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Research article

Comparison of CUMS at different pregnancy stages, maternal separation, and their effects on offspring in postpartum depression mouse models

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ABSTRACT

Due to the diversity of postpartum depression (PPD) patients and the complexity of associated pathophysiological changes, most current animal models cannot accurately simulate PPD-like symptoms. In this study, we established a reliable animal model for PPD by inducing chronic unpredictable mild stress (CUMS) at different stages (pre-pregnancy, pregnancy, or postnatal) in female mice, followed by maternal separation (MS) from day 2–21 after delivery. The results for female mice subjected to pre-pregnancy stress were not statistically significant due to a lower conception rate. However, female mice exposed to CUMS during either the gestational or postnatal stage, followed by MS, successfully exhibited PPD-like symptoms. The models were deemed effective based on observed behavioral abnormalities, impaired hippocampal neuron functioning, and reduced serum concentrations of neurotransmitters (5-HT, GABA, and NE). Additionally, mice that underwent gestational CUMS followed by MS displayed a more dysfunctional hypothalamic-pituitary-adrenal (HPA) axis and more severe uterine inflammation. The study also investigated the impact of PPD on the behavior and neurodevelopment of adolescent offspring through behavioral tests, enzyme-linked immunosorbent assay (ELISA), hematoxylin-eosin (HE) staining, and western blotting (WB). The results indicated that adolescent offspring of mothers with PPD exhibited behavioral and neurodevelopmental disorders, with male offspring being more susceptible than females. Female mice exposed to both CUMS and MS during the postnatal period had more severe adverse effects on their offspring compared to the other model groups.

1. Introduction

Postpartum depression (PPD) is a condition affecting mothers after childbirth, characterized by symptoms such as depressed mood, loss of interest and pleasure, fatigue, loss of appetite and weight, poor concentration, and sleep disturbances [1,2]. The global

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prevalence of PPD is 10%–15 %, typically occurring within six weeks postpartum [2]. Notably, women in the Middle East (26 %) and Asia (16 %) experience higher PPD prevalence compared to those in Western countries (8%–15 %) [3,4]. The increased risk of PPD in Asian women may be attributed to cultural practices, negative mother-in-law–daughter-in-law relationships, and greater reliance on social support during the postnatal period [3]. According to the *2022 Blue Book on National Depression*, one in five mothers in China suffers from depression, with 63 % experiencing PPD at some point [5]. Suicide is a severe consequence of PPD, ranking second among maternal mortality factors [6]. PPD is significantly associated with high infant mortality [7]. Despite the high prevalence and mortality, the willingness to seek help is low; even among those treated, less than 5 % achieve remission [8].

PPD adversely affects maternal health, family dynamics, mother-infant relationships, and the growth and development of infants and children [9]. Women with a history of depression during pregnancy may have abnormal immune function, leading to stunted growth and poor neurobehavioral outcomes in their offspring [10]. Perinatal depression impairs fetal emotional development and results in significant behavior, learning, and attention deficits in offspring. Neuroimaging studies have shown increased amygdala volume and decreased hippocampal volume in adolescents exposed to maternal PPD [11,12]. Mothers with PPD are less sensitive and responsive to their infants, lacking affection and emotional communication, which hampers the formation of healthy attachments essential for infant growth. These maternal behaviors lead to delayed language development, lower cognitive levels, sleep disturbances, and communication deficits in their offspring [13]. Maternal depression in animal models, such as the maternal separation (MS) model, has been shown to result in anxiety symptoms in infancy and altered neurotransmitter concentrations in the hypothalamus, hippocampus, and striatum in adulthood [14].

Animal models play a crucial role in exploring human development and disease [15]. Commonly used animal models for postpartum depression (PPD) include hormone-induced, stress-induced, and emerging models [16,17]. Hormone-induced models, such as those involving hormone withdrawal and chronic corticosterone treatment, have successfully replicated some PPD-like symptoms in rodents. However, these models are not entirely specific or convincing, as they only simulate postpartum hormonal fluctuations rather than the actual postpartum state after pregnancy and delivery. Additionally, these models do not allow for observation of effects on offspring.

