



Research Article

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Shuyu capsule improves estrous cycle-dependent depression-like behavior in premenstrual dysphoric disorder (PMDD) mice by increasing GABAergic neuronal activation and downregulating GABA_AR δ subunit expression in the dorsal periaqueductal gray (dPAG) region

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Abstract: Premenstrual dysphoric disorder (PMDD), a subtype of premenstrual syndrome (PMS), involves physical and emotional symptoms that impact patients' daily lives and productivity. A reliable, side-effect-free clinical intervention is needed. Shuyu capsule is an effective traditional Chinese medicine preparation for PMDD used in the clinics, but its therapeutic mechanism remains unclear. Previous research has suggested that the γ -aminobutyric acid (GABA)ergic system in the periaqueductal gray (PAG) may play a role in treating PMDD with traditional Chinese medicine, but there is a lack of functional verification. This study aims to reveal the potential mechanism of the Shuyu capsule in treating PMDD. The study employed an experimental design using female C57BL/6J and Vgat-Cre mice to assess the effects of Shuyu capsules on PMDD, with a focus on the GABAergic system in the dorsal PAG (dPAG). Assessments were conducted using the forced swimming test (FST) to gauge depression-like behaviors and western blot (WB) and immunofluorescence (IF) to measure the numbers of active GABAergic neurons and the γ -aminobutyric acid type A receptor (GABA_AR) δ subunit (GABRD) expression. Chemogenetic techniques and adeno-associated virus were specifically used to activate GABAergic neurons and knock down the expression of subunits, respectively, providing insights into the neurobiological mechanisms underpinning the therapeutic effects of Shuyu capsules in treating PMDD. After being stressed by FST, the immobility duration of PMDD mice in the late dioestrus (LD) phase decreased after the Shuyu capsule intervention, implying that it can improve the estrous cycle-dependent depression-like phenotype in PMDD mice. Additionally, the application of Shuyu capsule can downregulate the expression of GABRD and reverse the downtrend of activated GABAergic neurons in the dPAG of PMDD model mice. We also found that single-target manipulation was enough to improve the depression-like behavior of PMDD model mice. Transgenic mice with *GABRD* knockout were established, and their behaviors were tested, revealing changes in their exploratory behaviors, indicating that the GABRD may be closely related to anxiety disorders. Shuyu

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capsule plays an anti-PMDD role by activating GABAergic neurons and downregulating the expression of GABRD in the dPAG. This provides a theoretical basis for the clinical treatment of PMDD with traditional Chinese medicine and promotes the development of drugs for treating PMDD.

Key words: Premenstrual dysphoric disorder; Periaqueductal gray; γ -Aminobutyric acidergic (GABAergic) neuron; γ -Aminobutyric acid receptor δ subunit (GABRD); Shuyu capsule; Depression-like behavior

1 Introduction

Premenstrual syndrome (PMS) is a common condition among women of childbearing age, and premenstrual dysphoric disorder (PMDD) is a serious subtype of PMS. Currently, the global prevalence rate of PMDD is 3%–8% (Kadian and O'Brien, 2012; Reid and Soares, 2018). PMDD patients typically experience somatic symptoms such as breast pain and headaches, accompanied by cognitive-emotional symptoms like anxiety or depression (First, 2013). Recognized as a special disease, PMDD was included in the 11th edition of the International Morbidity Statistics Classification by the World Health Organization in 2019, categorized as a genitourinary system disease, and cross-classified as a depressive disease (Sekhon and Gupta, 2024). At present, selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine (FLX), are the primary medications used to treat PMDD and severe emotional disorders (Ismaili et al., 2016). However, long-term usage of SSRIs can result in various persistent side effects, making it crucial to identify effective therapeutic targets and treatments with improved efficacy for PMDD.

The core symptoms of PMDD, which are estrous cycle-dependent, appear in the middle and late luteal phases when the levels of progesterone and its metabolite allopregnanolone (ALLO) significantly decrease. PMDD's pathogenesis is closely linked to progesterone (Smith et al., 2006) and ALLO (Girdler et al., 2001; Osborne et al., 2019). Recent research has focused on the pathological mechanism underlying PMDD from the perspective of the central nervous system. Studies in both women (Goldstein et al., 2005) and animal models (Devall and Lovick, 2010) have found that the periaqueductal gray (PAG) matter in the midbrain is a brain region with varying activity throughout the menstrual cycle. Research also suggested that PAG stimulation in PMDD model rats with anxiety and stress in late dioestrus (LD) can cause hyperalgesia (Devall et al., 2009). Since γ -aminobutyric acidergic (GABAergic) neurons are the main neuron type in PAG (Reichling

and Basbaum, 1990), their activity may potentially play a role in the effect of antidepressants (Griffiths and Lovick, 2005). In addition, GABAergic neurons in the PAG are involved in the regulation of emotions and behaviors, such as anxiety (Lowery-Gionta et al., 2018) and fear (Behbehani, 1995). These findings imply that GABAergic neurons in the PAG could contribute to PMDD's pathogenesis.

γ -Aminobutyric acid type A receptors (GABA_ARs) are widely distributed in the central nervous system, and their main function is to mediate inhibitory currents (Farrant and Nusser, 2005). According to the literature, the expression of some GABA_AR subunits changes with the fluctuation of ALLO (Jensen et al., 2002; Smith et al., 2004), especially the δ subunits, which are mainly distributed in the extrasynaptic space (Griffiths and Lovick, 2005; Rudolph et al., 2020). Our previous study confirmed that the expression of GABA type A receptor δ subunit (GABRD) in the PAG of PMDD model rats was significantly decreased after treatment with a Shuyu capsule (Geng et al., 2024). This indicates that the GABRD may be the Shuyu capsule's target in treating PMDD.

Various studies have shown that the Shuyu capsule, a traditional Chinese medicine preparation, can treat PMDD effectively (Gao et al., 2014; Zhang et al., 2023). Our previous research demonstrated its ability to alleviate emotional and physical symptoms in PMDD rats (Geng et al., 2024). However, previous studies on the intervention mechanism of traditional Chinese medicine in PMDD have mainly focused on rat models and lacked molecular regulation and neuron type verification of the target brain region. Therefore, this study aimed to establish a suitable mouse model of PMDD to verify the specificity of the Shuyu capsule's target molecules in treating PMDD based on the above research results. We sought to verify that in Shuyu capsule therapy, PMDD symptoms are relieved through the regulation of GABRD expression and activated GABAergic neurons in the dPAG.

2 Materials and methods

2.1 Animals

All C57BL/6J mice (11 weeks old, 20–25 g) used in this study were female and were randomly assigned to cages housing five mice each. The mice were maintained at (21 ± 2) °C and (45 ± 10) % relative humidity under a 12-h light/dark cycle, with ad libitum access to food and water, at Shandong University of Traditional Chinese Medicine, Jinan, China. Information on the strains and sources of the experimental mice is detailed in the supplementary materials and methods.

2.2 Experimental grouping and drug treatment

Before the experiment, vaginal smears were performed on the mice for a week, and the mice with stable and regular estrous cycles were screened for formal experiments. Then, mice in their estrus (E) and LD phases were stressed by the forced swimming test (FST). In accordance with the method used by Schneider and Popik (2007) and Wei et al. (2020), the immobility duration in the E phase was subtracted from the immobility duration in the LD phase, and the differences between the two periods were calculated. The top 30% with big differences in experimental mice were selected as PMDD model mice, and the last 30% were the control mice (Con). PMDD model mice were then separated into three groups, which were orally administered saline (Mod group; 10 mL/(kg·d)), FLX (FLX group; 3.738 mg/(kg·d)), or Shuyu capsule (Shuyu group; 571.2 mg/(kg·d)) (Gao et al., 2014), respectively. Mice in the control group were administered saline orally at a dose of 10 mL/(kg·d). Mice were gavaged at 9:00 a.m. each day continuously for 12 d. After the gavage, the behaviors of mice in each group were tested by FST as same as prior to intervention, and mice were sacrificed for sampling in the LD phase. Shuyu capsules were prepared by the Pharmacy Laboratory of the Shandong University of Traditional Chinese Medicine (BP: 2008L11169). FLX was purchased from Eli Lilly Company, IN, USA (BP, 20510A).

