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Research Article

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Shuyu capsule improves oestrous cycle-dependent depressive-like behaviour in PMDD mice by increasing GABAergic neuronal activation and down-regulating $GABA_AR$ δ subunit expression in the dPAG region

Jialing XU[1,2], Kun LIU[1,2], Yaru CUI[1,2], Hao ZHANG[1,2], Xinyu WANG[1,2], Minghui HU[1,2], Zifa LI[1,2], Peng GAO[3], Wei LIU4, Mingqi QIAO[5], Wenqiang CUI[6]✉, Xiwen GENG[1,2]✉, Sheng WEI[1,2]✉

[1]*Key Laboratory of Traditional Chinese Medicine Classical Theory, Ministry of Education/Shandong Provincial Engineering Research Center for the Prevention and Treatment of Major Brain Diseases with Traditional Chinese Medicine/Jinan Key Laboratory of the Prevention and Treatment of Major Brain Diseases with Traditional Chinese Medicine, Shandong University of Traditional Chinese Medicine, Ji'nan 250355, China*
[2]*Chinese Medicine and Brain Science Interdisciplinary Research Center, Shandong University of Traditional Chinese Medicine, Ji'nan 250355, China*
[3]*Institute of Pharmaceutical Research, Shandong University of Traditional Chinese Medicine, Ji'nan 250355, China*
[4]*Department of Encephalopathy, The Second Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Ji'nan 250001, China*
[5]*College of Traditional Chinese Medicine, Shandong University of Traditional Chinese Medicine, Ji'nan 250355, China*
[6]*Department of Neurology, Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Ji'nan 250014, China*

Abstract: Background: 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 clinics, but its therapeutic mechanism remains unclear. Previous research has suggested that the gamma-aminobutyric acid (GABAergic) system in the periaqueductal gray (PAG) may play a role in treating PMDD with traditional Chinese medicine, but functional verification is lacking. Purpose: This study aims to reveal the potential mechanism of the Shuyu capsule in treating PMDD. Study design: The study employed an experimental design using female C57BL/6J and Vgat Cre mice to assess the effects of Shuyu capsules on PMDD, focusing on the GABAergic system in the dorsal periaqueductal gray (dPAG). Methods: Assessments were conducted using the Forced Swimming Test (FST) to gauge depressive-like behaviours and Western blot (WB) and immunofluorescence (IF) to measure the numbers of active GABAergic neurons and the gamma-aminobutyric acid type A receptor ($GABA_AR$) δ subunit expressions. Chemogenetic techniques and adeno-associated virus were specifically used to activate GABAergic neurons

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✉Sheng WEI, weisheng@sdutcm.edu.cn
✉Xiwen GENG, xwgeng@sdutcm.edu.cn
✉Wenqiang CUI, wqcui16@fudan.edu.cn
Jialing XU, https://orcid.org/ 0000-0001-5812-8953
Kun LIU, https://orcid.org/ 0000-0002-5258-1658
Yaru CUI, https://orcid.org/0009-008-1617-3578
Hao ZHANG, https://orcid.org/ 0000-0001-6171-6174
Xinyu WANG, https://orcid.org/0000-0001-8612-2954
Minghui HU, https://orcid.org/0000-0003-2493-1718
Zifa LI, https://orcid.org/ 0000-0002-2782-3766
Peng GAO, https://orcid.org/0000-0003-4750-1008
Wei LIU, https://orcid.org/0000-0003-0477-1497
Mingqi QIAO, https://orcid.org/0000-0001-7310-1206
Wenqiang CUI, https://orcid.org/ 0000-0003-1825-0799
Xiwen GENG, https://orcid.org/ 0000-0002-6112-7385
Sheng WEI, https://orcid.org/ 0000-0002-1326-4625

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and knock down the expression of subunits, respectively, providing insights into the neurobiological mechanisms underpinning the therapeutic effects of Shuyu capsules in treating PMDD. Results: After being stressed by forced swimming, the immobility duration of PMDD mice in the late dioestrus (LD) phase increased after the Shuyu capsule intervention, implying that it can improve the oestrous cycle-dependent depression-like phenotype in PMDD mice. Additionally, the application of Shuyu can down-regulate the expression of GABA type A receptor δ subunit (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 behaviour of PMDD model mice. Transgenic mice with GABRD knockout were established, and their behaviours were tested, revealing changes in their exploratory behaviours, indicating that the $GABA_AR$ δ subunit may be closely related to anxiety diseases. Conclusion: Shuyu capsule plays an anti-PMDD role by activating GABAergic neurons and down-regulating 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; GABAergic neuro; Gamma-Aminobutyric Acid Receptor Delta Subunit; Shuyu Capsule; Depression-like Behaviors