Stress models, including gestational stress, chronic stress, maternal separation (MS), and pregestational stress models, use psychological and social stressors to simulate PPD. These models reflect the association between social situations, relationship disruptions, and the occurrence of PPD in women. Emerging models include the transgenic depression animal model (e.g., *KCC2/Crh* and *Gabrd* −/− mice) and the high-fat diet model, though these are less common due to complexity and expense.

Composite models, such as MS combined with restraint stress and late pregnancy gastric intubation combined with MS, have been

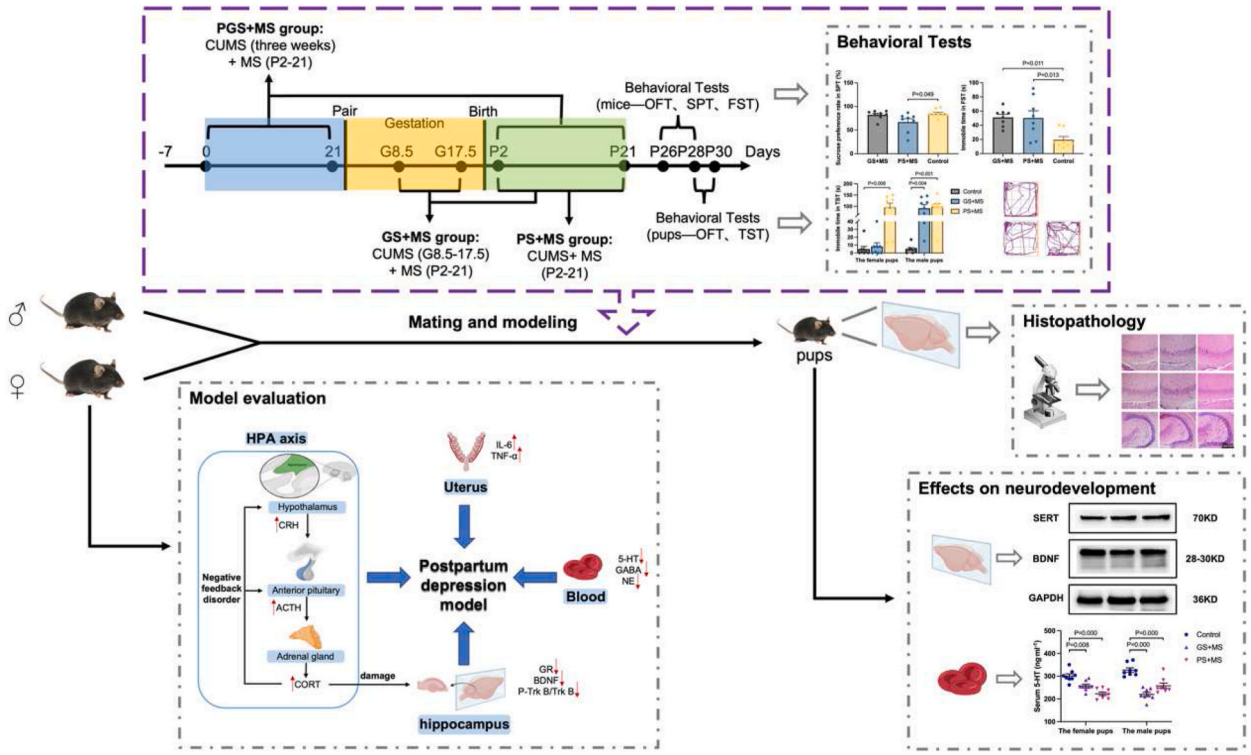

Fig. 1. The schematic design of this study. G: gestational day; P: postnatal day; CUMS: chronic unpredictable mild stress; MS: maternal separation; PGS + MS group: pregestational stress + maternal separation group; GS + MS group: gestational stress + maternal separation group; PS + MS group: postpartum stress + maternal separation group; OFT: open filed test; SPT: sucrose preference test; FST: forced swimming test; TST: tail suspension test; HPA axis: hypothalamic-pituitary-adrenal axis.

suggested to better simulate the natural onset of depression [18,19]. However, these models primarily address PPD-like symptoms caused by stress during gestation and the postnatal period, and the stress paradigms used, such as chronic restraint or gastric intubation, are limited in replicating the real-life situations faced by women.