2.3 Extraction and component analysis of Shuyu capsule

The method of extraction for the Shuyu capsule was the same as described in our previous work (Geng et al., 2024).

2.4 Measurement of allopregnanolone in mouse serum

Serum samples were collected from the infraorbital orbital venous plexus. The ALLO concentration in the serum was detected using an enzyme-linked immunosorbent assay (ELISA) kit (Cusabio Biotech, Wuhan, China; CSB-E05104m), following a detailed protocol.

2.5 Vaginal smear test

For the vaginal smear procedure, the vaginal orifices of the mice were fully exposed. Saline was drawn into a pipette, and the tip of the pipette was then placed into the vagina of the mouse, and the saline was flushed in and out several times. The vaginal perfusion solution fluid was then drawn back into the pipette and spread evenly on a sticky glass slide. The slides were left to air dry, and then the dried slides were put into a 4% (0.04 g/mL) paraformaldehyde solution (Beyotime, Shanghai, China; P0099). After a few minutes, the slides were taken out and washed with pure water, and then put into a crystal violet staining solution (Beyotime; C0121) for 10 min. After washing with pure water again, the slides were placed under the microscope for observation of vaginal epithelial cell morphology.

2.6 Forced swimming test

The FST was conducted every morning in an environment with red light illumination, and the experimental mice adapted to the environment for 30 min before the experiment commenced. The mice were then placed into a plexiglass bucket with an inner diameter of 11 cm and a depth of 18 cm, which contained water with a temperature of (23 ± 1) °C. Mice were allowed to swim freely for 6 min. The activity of the mice during the experiment was recorded, and the number of immobile mice and the duration of immobility in the water were noted. An immobile state is defined as no movement except breathing and slightly paddling the front paws to keep balance (Yankelevitch-Yahav et al., 2015). After the experiment, the mice were fished out of the bucket, dried, and returned to the home cage. After each experiment, the water was changed and the bucket was washed.

2.7 Location of the dorsal periaqueductal grey matter and virus injection

The methods for locating the dorsal PAG (dPAG) and injecting the virus were the same as those described

in our previous work (Geng et al., 2024). We marked the standard surgery dPAG site (anterior-posterior (AP): -4.36 mm; medial-lateral (ML): ± 0.3 mm; dorsal-ventral (DV): -2 mm) and then drilled a small hole in the skull. To avoid vertical needle insertion compressing blood vessels and causing massive bleeding, we adjusted the needle insertion angle to 30° (the site was adjusted to AP: -4.36 mm; ML: ± 1.08 mm; DV: -2.4 mm). Details about the virus are as follows: adeno-associated virus (AAV)-hSyn-DIO-mCherry (BrainVTA Co., Ltd., Wuhan, China; PT-0115); AAV-hSyn-DIO-hM3D (Gq)-mCherry (BrainVTA; PT-0019); AAV-U6-short hairpin RNA (shRNA) (scramble)-CMV-enhanced green fluorescent protein (EGFP)-pA (BrainVTA; PT-0916); AAV-U6-shRNA (GABARD)-CMV-EGFP-pA (BrainVTA; PT-0360). The details of shRNA sequence information are shown in Table S1.

2.8 Intraperitoneal injection of Clozapine N-oxide

After two weeks of virus expression, Clozapine N-oxide (CNO; APExBIO, Shanghai, China; A3317) powder, after full centrifugation, was dissolved in a saline solution containing 5% (0.05 g/mL) dimethyl sulfoxide (DMSO) (Aladdin; D2107139). After determining the estrous cycle phase, mice were intraperitoneally injected with CNO (5 mg/kg) (Hu et al., 2022) in the E and LD phases, and the FST was performed 1 h later.

2.9 Western blot

The methods for locating the dPAG and injecting the virus were the same as those described in our previous work (Geng et al., 2024). The primary and secondary antibody information is as follows: GABA_AR $\alpha 4$ (Immunoway, Suzhou, China; YT1822, 1:1000 (volume ratio, the same bellow)); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Proteintech, Wuhan, China; 60004-1-Ig, 1:5000); GABA_AR $\beta 1$ (ThermoFisher, Shanghai, China; PA5-116439, 1:1000); GABA_AR δ (ThermoFisher; PA5-101598, 1:1000); horseradish peroxidase (HRP) goat anti-rabbit immunoglobulin G (IgG) (heavy chain (H)+light chain (L)) (ABclonal, Wuhan, China; AS014, 1:5000); and HRP goat anti-mouse IgG (H+L) (ABclonal; AS003, 1:5000).

2.10 Immunofluorescence staining

Brain tissue slices were put into a $1\times$ citrate antigen retrieval solution (Beyotime; P0081) and heated

at 95°C for 5–10 min. They were then washed and blocked using phosphate-buffered saline (PBS), which contains 5% (volume fraction) goat serum and 1% (volume fraction) Triton X-100 (Solarbio, Beijing, China; T8200), at room temperature for 2 h. Then, slices were put into the primary and secondary antibody incubation solutions. After incubation, slices were stuck to the glass slide and air-dried, evenly mounted with an anti-fade medium with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Solarbio; S2110), and then immediately observed and imaged under a fluorescent microscope. The primary and secondary antibody information is as follows: glutamate decarboxylase 67 (GAD67) (Millipore, Shanghai, China; MAB5406, 1:200); cellular Finkel-Biskis-Jenkins murine osteosarcoma viral oncogene homolog (c-Fos) (Abcam, Shanghai, China; ab208942, 1:1000); goat anti-rabbit IgG H+L Alexa Fluor[®] 647 (Abcam; ab150083, 1:500); and goat anti-mouse IgG H+L Alexa Fluor[®] 488 (Abcam; ab150113, 1:1000).

2.11 Home cage behavior test

Using the method referenced by Kyzar et al. (2012), we identified the behaviors of *Gabrd*^{+/+} and *Gabrd*^{-/-} mice in detail. Each animal was placed separately in a transparent, open cage with proper padding height. There was a dining and drinking area on the cage cover, but nothing else in the cage. The video capture facilities were placed in front of the cage, and the experimental environment was equipped with ventilation and lighting facilities that were isolated from external stimuli. The recording started at 9:00 a.m. every day and lasted 24 h. The environment's lights were turned off at 9:00 a.m. and turned on at 9:00 p.m. In the dark phase, the experiment was conducted under red light with a brightness of <25 lux. The video was recorded in AVI format and was analyzed using HomeCageScan software (Clever Sys, Inc., Guangzhou, China). The activity data of the mice were expressed as the percentage of time they spent engaging in the behaviors.