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. At present, 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). Recognised as a special disease, PMDD was included in the 11th edition of the International Morbidity Statistics Classification by the World Health Organization in 2019, categorised 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, 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 oestrous cycle-dependent, appear in the middle and late luteal phase 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 matter (PAG) in the midbrain is a brain region with varying activity throughout the menstrual cycle. Research also suggests that PAG stimulation in PMDD model rats with anxiety and stress in late dioestrus (LD) can cause hyperalgesia (Devall et al., 2009). Since 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 behaviours, 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.

Gamma-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 also 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 $GABA_AR$ δ subunit may be the Shuyu capsule's target in treating PMDD.

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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 also 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 $GABA_AR$ δ subunit expression and activated GABAergic neuron in the dPAG.

2 Materials and methods

2.1 Animals

All mice (11 weeks old, 20–25g) 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 light/dark cycle of 12:12 h, with ad libitum access to food and water, at Shandong University of Traditional Chinese Medicine [Laboratory animal use licence 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 Licence number: SDUTCM20230211001). Information on the strains and sources of the experimental mice is detailed in the *supplementary material*.

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 oestrous cycles were screened for formal experiments. Then, mice in their oestrus (E) and LD phases were stressed by forced swimming. In accordance with the method used by Schneider (Schneider and Popik, 2007) and Wei (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 of 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, each of which was orally administered saline (Mod group; 10 ml•kg-1•d-1), fluoxetine (FLX group, 3.738 mg•kg-1•d-1), or a Shuyu capsule (Shuyu group, 571.2 mg•kg-1•d-1) (Gao, et al., 2014). Mice in the Con group were orally administered saline at a dose of 10 ml•kg-1•d-1. Mice were gavaged at 9:00 a.m. each day continuously for 12 days. After the gavage, the behaviours 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). Fluoxetine (FLX) was purchased from Eli Lilly and Company (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 ALLO in rat serum

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

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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 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% paraformaldehyde solution (Beyotime, P0099). After a few minutes, the slides were taken out and washed with pure water, 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 forced swimming test was conducted every morning in an environment with red light illumination, and the experimental mice adapted to the environment for 30 minutes 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 range of 23 ± 1 ℃. Mice were allowed to swim freely for 6 minutes. 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 washed.

2.7 Location of the dorsal periaqueductal grey matter (dPAG) and virus injection

The methods for locating the dPAG and injecting the virus were similar to those described in our previous work (Geng, et al., 2024). We marked the standard surgery dPAG site (AP: -4.36mm; ML: ±0.3mm; DV: -2mm) 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: -4.36mm; ML: ±1.08mm; DV: -2.4mm). Details about the virus are as follows: AAV-hSyn-DIO-mCherry (Brain VTA, PT-0115), AAV-hSyn-DIO-hM3D(Gq)-mCherry (Brain VTA, PT-0019), AAV-U6-shRNA(scramble)-CMV-EGFP-pA (Brain VTA, PT-0916), AAV-U6-shRNA (Gabard)-CMV-EGFP-pA (Brain VTA, PT-0360. See Table S1 for details of ShRNA sequence information.).

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2.8 Intraperitoneal injection of CNO

After two weeks of virus expression, Clozapine N-oxide (CNO, ApexBio, A3317) powder, after full centrifugation, was dissolved in a saline solution containing 5% DMSO (Aladdin, D2107139). After determining the oestrous cycle phase, mice were intraperitoneally injected with CNO (5 mg/kg) (Hu et al., 2022) in the E and LD phases, respectively, and the forced swimming test was performed 1 hour later.

