depression [23]. It has been reported that activation of the central Ghrelin/GHS-R system can enhance the release of 5-HT [41,42]. Recent studies have reported that the Ghrelin/GHS-R system can modulate the midbrain dopamine system [43]. As a result, we measured the levels of four neurotransmitters in the mouse brain, including 5-HT, GABA, Glu and DA, to preliminarily evaluate the effects of YJ on neurotransmitter changes. Our findings indicated that YJ could reverse the decrease in central 5-HT content caused by CUMS, possibly due to its sensitizing effect on GHS-R. Furthermore, it is worth noting that YJ application also resulted in changes in central DA and GABA levels. However, further investigation is needed to determine whether these changes are related to the effects of YJ on the Ghrelin/GHS-R system.

Nevertheless, our study is not without limitations. First, the mTOR/S6K signal pathway is complex and widespread throughout the body, and we targeted only this pathway in the gastric tissue. Second, although we demonstrated that YJ's modulation of the GHR/GHS-R system plays a key role in its treatment of depressed mice, further studies are needed to investigate the potential mechanisms of the GHR/GHS-R system in depression.

5. Conclusion

In conclusion, all the results strongly support YJ's antidepressant mechanism based on the following two points:1) YJ demonstrates its antidepressant activity by inhibiting the gastric mTOR/S6K signal pathway and improving the level of ghrelin. 2) YJ sensitized GHS-R in the hippocampus, increased the activation of the Ghrelin/GHS-R system, protected hippocampal neurogenesis, and increased the level of 5-HT. This study is the first to propose an explanation for YJ's antidepressant mechanism by considering its peripheral effects and provides a new perspective for understanding the treatment mechanisms of YJ.

Declarations of animal experiment ethics

Animal experiments were conducted under the approval and supervision of the Animal Ethics Experiment Committee of Jiangxi University of Traditional Chinese Medicine, with ethics number JZLLSC20210061.

Data availability statement

The data that support the findings of this study had not been deposited into a publicly available repository, and will be available from the corresponding author upon reasonable request.

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CRediT authorship contribution statement

Zhentao Zhang: Writing – review & editing, Writing – original draft, Data curation. **Dan Su:** Writing – review & editing, Methodology, Conceptualization. **Meixizi Lai:** Visualization, Validation, Software, Investigation, Data curation. **Yonggui Song:** Writing – review & editing, Project administration, Methodology, Conceptualization. **Huizhen Li:** Writing – review & editing, Visualization, Software, Investigation, Data curation. **Ming Yang:** Supervision. **Genhua Zhu:** Supervision, Funding acquisition. **Hong Liu:** Software, Methodology, Conceptualization. **Zhifu Ai:** Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37038.

References

[1] L. Cui, et al., Major depressive disorder: hypothesis, mechanism, prevention and treatment, Signal Transduct. Targeted Ther. 9 (1) (2024) 30.
[2] B. Short, et al., Side-effects associated with ketamine use in depression: a systematic review, Lancet Psychiatr. 5 (1) (2018) 65–78.
[3] Z. Dandan, Clinical Study on Rapid Improvement of Depressive Symptoms by Yueju Pill, vol. 48, Nanjing University of traditional Chinese Medicine, 2016.
[4] W. Xue, et al., Yueju pill rapidly induces antidepressant-like effects and acutely enhances BDNF expression in mouse brain, Evid Based Complement Alternat Med 2013 (2013) 184367.
[5] J. Tang, et al., Involvement of normalized NMDA receptor and mTOR-related signaling in rapid antidepressant effects of Yueju and ketamine on chronically stressed mice, Sci. Rep. 5 (2015) 13573.
[6] W. Xue, et al., PKA-CREB-BDNF signaling regulated long lasting antidepressant activities of Yueju but not ketamine, Sci. Rep. 6 (2016) 26331.
[7] N. Reich, C. Holscher, Beyond appetite: acylated ghrelin as a learning, memory and fear behavior-modulating hormone, Neurosci. Biobehav. Rev. 143 (2022) 104952.