2.12 Data analysis

All values are presented as mean \pm standard error of the mean (SEM) for the data on the serum level of ALLO, the results of the western blot (WB) analysis for the virus efficiency verification, and the home cage behavior test. An unpaired two-tailed Student's *t*-test

was applied for two-group comparisons. For behavioral test data, all values are presented as mean±standard error of the mean (SEM). A two-way repeated measure analysis of variance (ANOVA) was performed, and a Bonferroni comparisons test was used for data detection and comparison. For the immunofluorescence (IF) staining and WB analysis of the GABA_AR subunit expression data, all values are presented as mean±SEM. A repeated measure one-way ANOVA was performed, and a Bonferroni comparisons test was used to detect and compare data. All values underwent normality testing (Kolmogorov–Smirnov test) and homoscedasticity testing (Levene’s test) before analysis with a parametric test. The significance level was set at $P<0.05$. Differences between groups were examined for statistical significance using a two-tailed Student’s *t*-test (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$). GraphPad Prism 8.0.1 software was used to create graphs for the statistical analyses.

3 Results

3.1 Alleviative effect of Shuyu capsules on estrus cycle-dependent depression-like behavior in PMDD mice

We used mice with natural estrous cycles to simulate the fluctuation of gonadal hormones in a normal menstrual cycle. The stability and regularity of the estrous cycles of the experimental mice were confirmed. Then the mice were subjected to model screening, grouping, drug intervening, and tissue sampling (Fig. 1a). Since the estrous cycle of mice typically lasts 4–6 d (Erika and López-Rubalcava, 2022), we examined the morphology and proportion of their vaginal epithelial cells to select suitable experimental subjects (Brack and Lovick, 2007). In the proestrus (P) phase, epithelial cells were primarily nucleated and fully keratinized (indicated by the indigo arrow). During the E phase,

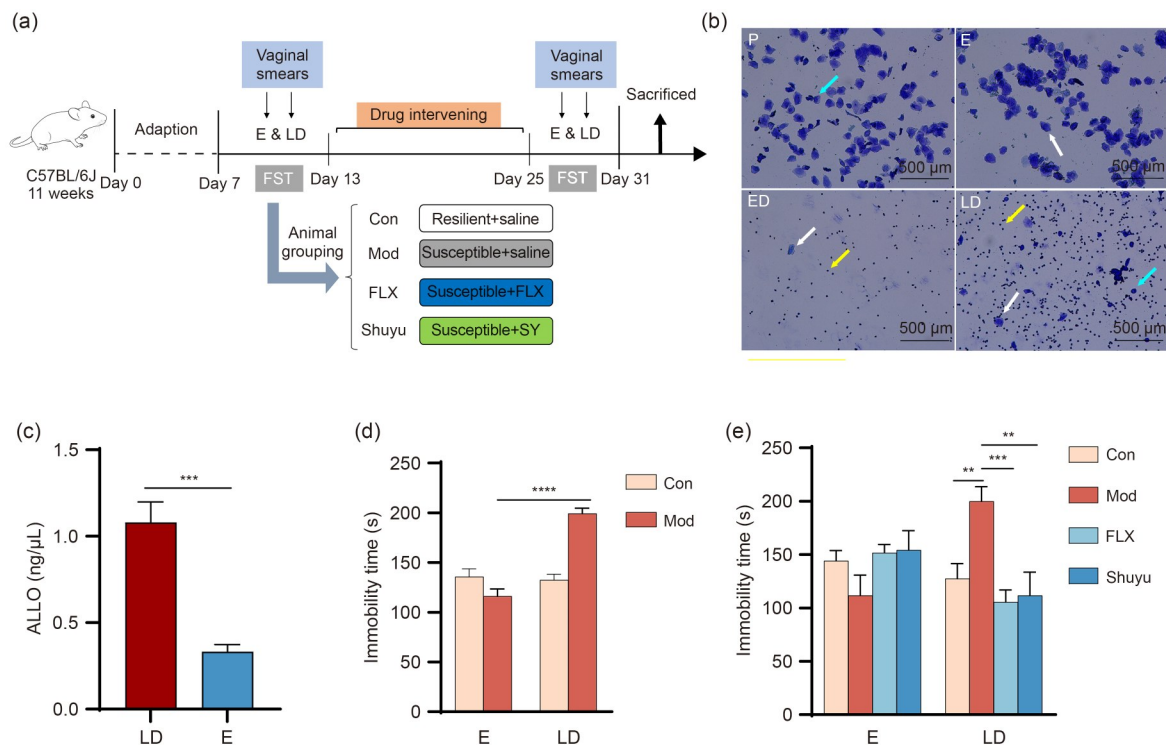


Fig. 1 Effect of Shuyu capsule on the depression-like behavior in the late dioestrus (LD) phase of premenstrual dysphoric disorder (PMDD) mice. (a) Design diagram of the experimental process. (b) Representative images of vaginal smears of mice at proestrus (P), estrous (E), early dioestrus (ED), and LD. (c) Concentration of allopregnanolone (ALLO) in the mouse serum. All values are expressed as mean±standard error of the mean (SEM). *** $P<0.001$, unpaired two-tailed Student’s *t*-test. (d) Immobility duration was detected via the forced swimming test (FST) in the E and LD phases before the Shuyu intervention. (e) FST evaluated the efficacy of Shuyu capsules by recording the immobility duration in the E and LD phases after the intervention. All values are expressed as mean±SEM. ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$, compared to baseline (con), two-way analysis of variance (ANOVA) with Bonferroni’s test ($n\geq 6$ per group). Con: control; Mod: saline; FLX: fluoxetine; Shuyu, SY: Shuyu capsule.

these cells evolved into nuclear-free keratinocytes (indicated by the white arrow). Leukocytes predominated by the early dioestrus (ED) phase (indicated by the yellow arrow). The pathological phenotype of PMDD manifested in the LD phase, characterized by an abundance of leukocytes and a scarcity of nucleated epithelial cells (Fig. 1b). Progesterone metabolite ALLO typically peaks in the early luteal phase in women and decreases sharply during the mid to late luteal phases (Sundström-Poromaa et al., 2020). We measured ALLO serum levels and confirmed hormonal fluctuations in the experimental mice. The results showed that ALLO levels were significantly higher in the LD phase than in the E phase (Fig. 1c), mirroring typical female hormonal patterns.

Exposure to stressful environments in animals leads to behaviors indicative of severe depression. Marván et al. (1997) demonstrated that rats exhibited significantly longer immobility time during the FST in non-receptive periods compared to receptive periods. Given the heightened stress sensitivity in PMDD patients during the luteal phase—akin to the non-receptive period in mice—the LD phase was selected as the non-receptive phase and the E phase as the receptive phase (Wei et al., 2020). FST was used to elicit depression-like behaviors in mice during the LD phase. Mice in the PMDD group displayed significantly longer immobility time in the LD phase than in the E phase, with no notable difference from the control group mice (Fig. 1d). Mice in the model group received treatments of Mod, FLX, and Shuyu, while the control group received the same volume of saline. Subsequently, all four groups repeated the FST across two periods to assess drug efficacy. We found that the PMDD mice exhibited significantly longer immobility time than control mice in the LD phase, which notably decreased following Shuyu treatment (Fig. 1e). No differences in the number of immobilities were observed among the groups (Fig. S1). Video analysis revealed that the changes were due to prolonged immobility time in the experiment. Overall, these results suggest that Shuyu capsules effectively mitigate depression-like behavior in PMDD mice.

3.2 Effect of Shuyu capsules on GABAergic neurons in the dPAG of PMDD mice

The PAG activity of PMDD patients differs from that of healthy women (Goldstein et al., 2005). Given

that GABAergic neurons are the predominant neuron type in the PAG (Reichling and Basbaum, 1990), Griffiths and Lovick (2005) have shown that the neurons of the dPAG may play a potential role in the effect of antidepressants. Our previous research demonstrated that the Shuyu capsule alleviates emotional and physical symptoms in PMDD rats by activating neurons in the dPAG (Geng et al., 2024). These findings suggest that GABAergic neurons in the dPAG are the key targets for treating PMDD with the Shuyu capsule.