2.9 Western blot

The methods for locating the dPAG and injecting the virus were similar to those described in our previous work (Geng, et al., 2024). The primary and secondary antibody information is as follows: $GABA_AR$ α4 (Immunoway, YT1822, 1:1000), GAPDH (Proteintech, 60004-1-Ig, 1:5000), $GABA_AR$ β1 (ThermoFisher, PA5-116439, 1:1000), $GABA_AR$ δ (ThermoFisher, PA5-101598, 1:1000), HRP Goat Anti-Rabbit IgG (H+L) (ABclonal, 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 1X citrate antigen retrieval solution (Beyotime, P0081) and heated at 95℃ for 5–10 min. They were then washed and blocked using phosphate buffer saline (PBS), which contains 5% goat serum and 1% tritonX-100 (Solarbio, T8200), at room temperature for 2 hours. 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 DAPI (Solarbio, S2110) and then immediately observed and imaged under a fluorescent microscope. The primary and secondary antibody information is as follows: GAD 67 (Millipore, MAB5406, 1:200), c-Fos (Abcam, ab208942, 1:1000), Goat Anti-Rabbit IgG H&L Alexa Fluor® 647 (Abcam, ab150083, 1:500), Goat Anti-Mouse IgG H&L Alexa Fluor® 488 (Abcam, ab150113, 1:1000).

2.11 Homecage behaviors test

Using the method referenced by Kyzar et al. (Kyzar et al., 2012), we identified the behaviours 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 hours. 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 analysed using HomeCage Scan software. The activity data of the mice were expressed as the percentage of time they spent engaging in the behaviours.

2.12 Data analysis

All values are presented as mean ± standard error of the mean (S.E.M) for the data on the serum level of ALLO, the results of the Western blot analysis for the virus efficiency verification, and the homecage behaviours test. An unpaired two-tailed Student's t-test was applied for two-group comparisons. For behavioural test data, all values are presented as mean ± S.E.M. A two-way repeated measures ANOVA was performed, and a Bonferroni comparisons test was used for data detection and comparison. For the IF staining and Western blot analysis of the $GABA_AR$ subunit expression data, all values are presented as mean ± S.E.M. A repeated measures 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.

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3 Results

3.1 Shuyu capsules alleviated oestrus cycle-dependent depression-like behaviour in PMDD mice

We used mice with natural oestrous cycles to simulate the fluctuation of gonadal hormones in a normal menstrual cycle. The stability and regularity of the oestrous cycles of the experimental mice were confirmed, and then the mice were subjected to model screening, grouping, drug intervention, and tissue sampling (Fig. 1a). Since the oestrous cycle of mice typically lasts 4–6 days (Erika and Lopez-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 keratinised (indicated by the indigo arrow). During oestrus (E), these cells evolved into nuclear-free keratinocytes (indicated by the white arrow). Leukocytes predominate by the early dioestrus (ED) phase (indicated by the yellow arrow). The pathological phenotype of PMDD manifests in the LD phase, characterised 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 phase (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 oestrus (E) phase

than in the LD phase (Fig. 1c), mirroring typical female hormonal patterns.

Exposure to stressful environments in animals leads to behaviours indicative of severe depression. Marvan and his colleagues (Marvan et al., 1997) demonstrated that rats exhibit significantly longer immobility times 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 of mice—the LD phase was selected as the non-receptive phase and the E phase as the receptive phase (Wei, et al., 2020). Forced swimming was used to elicit depression-like behaviours in mice during the LD phases. Mice in the PMDD group displayed significantly longer immobility times in the LD phase compared to the E phase, with no notable difference from the control group mice (Fig. 1d). Mice in the model group received treatments of saline (Mod), fluoxetine (FLX), and Shuyu capsule (Shuyu), while the control (Con) group received 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 times 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 times in the experiment. Overall, these results suggest that Shuyu capsules effectively mitigate depression-like behaviour in PMDD mice.

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Fig. 1 Shuyu capsule can improve the depression-like behaviour in the LD phase of PMDD mice. (a) Design diagram of the experimental process. (b) Representative images of vaginal smears of mice at proestrus (P), oestrous (E), early dioestrus (ED) and late dioestrus (LD). Scale bars, 500 μm. (c) The concentration of ALLO in the mouse serum. All values are expressed as mean ± S.E.M, *** p <0.001, unpaired two-tailed Student's *t*-test. (d) The immobility duration was detected via the FST in the E and LD phases before the Shuyu intervention. (e) The 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±standard error of the mean (SEM), $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$, two-way ANOVA compared to baseline, with Bonferroni's test ($n\geq6$ per group).

3.2 Shuyu capsules increased the number of activated GABAergic neurons in dPAG in 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), studies, including one by Devall (Griffiths and Lovick, 2005) and colleagues, have shown that the neurons of the dPAG may play a potential role in the effect of antidepressants. Our previous research also 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 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 gene 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 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.