[8] I. Sakata, et al., Ghrelin-producing cells exist as two types of cells, closed- and opened-type cells, in the rat gastrointestinal tract, Peptides 23 (3) (2002) 531–536.
[9] M. Shintani, et al., Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway, Diabetes 50 (2) (2001) 227–232.
[10] G. Xu, et al., Gastric mammalian target of rapamycin signaling regulates ghrelin production and food intake, Endocrinology 150 (8) (2009) 3637–3644.
[11] G. Xu, et al., Regulation of gastric hormones by systemic rapamycin, Peptides 31 (12) (2010) 2185–2192.
[12] G. Xu, et al., Ghrelin contributes to derangements of glucose metabolism induced by rapamycin in mice, Diabetologia 55 (6) (2012) 1813–1823.
[13] A. Cabral, et al., Is ghrelin synthesized in the central nervous system? Int. J. Mol. Sci. 18 (3) (2017).
[14] M. Perello, et al., Brain accessibility delineates the central effects of circulating ghrelin, J. Neuroendocrinol. 31 (7) (2019) e12677.
[15] H.J. Huang, et al., Ghrelin alleviates anxiety- and depression-like behaviors induced by chronic unpredictable mild stress in rodents, Behav. Brain Res. 326 (2017) 33–43.
[16] M. Lutter, et al., The orexigenic hormone ghrelin defends against depressive symptoms of chronic stress, Nat. Neurosci. 11 (7) (2008) 752–753.
[17] N. Sun, et al., Ghrelin attenuates depressive-like behavior, heart failure, and neuroinflammation in postmyocardial infarction rat model, Eur. J. Pharmacol. 901 (2021) 174096.
[18] M.V. Masule, et al., Ghrelin mediated regulation of neurosynaptic transmitters in depressive disorders, Curr Res Pharmacol Drug Discov 3 (2022) 100113.
[19] H.J. Huang, et al., The protective effects of Ghrelin/GHSR on hippocampal neurogenesis in CUMS mice, Neuropharmacology 155 (2019) 31–43.
[20] V. Morin, F. Hozer, J.F. Costemale-Lacoste, The effects of ghrelin on sleep, appetite, and memory, and its possible role in depression: a review of the literature, Encephale 44 (3) (2018) 256–263.
[21] B.K. Mani, et al., Neuroanatomical characterization of a growth hormone secretagogue receptor-green fluorescent protein reporter mouse, J. Comp. Neurol. 522 (16) (2014) 3644–3666.
[22] J.M. Zigman, et al., Expression of ghrelin receptor mRNA in the rat and the mouse brain, J. Comp. Neurol. 494 (3) (2006) 528–548.
[23] M. Kluge, et al., Effects of ghrelin on psychopathology, sleep and secretion of cortisol and growth hormone in patients with major depression, J. Psychiatr. Res. 45 (3) (2011) 421–426.
[24] L. Guo, et al., GHS-R1a deficiency alleviates depression-related behaviors after chronic social defeat stress, Front. Neurosci. 13 (2019) 364.
[25] E.S. Harmatz, et al., Central ghrelin resistance permits the overconsolidation of fear memory, Biol. Psychiatr. 81 (12) (2017) 1003–1013.
[26] Y. Song, et al., Raw and wine processed Schisandra chinensis attenuate anxiety like behavior via modulating gut microbiota and lipid metabolism pathway, J. Ethnopharmacol. 266 (2021) 113426.
[27] C.M. Cooper, et al., Discovery and replication of cerebral blood flow differences in major depressive disorder, Mol. Psychiatr. 25 (7) (2020) 1500–1510.
[28] T. Wang, et al., Pulsatilla chinensis saponins ameliorated murine depression by inhibiting intestinal inflammation mediated Ido1 overexpression and rebalancing tryptophan metabolism, Phytomedicine 116 (2023) 154852.
[29] H. Schellekens, et al., Ghrelin signalling and obesity: at the interface of stress, mood and food reward, Pharmacol. Ther. 135 (3) (2012) 316–326.
[30] Z. Li, et al., mTOR signaling in X/A-Like cells contributes to lipid homeostasis in mice, Hepatology 69 (2) (2019) 860–875.
[31] J. Tian, et al., Rapamycin attenuates anxiety and depressive behavior induced by Helicobacter pylori in association with reduced circulating levels of ghrelin, Neural Plast. 2022 (2022) 2847672.
[32] C.S. Jernigan, et al., The mTOR signaling pathway in the prefrontal cortex is compromised in major depressive disorder, Prog. Neuro-Psychopharmacol. Biol. Psychiatry 35 (7) (2011) 1774–1779.
[33] A. Chandran, et al., Reduced phosphorylation of the mTOR signaling pathway components in the amygdala of rats exposed to chronic stress, Prog. Neuro-Psychopharmacol. Biol. Psychiatry 40 (2013) 240–245.
[34] W.L. Zhu, et al., Glycine site N-methyl-D-aspartate receptor antagonist 7-CTKA produces rapid antidepressant-like effects in male rats, J. Psychiatry Neurosci. 38 (5) (2013) 306–316.
[35] Z.M. Ignacio, et al., New perspectives on the involvement of mTOR in depression as well as in the action of antidepressant drugs, Br. J. Clin. Pharmacol. 82 (5) (2016) 1280–1290.
[36] S. Diano, et al., Ghrelin controls hippocampal spine synapse density and memory performance, Nat. Neurosci. 9 (3) (2006) 381–388.
[37] W. Pan, H. Tu, A.J. Kastin, Differential BBB interactions of three ingestive peptides: obestatin, ghrelin, and adiponectin, Peptides 27 (4) (2006) 911–916.
[38] A. Cabral, et al., Divergent neuronal circuitries underlying acute orexigenic effects of peripheral or central ghrelin: critical role of brain accessibility, J. Neuroendocrinol. 26 (8) (2014) 542–554.
[39] M. Uriarte, et al., Evidence supporting a role for the blood-cerebrospinal fluid barrier transporting circulating ghrelin into the brain, Mol. Neurobiol. 56 (6) (2019) 4120–4134.
[40] W. Liu, et al., The role of neural plasticity in depression: from Hippocampus to prefrontal cortex, Neural Plast. 2017 (2017) 6871089.
[41] M. Alvarez-Crespo, et al., The amygdala as a neurobiological target for ghrelin in rats: neuroanatomical, electrophysiological and behavioral evidence, PLoS One 7 (10) (2012) e46321.
[42] C. Hansson, et al., Influence of ghrelin on the central serotonergic signaling system in mice, Neuropharmacology 79 (2014) 498–505.
[43] J.A. Engel, et al., Ghrelin activates the mesolimbic dopamine system via nitric oxide associated mechanisms in the ventral tegmental area, Nitric Oxide 131 (2023) 1–7.