IF staining explored whether Shuyu capsules treat PMDD by influencing GABAergic neuron activity in the dPAG. Activation of these neurons was assessed by counting cells co-labelled with the immediate early protein c-Fos, an indicator of neuron activation in the dPAG (Fig. 2a). IF results revealed that the number of co-labelled neurons in the dPAG of PMDD mice was significantly lower than that in control mice. However, following treatment with Shuyu capsules, the number of co-labelled neurons in PMDD mice increased (Fig. 2b). This supports our hypothesis that Shuyu capsules modulate the activity of GABAergic neurons in the dPAG of PMDD mice.

3.3 Effect of specific dPAG GABAergic neuron activation on estrous cycle-dependent depression-like behavior in PMDD mice

To determine whether GABAergic neurons in the dPAG are the targets for treatment of PMDD using Shuyu capsules, a chemogenetic experiment was conducted, as depicted in Fig. 3a. Vgat-Cre female mice with regular estrous cycles were selected. To mimic the effects of Shuyu capsules on GABAergic neurons, AAV-DIO-hM3Dq-mCherry and AAV-DIO-mCherry were injected into the dPAG of PMDD and control mice, respectively (Figs. 3b and 3c). Before virus injection, the mice showing a significant difference in immobility duration between the LD and E phases were classified as PMDD mice, whereas no such difference was observed in the control mice (Fig. 3d). After the virus was completely expressed, different groups of mice were intraperitoneally injected with CNO, and the FST was repeated in the LD phase. We assessed the immobility duration in various groups of mice during the test and discovered that chemogenetically activated GABAergic neurons in the dPAG of PMDD mice reduced immobility time in the LD phase when performing the FST. However, there was no significant

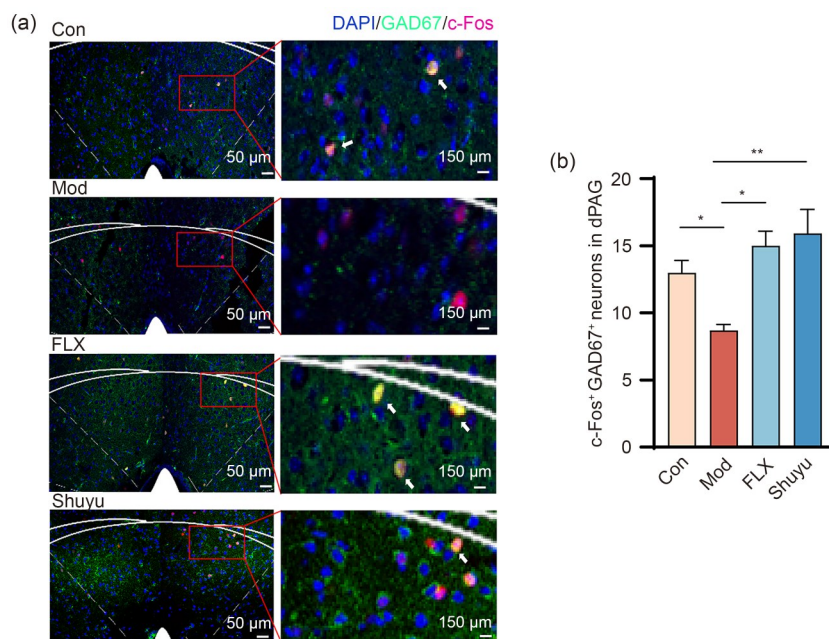


Fig. 2 Shuyu capsule can increase the number of activated γ -aminobutyric acidergic (GABAergic) neurons of dorsal periaqueductal gray (dPAG) in premenstrual dysphoric disorder (PMDD) mice. (a) Representative images of cellular Finkel-Biskis-Jenkins murine osteosarcoma viral oncogene homolog (c-Fos)-positive GABAergic neurons of dPAG obtained using immunohistochemical staining. (b) The number of GABAergic neuronal activations in the dPAG of mice in different groups. All values are expressed as mean \pm standard error of the mean (SEM). * $P < 0.05$, ** $P < 0.01$, repeated measures and one-way analysis of variance (ANOVA) with Bonferroni's test ($n \geq 3$ per group). GAD67: glutamate decarboxylase 67; DAPI: 4',6-diamidino-2-phenylindole dihydrochloride; Con: control; Mod: saline; FLX: fluoxetine; Shuyu: Shuyu capsule.

effect on the depression-like behavior of PMDD mice injected with the control virus (Fig. 3e). Similarly, activating GABAergic neurons in the dPAG of control mice did not significantly alter their behavior (Fig. 3e). These findings suggest that activating GABAergic neurons in the dPAG is sufficient to ameliorate the estrous cycle-dependent depression-like behavior in PMDD mice. Shuyu capsules could improve these depression-like symptoms by targeting the specific neurons.

3.4 Effect of Shuyu capsules on the expression of GABRD of dPAG in PMDD mice

GABA_ARs have heterogeneous pentameric structures composed of two α subunits, two β subunits, and a γ or δ subunit. Those containing the δ subunit are mostly located in the extrasynaptic space and are known for their high conductivity, facilitating large conductance and managing substantial inhibitory currents (Koh et al., 2023). They are highly sensitive to GABA (Lovick et al., 2005; Möhler, 2006). ALLO, closely associated with the pathogenesis of PMDD, acts as a positive-allosteric modulator of GABA at GABA_ARs (Pinna et al., 2000). It promotes the desensitization of GABA_ARs,

enhancing neurogenesis and producing anti-depression and anti-anxiety effects (Puia et al., 2003; Carver and Reddy, 2013). It has been observed that GABA_ARs influence estrous cycle-dependent brain activity, with δ subunit-containing GABA_ARs mediating tonic inhibitory currents over three times stronger than phase inhibitory currents (Mody and Pearce, 2004). The δ subunit expression of GABA_AR has been shown to correlate directly with progesterone levels (Griffiths and Lovick, 2005). Research indicates that GABA_ARs containing δ subunits are associated with anxiety and stress responses (Rudolph et al., 2020; Zhang et al., 2023). Additionally, our previous study confirmed that Shuyu capsules alter the expression of GABA_AR subunits in the PAG of PMDD mice (Geng et al., 2024). Based on these findings, we hypothesize that the GABRD in the dPAG may play a role in how Shuyu capsules alleviate depression-like behaviors in PMDD.

The $\alpha 4$ subunit is sensitive to progesterone withdrawal (Gulinello et al., 2002), while the plasticity of the $\beta 1$ subunit is influenced by benzodiazepines, commonly used to treat anxiety disorders (Holt et al., 1996). To investigate this, WB was employed to measure the