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Fig. 2 Shuyu capsule can increase the number of activated GABAergic neurons of dPAG in PMDD mice. (a) Representative images of c-Fos positive GABAergic neurons of dPAG obtained using immunohistochemical staining. Scale bars, 50 μm. (b) The numberof GABAergic neuronal activation in dPAG of mice in different groups. All values are expressed as mean ± S.E.M, * p <0.05, ** p <0.01, repeated measures, and one-way ANOVA with Bonferroni's test (n≥3 per group).

3.3 Specific activation of GABAergic neurons in dPAG can significantly improve the oestrous cycle-dependent depression-like behaviour of PMDD mice

To determine whether GABAergic neurons in the dPAG are the target for treatment of PMDD using Shuyu capsules, a chemogenetic experiment was conducted, as depicted in Fig. 3a. Vgat Cre female mice with regular oestrous 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 divided into PMDD mice, with no difference observed in control mice (Fig. 3d). After the virus was completely expressed, different groups of mice were intraperitoneally injected with Clozapine N-oxide (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 times in the LD phase when performing the FST. However, there was no significant effect on the depression-like behaviour 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 behaviour (Fig. 3e). These findings suggest that activating GABAergic neurons in the dPAG is sufficient to ameliorate the oestrous cycle-dependent depression-like behaviour in PMDD mice. Shuyu capsules likely improve these depression-like symptoms by targeting these specific neurons.

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Fig. 3. Chemogenetically specific activation of GABAergic neurons in the dPAG can effectively improve depression-like behaviour in PMDD mice. (a) Design diagram of the experimental process. (b) Schematic diagram of virus injection and information on viruses. (c) Representative images of fluorescence of the virus, which is expressed in the dPAG. Scale bars, 200 μm. (d) The immobility duration was detected via the FST in the E and LD phases of Vgat mice before virus injection. (e) The efficacy of the activated GABAergic neurons in different groups of mice evaluated according to the immobility duration in the FST in LD phases after the virus was fully expressed. All values are

expressed as mean ±S.E.M, * p<0.05, ** p <0.01, repeated measures, two-way ANOVA with Bonferroni's test (n≥5 per group).

3.4 Shuyu capsules reduced the expression of $GABA_AR$ δ subunit of dPAG in PMDD mice

$GABA_ARs$ are 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 also highly sensitive to GABA (Lovick et al., 2005; Mohler, 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 desensitisation 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 oestrous 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 hypothesise that the $GABA_AR$ δ subunit in the dPAG may play a role in how Shuyu capsules alleviate depression-like behaviours in PMDD.

The α4 subunit is sensitive to progesterone withdrawal (Gulinello et al., 2002), while the plasticity of the β1 subunit is influenced by benzodiazepines, commonly used to treat anxiety disorders (Holt et al., 1996). To investigate this, WB was employed to measure the expression of $GABA_AR$ containing α4 (Fig. 4a), β1 (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 α4 (Fig. 4d) and β1 (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 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.