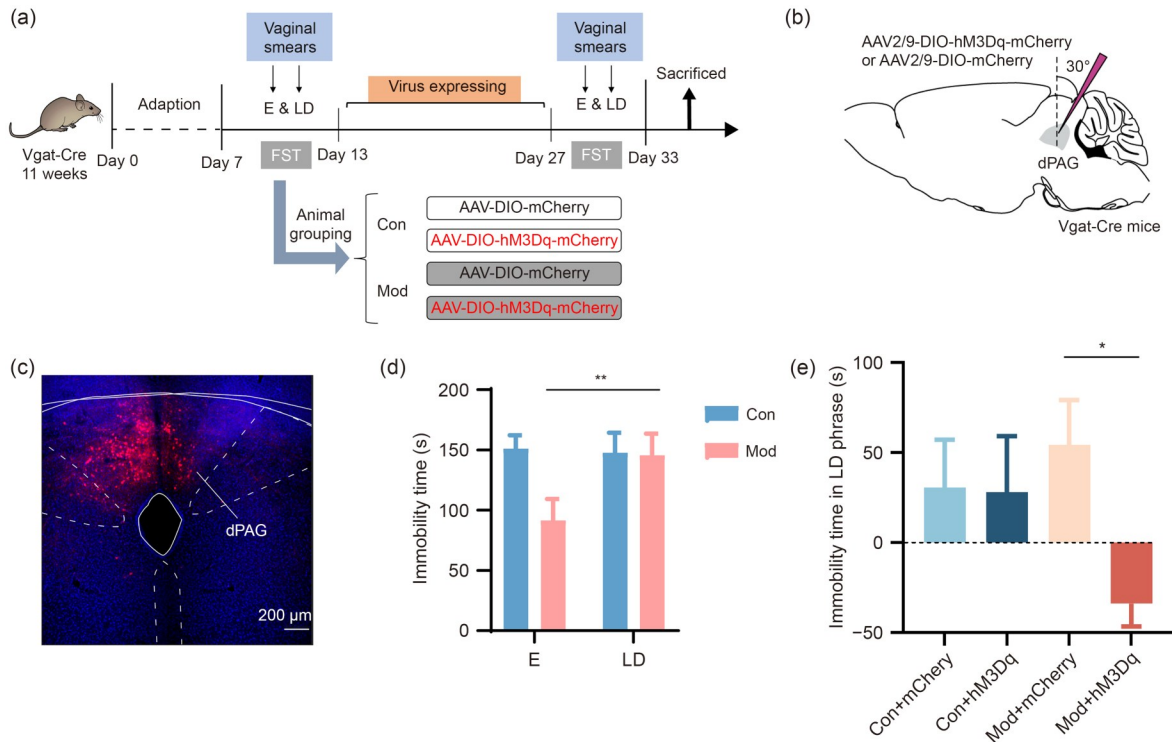


Fig. 3 Chemogenetically specific activation of γ -aminobutyric acidergic (GABAergic) neurons in the dorsal periaqueductal gray (dPAG) can effectively improve depression-like behavior in premenstrual dysphoric disorder (PMDD) mice. (a) Design diagram of the experimental process. (b) Schematic diagram of virus injection and information on viruses. (c) Representative fluorescence image of the virus, which is expressed in the dPAG. (d) Immobility duration was detected via the forced swimming test (FST) in the estrus (E) and late dioestrus (LD) phases of Vgat-Cre mice before virus injection. (e) Efficacy of the activated GABAergic neurons in different groups of mice was evaluated according to the immobility duration in the FST in the LD phase after the virus was fully expressed. All values are expressed as mean \pm standard error of the mean (SEM). * $P < 0.05$, ** $P < 0.01$, repeated measures and two-way analysis of variance (ANOVA) with Bonferroni's test ($n \geq 5$ per group). Con: control; Mod: saline; AAV: adeno-associated virus.

expression of GABA_AR containing $\alpha 4$ (Fig. 4a), $\beta 1$ (Fig. 4b), and δ (Fig. 4c) subunits in the dPAG of PMDD mice treated with Shuyu capsules. WB results indicated a significant increase in the δ subunit in PMDD mice compared to controls, with a decrease post-Shuyu capsule intervention (Fig. 4f). However, the expression levels of $\alpha 4$ (Fig. 4d) and $\beta 1$ (Fig. 4e) subunits showed no significant differences across the groups. To further confirm the expression and distribution of the δ subunit in the dPAG, we performed IF staining of the GABRD protein in the dPAG (Fig. 4g). The IF results were consistent with those from the WB. Compared to the control mice, PMDD mice exhibited a significant increase in labelled GABRD in the dPAG, which decreased following treatment with Shuyu capsules (Fig. 4h). These findings confirm that Shuyu capsules influence GABRD expression in the dPAG of PMDD mice.

3.5 Effect of specific knockdown of GABRD in the dPAG on the estrous cycle-dependent depression-like behavior of PMDD mice

Considering the critical role of GABA_ARs in the central nervous system, we hypothesized that the anti-PMDD effects of Shuyu capsules might be linked to the expression of GABA_ARs in the dPAG. To test this theory, we used AAV to knock down the expression of GABRD in the dPAG, simulating the effects of Shuyu capsules on PMDD mice and examining whether reduced δ subunit expression could improve estrous cycle-dependent depression-like behavior (Fig. 5a). We injected AAV-U6-shRNA (GABRD)-CMV-EGFP and AAV-U6-shRNA (scramble)-CMV-EGFP into the dPAG of mice in the different groups (Fig. 5b). After full expression of the viruses, tissue from the dPAG was collected for WB analysis to confirm the knockdown efficiency (Fig. S2). After confirming the efficacy of the

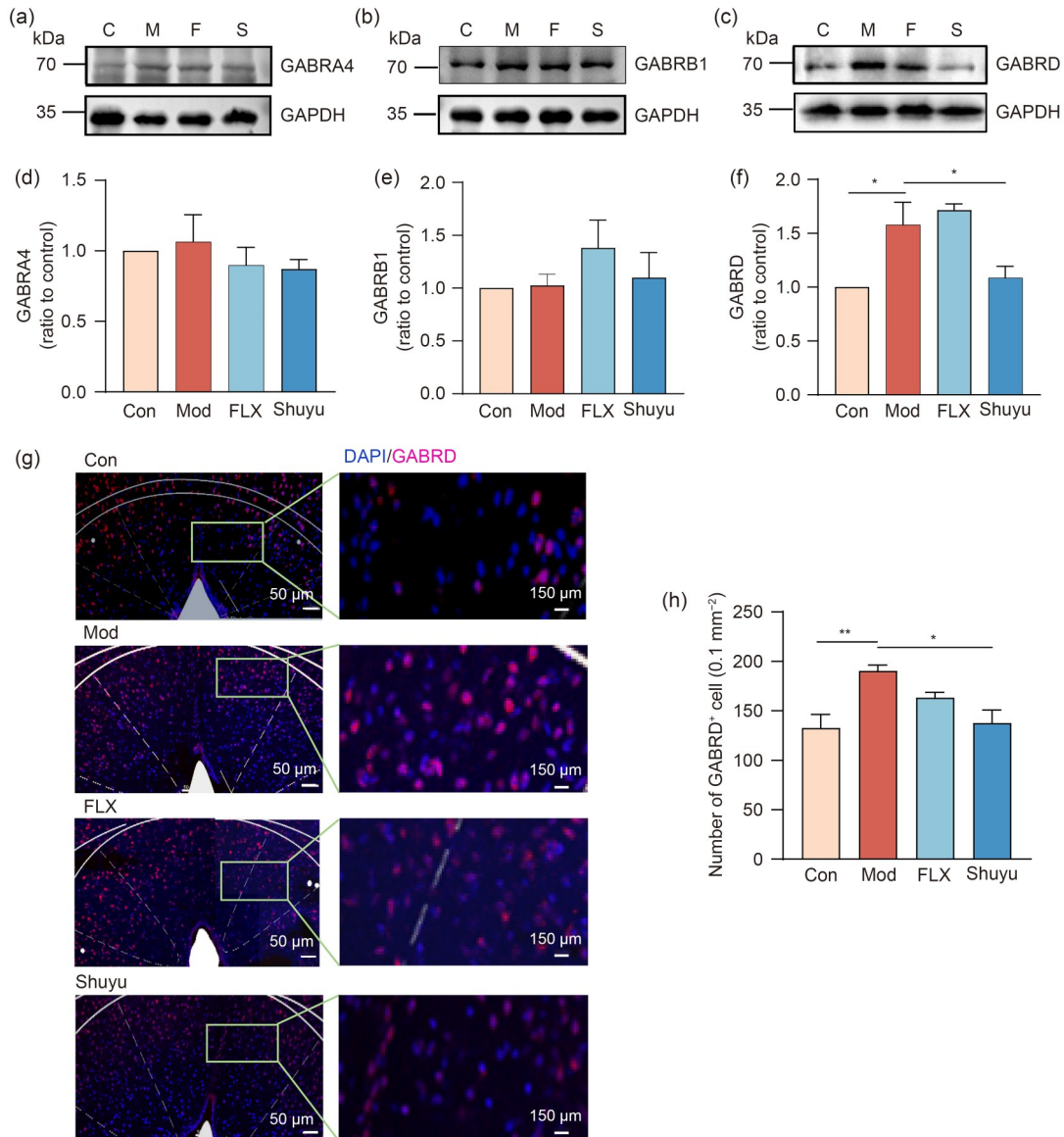


Fig. 4 Shuyu capsule can reduce the expression of the γ -aminobutyric acid type A receptor (GABA_AR) δ subunit (GABRD) of dorsal periaqueductal gray (dPAG) in premenstrual dysphoric disorder (PMDD) mice. (a–c) Representative images of a western blot (WB) experiment showing Shuyu capsule's effect on the protein levels of GABA_AR α 4 (GABRA4) (a), GABA_AR β 1 (GABRB1) (b), and GABRD (c) of the dPAG in PMDD mice. (d–f) Results of the WB experiment showing the protein levels of GABRA4 (d), GABRB1 (e), and GABRD (f) of the dPAG. (g) Representative images of the immunofluorescence (IF) experiment showing the effect of Shuyu capsule on the GABRD-positive cells of the dPAG in PMDD mice. (h) Results of the IF experiment showing the GABRD-positive cells in the dPAG. All values are expressed as mean \pm standard error of the mean (SEM). * $P < 0.05$, ** $P < 0.01$, repeated measures and one-way analysis of variance (ANOVA) with Bonferroni's test ($n \geq 3$ per group). DAPI: 4',6-diamidino-2-phenylindole dihydrochloride; Con (C): control; Mod (M): saline; FLX (F): fluoxetine; Shuyu (S): Shuyu capsule.

virus, PMDD mice in FST with longer immobility durations in the LD phase compared to the E phase were identified using the FST (Fig. 5c). Then, PMDD mice received injections of the two types of virus into the dPAG. Following full viral expression, they underwent FST in both the E and LD phases. The FST results

indicated that the knockdown of the GABRD significantly reduced the immobility duration difference between the model mice's LD and E phases (Fig. 5d).

The mice showing no difference in immobility duration between the two phases in the FST were screened as a control group (Fig. 5e). In contrast, the knockdown

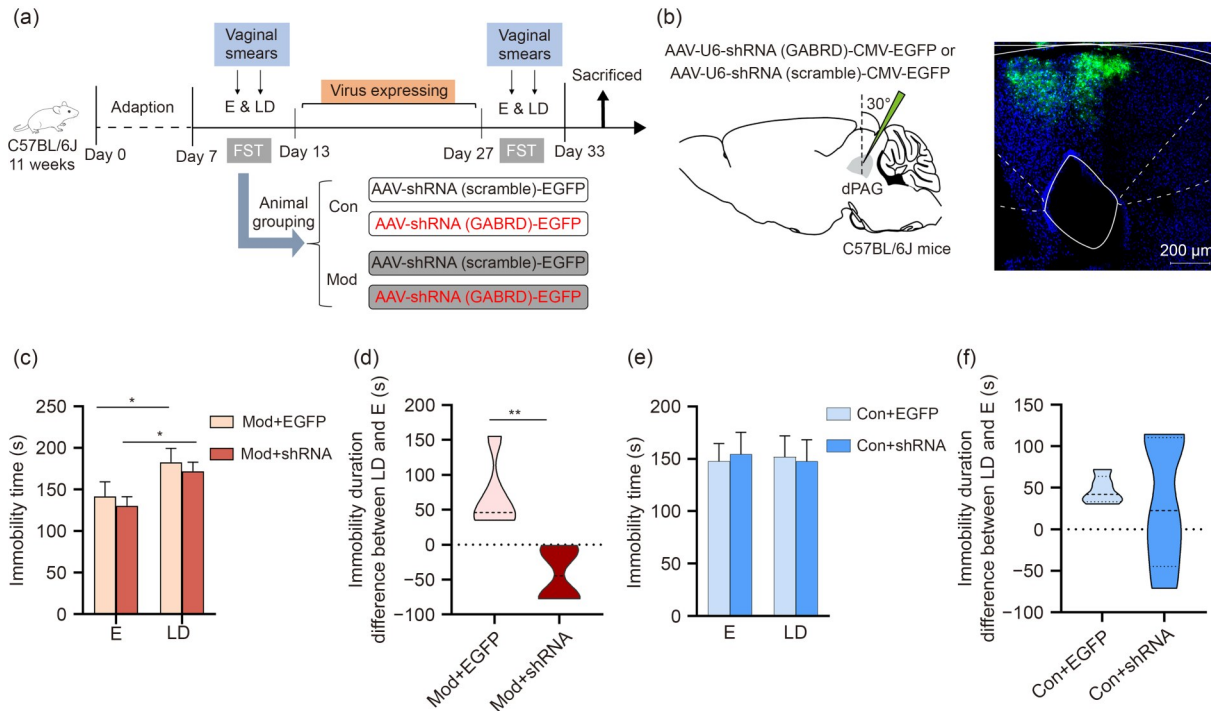


Fig. 5 Adeno-associated virus-specific knockdown of the expression of γ -aminobutyric acid type A receptor ($GABA_A$ R) δ subunit (GABRD) in dorsal periaqueductal gray (dPAG), which can effectively improve the depression-like behavior of premenstrual dysphoric disorder (PMDD) mice. (a) Design diagram of the experimental process. (b) Left: schematic diagram of virus injection and information on viruses. Right: representative fluorescence image of the virus, which is expressed in the dPAG. (c) Immobility duration of PMDD mice was detected via the forced swimming test (FST) in the estrus (E) and late dioestrus (LD) phases of *Vgat-Cre* mice before virus injection. (d) Immobility duration of PMDD mice was detected via the FST in the E and LD phases of *Vgat-Cre* mice after the virus was fully expressed. (e) Immobility duration of control mice was detected via the FST in the E and LD phases of *Vgat-Cre* mice before virus injection. (f) Immobility duration of control mice was detected via the FST in the E and LD phases of *Vgat-Cre* mice after the virus was fully expressed. All values are expressed as mean \pm standard error of the mean (SEM). * $P < 0.05$, ** $P < 0.01$, repeated measures and two-way/one-way analysis of variance (ANOVA) with Bonferroni's test ($n \geq 5$ per group). AAV: adeno-associated virus; shRNA: short hairpin RNA; EGFP: enhanced green fluorescent protein; Con: control; Mod: saline; CMV: cytomegalovirus promoter.

virus did not affect the phenotype of the control mice (Fig. 5f). The experimental manipulation of GABRD demonstrated that the effects of the Shuyu capsule on PMDD could be replicated. Specifically, downregulating the expression of GABRD in the dPAG of PMDD mice led to improvements in estrous cycle-dependent depression-like behavior.