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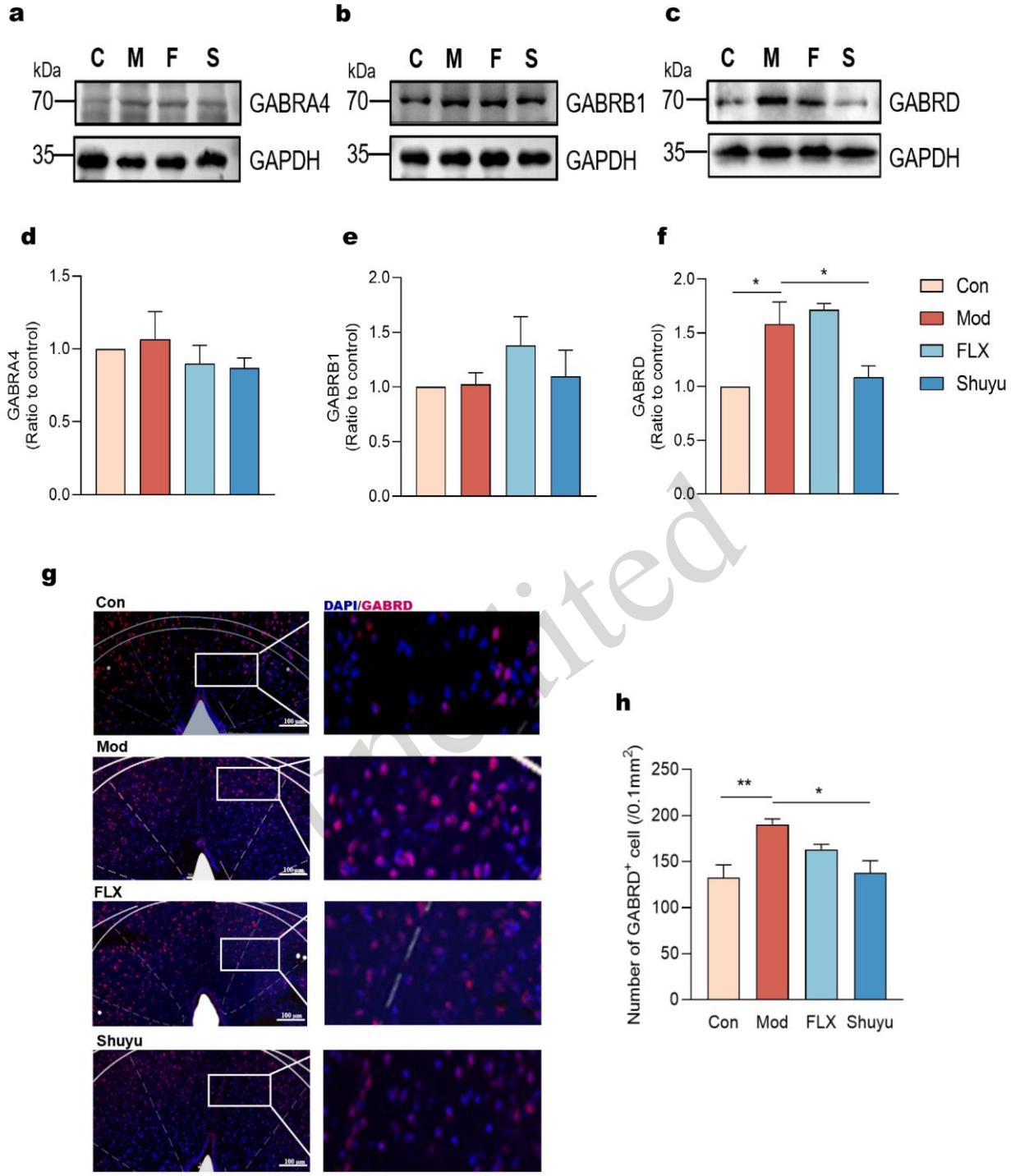

Fig. 4 Shuyu capsule can reduce the expression of the GABA$_A$R δ subunit of dPAG in PMDD mice. (a-c) Representative images of WB experiment showing Shuyu capsule's effect on the protein levels of GABA$_A$R α4 (a), GABA$_A$R β1 (b), and GABA$_A$R δ (c) of the dPAG in PMDD mice. (d–f) The results of the WB experiment showing the protein levels of GABA$_A$R α4 (d), GABA$_A$R β1 (e), and GABA$_A$R δ (f) of the dPAG (Gg). Representative images of the IF experiment showed the effect of Shuyu capsule on the GABRD-positive cells of the dPAG in PMDD mice. Scale bars, 50 μm. (h) The results of the IF experiment showing the GABRD-positive cells in the dPAG. All values are expressed as mean ± S.E.M, * p <0.05, ** p <0.01, repeated measures, and one-way ANOVA with Bonferroni's test (n≥3 per group).

3.5 Specific knockdown of the expression of GABRD in the dPAG can significantly improve the oestrous cycle-dependent depression-like behaviour of PMDD mice

Considering the critical role of $GABA_AR$s in the central nervous system, we hypothesised that the anti-PMDD effects of Shuyu capsules might be linked to the expression of $GABA_AR$s in the dPAG. To test this theory, we used adeno-associated virus (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 oestrous cycle-dependent depression-like behaviour (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 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 knocking down 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 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 oestrous cycle-dependent depression-like behaviour.