3.6 Systemic knockout of GABRD triggering behavioral changes in mice

To gain insight into the contribution of the GABRD to behavioral change in mice, we constructed transgenic mice with systemic GABRD knockout. The *Gabra*^{+/+} (wild-type, WT) and *Gabra*^{-/-} (homozygous, HO) mice were selected and underwent FST, but we found that there was no significant difference in immobility duration (Fig. S3a) or number (Fig. S3b) between the two

groups. To expand the detection surface of mouse behaviors, two genotypes of mice underwent a home cage behavior test lasting 24 h, and then we compared more than 30 behaviors between the two genotypes of mice (Fig. S3c). The results of behavior detection showed that there were significant differences in the “come down” behavior between the two groups (Fig. S3d), and notably, there was also a different trend in “rear up from partial” (Fig. S3e).

4 Discussion

PMDD, a serious premenstrual disease in women of childbearing age, significantly affects the physical and mental health of patients. Although FLX, a type of SSRI, is commonly used to treat PMDD, it has been

associated with various side effects, such as nausea, insomnia, headache, cardiovascular dysfunction, and reproductive system dysfunction (Klatzkin et al., 2006; Spindelegger et al., 2015). Therefore, there is a pressing need to develop a treatment for PMDD that is not only effective but also associated with minimal side effects.

Traditional Chinese medicine, known for its stable and significant therapeutic effects, is now widely accepted. One such compound, the Shuyu capsule, is commonly used in clinics for treating PMDD and contains Radix Bupleuri, Radix Paeoniae Alba, Rhizoma Cyperi, and Radix Glycyrrhizae (Table S2). Studies have shown that the Shuyu capsule has a remarkable curative effect on various depression-like mood disorders (Li et al., 2016; Wang et al., 2018; Zhang et al., 2023). However, the underlying therapeutic mechanism remains unclear.

In our study, we found that the Shuyu capsule intervention had a therapeutic effect on estrous cycle-dependent depression-like behavior in PMDD mice. We used multiple neurobiological technologies to demonstrate that the Shuyu capsule affects the GABAergic system, activating GABAergic neurons and decreasing the expression of GABRD in the dPAG to improve depression-like behavior in PMDD mice.

4.1 Shuyu capsule treatment improving estrous cycle-dependent depression-like behavior in PMDD mice

Mice were selected for PMDD model screening to improve mechanism verification. The FST was chosen as the screening paradigm because female rats exhibit estrous cycle-dependent depression-like behavior during the test (Marván et al., 1997) and because of its favorable predictive validity. In the LD phase, the immobility duration of PMDD mice was significantly increased compared to that of the control group, while there was no difference in the E phase (Marván et al., 1997; Wei et al., 2020). Shuyu treatment improved depression-like behavior in PMDD mice during the LD phase (Fig. 1). These results demonstrate that Shuyu capsule intervention can alleviate estrous cycle-dependent depression-like behavior in PMDD mouse models.

4.2 Shuyu capsule treatment modulating dPAG GABAergic neuron activity in PMDD

Both physical and emotional symptoms accompany PMDD. The PAG is a brain region involved in

pain perception (Campion et al., 2016; Chung et al., 2020) and negative emotional and behavioral regulation (Devall et al., 2009; Lowery-Gionta et al., 2018). The Shuyu capsule has been confirmed to regulate the sensitivity of the PAG in PMDD rats (Geng et al., 2024). It has also been demonstrated that both FLX and Shuyu capsules can affect PAG activity in rats (Devall et al., 2015; Geng et al., 2024). Based on these findings, to determine whether the Shuyu capsule intervention in PMDD mice affects the activity of GABAergic neurons in the dPAG and whether specific activation of this target can play a role in treating PMDD, IF staining was used to measure the number of activated neurons in the dPAG. We found significantly more activated neurons in the dPAG of PMDD mice than in that of the control mice. The Shuyu intervention could effectively reverse this downward trend (Fig. 2). The outcomes of our study align with those by Devall et al. (2015), who discovered that rats subjected to stress exhibited fewer cells labelled with LD during the estrous cycle.

Additionally, stressed rats showed a significant increase in c-Fos-positive cells in the PAG compared to saline-treated rats. However, our work differs from the study by Devall et al. (2015), in which we screened the phenotypes of the experimental mice while their subjects were normal rats. This allows us to maintain a higher level of clinical consistency and face validity in our model, as not all women are PMDD patients. In addition, Devall et al. (2015) did not identify the type of neurons in the PAG and did not conduct further functional verification research. To better understand Shuyu's treatment of PMDD, we used chemogenetic methods to manipulate the activity of GABAergic neurons in the dPAG. Our results showed that the activation of GABAergic neurons in the dPAG was able to improve depression-like behaviors in PMDD mice (Fig. 3). These results suggest that the Shuyu capsule exerts a therapeutic effect on PMDD mice by activating GABAergic neurons in the dPAG.

4.3 Shuyu capsule treatment ameliorating PMDD by downregulating the expression of GABRD in dPAG

The ALLO is considered a positive-allosteric modulator of the action of GABA at GABA_ARs (Pinna et al., 2000); the fluctuation of ALLO changes the expression of GABA_AR subunits, especially GABA_AR with δ subunit, which is highly sensitive to GABA and

is located outside of the synapse (Möhler, 2006). In our previous study, we used WB and immunohistochemistry techniques to demonstrate that Shuyu capsule intervention in PMDD rats impacted the expression of GABA_AR subunits in the PAG (Geng et al., 2024). To determine whether this change also occurred in mice, we conducted a WB experiment to measure the expression of GABA_AR subunits in dPAG. We observed that GABRD expression increased in the dPAG of the model mice, but this alteration was reversed after administering Shuyu capsules (Fig. 4f). To further confirm this conclusion, IF was used to stain the GABRD in the dPAG of mice. Consistent with the previous conclusions, the Shuyu capsule intervention could reverse the increased trend of GABRD in the dPAG of model mice (Fig. 4h). To prove that the GABRD is the target of the Shuyu capsule in treating PMDD, we knocked down the expression of GABRD in the dPAG of the model mice. We found that the depression-like phenotype of PMDD mice was improved (Fig. 5). These results substantiate the hypothesis that the GABRD of the dPAG is the target of the Shuyu capsule in treating PMDD.

4.4 Potential role of GABRD in anxiety-like behaviors in mice

Many studies have shown that ovarian hormone fluctuation and GABRD expression affect rodents' behavioral phenotype. Since we constructed a knock-out mouse, which systemically knocks out the *Gabrd* gene in mice, we performed FST experiments to verify whether the GABRD affects mice's estrous cycle-dependent depression-like behaviors. Surprisingly, there was no difference in behavior between the mice in the FST (Fig. S3a). This is different from the effect of GABRD knockdown in dPAG on depression-like behavior in PMDD mice mentioned above. It may be because the effects of the systemic knockout of the *Gabrd* gene on mice's behaviors are different from those of the reduced expression of GABRD in emotion-related brain regions. We speculate that this paradigm may not be suitable for identifying estrous cycle-dependent behavior changes in *Gabrd*^{-/-} mice.