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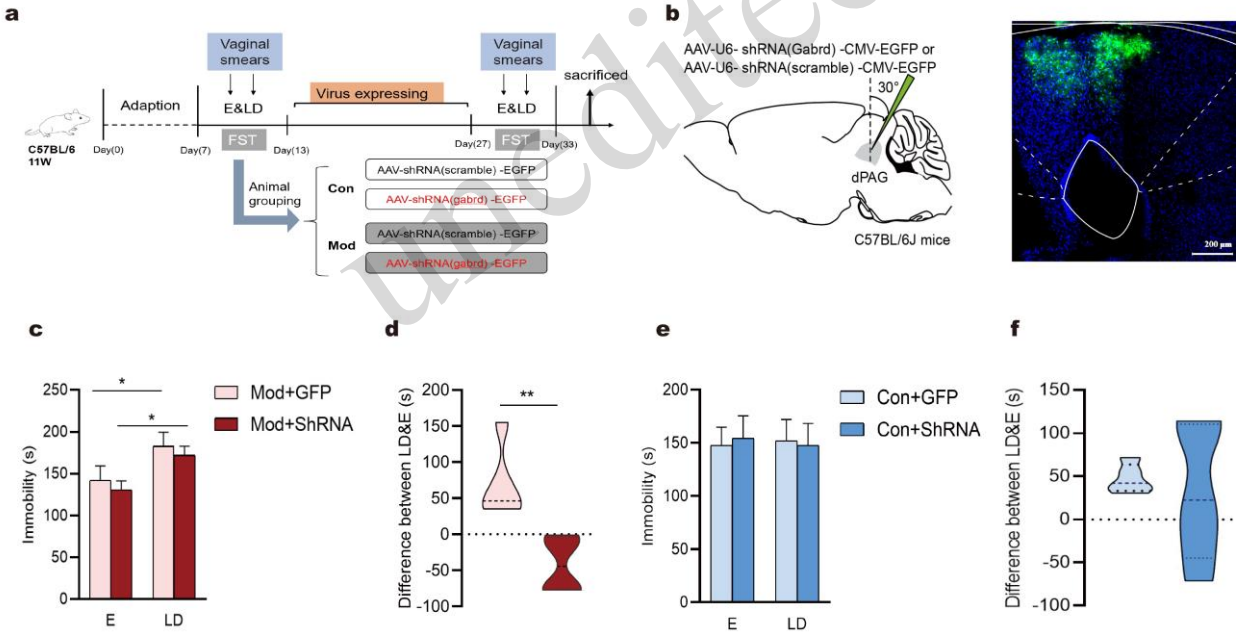

Fig. 5 Adeno-associated virus-specific knockdown of the expression of $GABA_AR$ δ subunit in dPAG, which can effectively improve the depression-like behaviour of PMDD mice. (a) Design diagram of the experimental process. (b) Left: Schematic diagram of virus injection and information on viruses. Right: Representative images of fluorescence of the virus, which is expressed in the dPAG. Scale bars, 500 μm. (c) The immobility duration of PMDD mice was detected via the FST in the E and LD phases of Vgat mice before virus injection. (d) The immobility duration of PMDD mice was detected via the FST in the E and LD phases of Vgat mice after the virus was fully expressed. (e) The immobility duration of control mice was detected via the FST in the E and LD phases of Vgat mice before virus injection. (f) The immobility duration of control mice was detected via the FST in the E and LD phases of Vgat mice after the virus was fully expressed. All values

are expressed as mean ± S.E.M, *p<0.05, **p<0.01, repeated measures, and two-way/ one-way ANOVA with Bonferroni's test (n≥5 per group).

3.6 Systemic gene knockout of the GABRD gene in mice triggers behavioural changes

To gain insight into the contribution of the $GABA_AR$ δ subunit to behavioural change in mice, we constructed transgenic mice with systemic GABRD knockout. The GABRD+/+ (Wild Type, WT) and GABRD-/- (Homozygous, HO) mice were selected and underwent FST, but we found that there was no significant difference in immobility duration (Fig. S3a) and number (Fig. S3b) between the two groups. To expand the detection surface of mouse behaviours, two genotypes of mice underwent a homecage behaviours test lasting 24 hours, and then we compared more than 30 behaviours between the two genotypes of mice (Fig. S3c). The results of behaviour detection showed that there were significant differences in the "come down" behaviour between the two groups (Fig. S3d), and notably, there was also a difference trend in "rear up from partial" (Fig. S3e) "remain rear up" and "come down to partially reared."

4 Discussion

PMDD, a serious premenstrual disease in women of childbearing age, significantly affects the physical and mental health of patients. Although fluoxetine, 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 both effective and has 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 oestrous cycle-dependent depression-like behaviour 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 behaviour in PMDD mice.