One study found that female rats in the acceptance period are less anxious compared to when they are in the nonacceptance period (Lovick et al., 2005). Another study verified that ovarian cycle-linked changes in GABA_ARs mediate tonic inhibition, altering seizure

susceptibility and anxiety (Mody and Pearce, 2004). Additionally, another study suggested that *Gabrd*^{+/-} and *Gabrd*^{-/-} mice constitute a mouse model of postpartum depression (Maguire and Mody, 2008). Ovarian hormone modulation of GABA_ARs is thought to play a role in depression, anxiety, and mood disorders. The connection between PMDD and postpartum depression and the rapid decline of ovarian hormones suggests that these two diseases share common pathogenic mechanisms (Noble, 2005). Studies indicate that phenotypic changes may accompany ovarian hormones' effect on the GABRD, and further investigation with alternative paradigms is necessary. We decided to conduct a 24-h home cage behavior test to record and analyze behavioral changes in mice. We found differences in the "come down," "rear up from partial," "remain rear up," and "come down to partially reared" behaviors between the two groups (Fig. S3). These behaviors are exploration-related behaviors of mice (Roughan et al., 2009). Our results align with those of a study investigating behavioral changes in female C57BL/6J mice, which found that subtle shifts in the exploratory behavior of each mouse were related to their estrous phase (Levy et al., 2023). Exploratory behaviors in mice are often associated with curiosity (Berlyne, 1966; Hsiung et al., 2023). Tools like the open field and elevated plus maze are used to evaluate anxiety-like behaviors in rodents by measuring the animal's curiosity and psychological states of anxiety and fear (Walz et al., 2016). In addition, another study using muscimol (a GABA_AR agonist) in mice showed that agitating GABA_AR could improve their anxiety-like behavior in elevated cross-maze experiments (Rodgers and Dalvi, 1997). The above results suggest that GABA_AR expression or activity may be closely related to anxiety-like and depression-like behaviors in rodents.

5 Conclusions

We proposed that the Shuyu capsule reduced the expression of GABRD in the GABAergic interneurons of the dPAG, which increased their activity. This led to a decrease in the excitability of vertebral neurons, resulting in a therapeutic effect. This effect is caused by the enhanced inhibitory effect of GABAergic interneurons on pyramidal neurons and the decreased excitability in brain regions (Fig. 6).

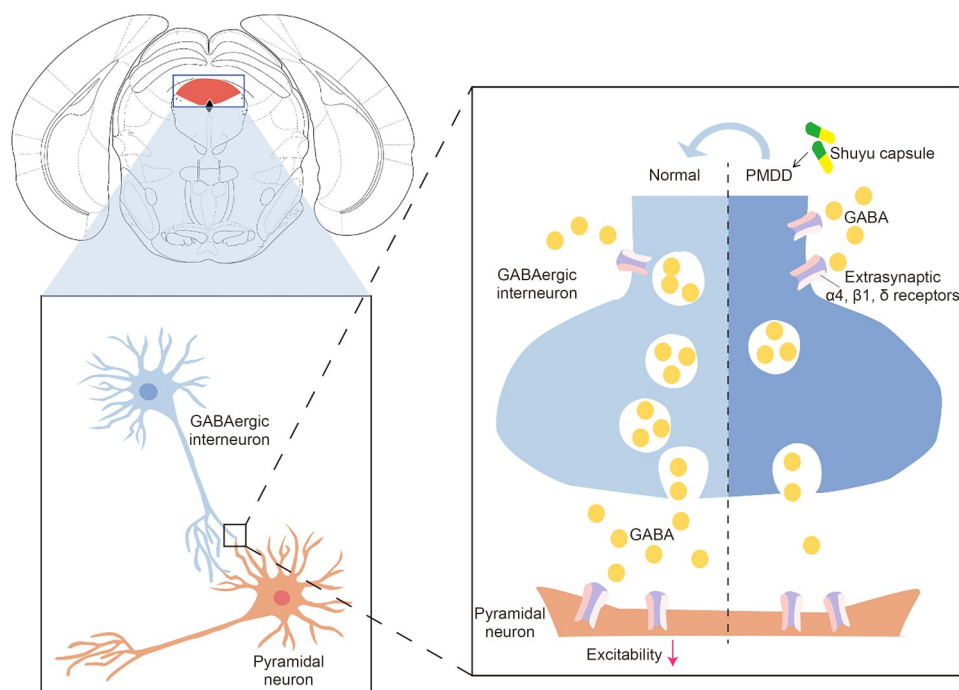


Fig. 6 Mechanism diagram illustrating that Shuyu capsule exerts a therapeutic effect on the γ -aminobutyric acidergic (GABAergic) system of the dorsal periaqueductal gray (dPAG) in premenstrual dysphoric disorder (PMDD) mice.

In conclusion, our study's outcomes clarified the potential mechanism through which Shuyu capsules may improve estrous cycle-dependent depression-like behavior in PMDD mice. Nonetheless, our research has the following limitations. (1) Although the Shuyu capsule's intervention on GABRD is suspected to be on GABAergic interneurons, this has not been experimentally validated. To confirm this, future research should include colocalization staining of GABAergic neurons and GABRD in the dPAG. (2) The therapeutic effects of the Shuyu capsule through the δ subunit-containing GABA_AR remain unclear. Our study aims to clarify the link between the chloride ion channel current in the GABRD and the intervention of the Shuyu capsule using the patch clamp technique. (3) To better comprehend the central nervous system's role in PMDD, we must investigate the upstream and downstream brain regions of GABAergic neurons in the dPAG and manipulate these circuits to uncover the underlying mechanism of PMDD. (4) Our research focuses only on neurons, which is limited to mental disease research. Reactive astrocytes have been associated with increased GABA synthesis, leading to enhanced tonic inhibition in affected brain regions, which can contribute to the onset of various disorders (Koh and Lee, 2024). It would be valuable to continue investigating the effect

of Shuyu capsule on the expression of astrocytes in the treatment of PMDD, as well as the relationship between reactive astrocytes and GABRD in the pathogenesis of PMDD.

Our study found that Shuyu capsules can improve estrous cycle-dependent depression-like behavior in PMDD mice by activating GABAergic interneurons and downregulating the expression of GABRD in the dPAG. Using mice as experimental objects, we further verified the study results on rats, thereby providing more evidence to support the pharmacodynamic mechanism of Shuyu capsules in treating PMDD. Our findings, which are based on the common pathological mechanism underlying postpartum depression, menstrual epilepsy, menopausal syndrome, and PMDD, could offer insights into the pathology and drug intervention mechanisms of these diseases. In addition, previous research has identified valuable monomers in drug components, which can further enrich the explanation of the mechanism underlying the Shuyu capsule's efficacy in treating PMDD. Since the Shuyu capsule has already been approved for clinical use, conducting extensive clinical trials to obtain sufficient treatment evidence may provide greater hope for PMDD patients and future drug development.

Data availability statement

The datasets used or analyzed during the current study are available from the corresponding author upon reasonable request.

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Author contributions

Jialing XU, Xiwen GENG, and Sheng WEI designed and led the project. Jialing XU, Kun LIU, and Yaru CUI conducted the literature research and data curation. Jialing XU, Minghui HU, and Hao ZHANG performed statistical analysis of the behavioral results. Xinyu WANG, Peng GAO, Wei LIU, and Wenqiang CUI gave constructive comments on the revision of the manuscript. Zifa LI, Mingqi QIAO, and Sheng WEI supervised the whole experimental process. Jialing XU prepared the manuscript. Wenqiang CUI, Xiwen GENG, and Sheng WEI assisted with the revision of the article. All authors have read and approved the final version of the manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Jialing XU, Kun LIU, Yaru CUI, Hao ZHANG, Xinyu WANG, Minghui HU, Zifa LI, Peng GAO, Wei LIU, Mingqi QIAO, Wenqiang CUI, Xiwen GENG, and Sheng WEI declare that they have no conflicts of interest.

All mice used in this study were housed and maintained at Behavioral Phenotyping (Chinese Medicine and Brain Science) Core Facility, Shandong University of Traditional Chinese Medicine (laboratory animal use license number: SYXK(Lu) 20220009). All experiments followed the guidelines outlined in the Guide for the Care and Use of Laboratory Animals, approved by the National Research Council (US) Committee (2011). Ethics approval for the experiments was obtained from the Ethics Review Board of Shandong University of Traditional Chinese Medicine (welfare ethics license number: SDUTCM20230211001).

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Supplementary information

Tables S1 and S2; Figs. S1–S3; Materials and methods