4.1 Shuyu capsule improves oestrous cycle-dependent depression-like behaviour in PMDD mice

Mice were selected for PMDD model screening to improve mechanism verification. The FST was chosen as the screening paradigm due to female rats exhibiting oestrous cycle-dependent depression-like behaviour during the test (Marvan, et al., 1997) and because of its favourable 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 (Marvan, et al., 1997; Wei, et al., 2020). Shuyu treatment improved depression-like behaviour in PMDD mice during the LD phase (Fig. 1). These results demonstrate that Shuyu capsule intervention can also alleviate oestrous cycle-dependent depression-like behaviour in PMDD mouse models.

4.2 The activity of GABAergic neurons of dPAG is involved in Shuyu capsule treatment with PMDD

Both physical symptoms 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 behavioural 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 fluoxetine and Shuyu capsules can affect PAG activity in rats (Devall et al., 2015; Geng, et al., 2024). Based on these

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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 control mice. The Shuyu intervention could effectively reverse this downward trend (Fig. 2). The outcomes of our study align with those of Devall and colleagues (Devall, et al., 2015), who discovered that rats subjected to stress exhibited fewer cells labelled with LD during the oestrous 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 Devall's in that 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's study 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 behaviours in PMDD mice (as shown in 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 plays a role in treating PMDD by downregulating the expression of $GABA_AR$ δ subunit 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 located outside of the synapse (Mohler, 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). Again, to further confirm this conclusion, IF was used to stain the $GABA_AR$ δ subunit in the dPAG of mice. Consistent with the previous conclusions, the Shuyu capsule intervention could reverse the increased trend of $GABA_AR$ δ subunit in the dPAG of model mice (Fig. 4h). To prove that the $GABA_AR$ δ subunit 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 $GABA_AR$ δ subunit of the dPAG is the target of the Shuyu capsule in treating PMDD.

4.4 The GABRD gene may be related to anxiety-like behaviours in mice

Many studies have shown that ovarian hormone fluctuation and $GABA_AR$ δ subunit expression affect rodents' behavioural phenotype. Since we constructed a knockout mouse, which systemically knocks out the GABRD gene in mice, we performed FST experiments to verify whether the $GABA_AR$ δ subunit affects mice's oestrous cycle-dependent depression-like behaviours. Surprisingly, there was no difference in behaviour between the mice in the FST (Figs. S3a and 6b). This is different from the effect of $GABA_AR$ δ subunit knockdown in dPAG on depression-like behaviour in PMDD mice mentioned above. It may be because the effects of the systemic knockout of the GABRD gene on mice behaviours 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 oestrous cycle-dependent behaviour 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 alter seizure susceptibility and anxiety (Mody and Pearce, 2004). Additionally, another study suggested that GABRD+/- and GABRD- /- mice constitute a mouse model of

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postpartum depression (Maguire and Mody, 2008). Ovarian hormone modulation of $GABA_AR$s 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 hormone's effect on the $GABA_AR$ δ subunit, and further investigation with alternative paradigms is necessary. We decided to conduct a 24-h homecage behaviours test to record and analyse behavioural changes in mice. We found differences in the "come down," "rear up from partial," "remain rear up," and "come down to partially reared" behaviours between the two groups (Fig. S3). These behaviours are exploration-related behaviours of mice (Roughan et al., 2009). Our results align with those of a study investigating behavioural changes in female C57BL/6J mice, which found that subtle shifts in the exploratory behaviour of each mouse were related to their oestrous phase (Levy et al., 2023). Exploratory behaviours 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 behaviours 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 behaviour 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 behaviours in rodents.

4.5 Conclusions

We proposed that the Shuyu capsule reduced the expression of $GABA_AR$ containing the δ subunit 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 (see Fig 6).

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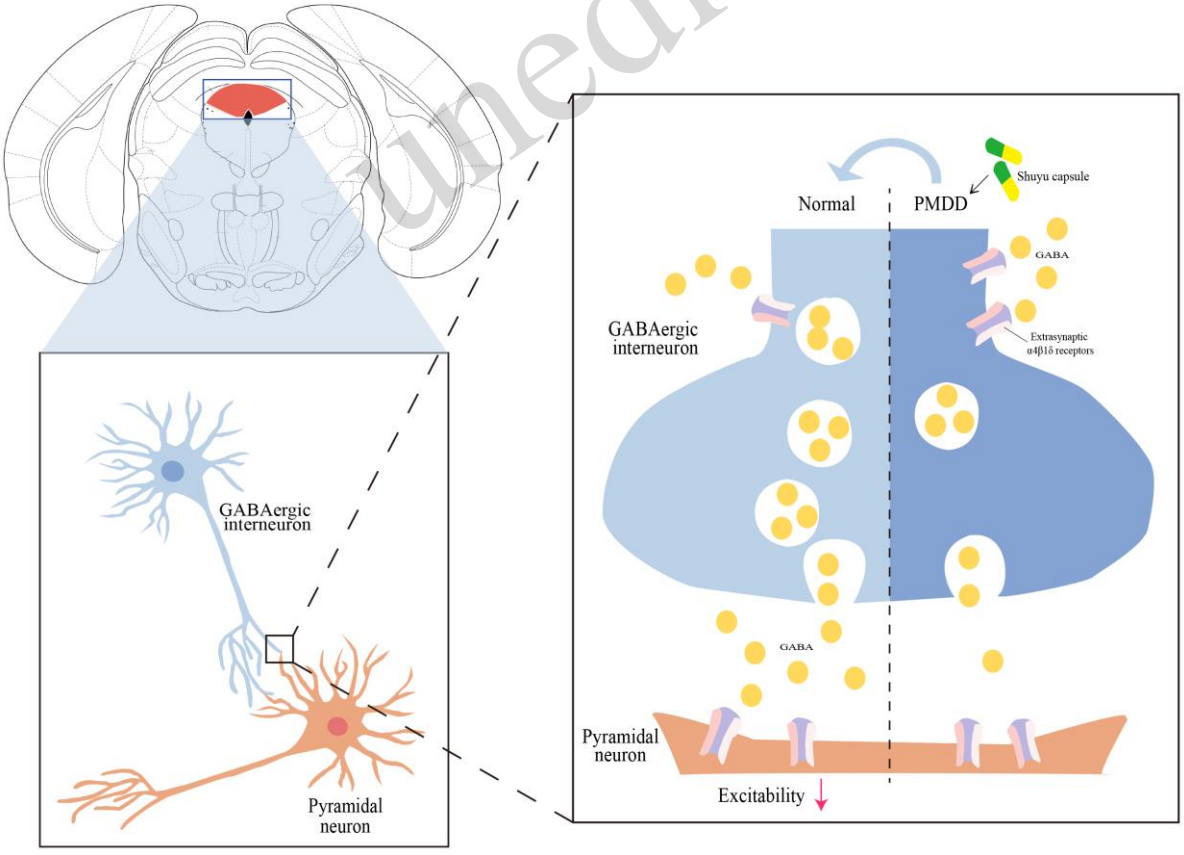

Fig. 6 Mechanism diagram illustrating that Shuyu capsule exerts a therapeutic effect on the GABAergic system of the

dPAG in PMDD mice.

In conclusion, our study's outcomes clarified the potential mechanism through which Shuyu capsules may improve oestrous cycle-dependent depressive-like behaviour in PMDD mice. Nonetheless, our research has the following limitations: 1. Although the Shuyu capsule's intervention on $GABA_AR$ containing δ subunit is suspected to be on GABAergic interneurons, this has not been experimentally validated. To confirm this, future research should include colocalisation staining of GABAergic neurons and $GABA_AR$ δ subunit 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 $GABA_AR$ δ subunit 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 oestrous cycle-dependent depressive-like behaviour in PMDD mice by activating GABAergic interneurons and downregulating the expression of $GABA_AR$ containing the δ subunit 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 shared pathological mechanism between 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.

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Data Availability statement

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

Acknowledgements

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Author contributions

Jialing XU, Xiwen GENG, Sheng WEI designed and led the project. Jialing XU, Kun LIU, 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, Wenqiang CUI gave constructive comments on the revision of the manuscript. Zifa LI, Mingqi QIAO, Sheng WEI Supervised the whole experimental process. Jialing XU prepared the manuscript. Wenqiang CUI, Xiwen GENG, Sheng WEI assisted with 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.

Declaration of Competing Interest

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 conflict of interest.

All mice used in this study were randomly assigned to cages housing five mice each. The mice were maintained at 21 ± 2°C and 45 ± 10% relative humidity under a light/dark cycle of 12:12 h, with ad libitum access to food and water, at Shandong University of Traditional Chinese Medicine [Laboratory animal use licence 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 Licence number: SDUTCM20230211001).

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Supplementary information:

Table S1-S2; Figs. S1-S3; Materials and methods

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