In the study, we assessed PPD models induced by chronic unpredictable mild stress (CUMS) at different stages (pre-pregnancy, pregnancy, or postnatal) followed by MS to establish a scientifically robust animal model for future PPD research. Given the negative impact of maternal depression on the physical and mental development of offspring, we also investigated the effects of PPD on the behavioral and neurological development of adolescent mice. This complements existing research on PPD-induced depression in offspring and provides a theoretical basis for preventing and treating the effects of PPD on the onset of depression in offspring. The schematic design of the study is depicted in Fig. 1.

2. Materials and methods

2.1. Animals

We obtained sexually naive female C57BL/6 mice aged 5–6 weeks and male C57BL/6 mice aged 7–8 weeks (SCXK (Zhe) 2019–0004) from Hangzhou Ziyuan Experimental Animal Technology Co. Ltd., Hangzhou, China. All experimental mice were housed (five per cage) in the barrier lab of the Experimental Animal Center at Zhejiang University of Technology. The laboratory conditions were maintained at 25 ± 1 °C, 60 % relative humidity, and a 12-h light cycle. All experimental procedures (animal ethics number: 20220715067) were conducted in accordance with the Guide for the Care and Use of Laboratory Animals at Zhejiang University of Technology, Hangzhou, China, and conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1996).

2.2. Experimental design

Female mice were randomly divided into four groups (n = 10). The control group underwent normal mating and birth without exposure to CUMS or MS. The pregestational stress (PGS) + MS group was subjected to CUMS three weeks prior to mating, followed by maternal separation from day 2–21 post-delivery (P 2–21). To reduce the rate of abortion and preterm delivery, the duration of CUMS during gestation was limited to 10 days, as referenced in previous studies [20,21]. The gestational stress (GS) + MS group was subjected to CUMS from days 8.5–17.5 of gestation (G 8.5–17.5), followed by maternal separation from day 2–21 post-delivery (P 2–21). The postpartum stress (PS) + MS group underwent normal mating, with CUMS and MS induced from day 2–21 post-delivery (P 2–21). All females aged 9–10 weeks were mated with unstressed males at a 2:1 ratio, and vaginal smears were examined the following day (after 12 h) to confirm pregnancy. The presence of sperm marked day 0.5 of pregnancy (G 0.5), and pregnant females were housed singly.

Female mice were exposed to CUMS at different stages as follows [22]: swimming in 40 °C water (2 min), behavioral restraint (3 h), overcrowding (24 h), tail clipping (2 min), exposure to foreign object (24 h), overnight light, fasting (12 h), tilted cage at 45° (24 h), no bedding (24 h), solitary (24 h), wet bedding (24 h), and no water (12 h). Stressors were non-repetitive and irregular to prevent adaptation and maintain responsiveness. All female mice, except those in the control group, underwent a 4-h MS from day 2–21 post-delivery. Females were placed individually in separation cages during MS, and heating mats (maintained at approximately 25 °C) were used to ensure optimal pup growth conditions. Mother and pup cages were positioned closely to allow females to smell and hear their pups. After the separation period, females were returned to their original cages [23].

Only two female and two male pups were selected per female to ensure consistent maternal care for each pup. All pups were weaned on day 22 post-delivery and separated into male and female groups. Eight female and eight male pups were randomly selected from each group as the offspring group. The offspring groups were categorized as follows: the control offspring group (Ctl-F1-M group, n = 8; Ctl-F1-F group, n = 8), the pregestational stress + maternal segregation offspring group (PGS + MS-F1-M group, n = 8; PGS + MS-F1-F group, n = 8), the pregnancy stress + maternal segregation offspring group (GS + MS-F1-M group, n = 8; GS + MS-F1-F group, n = 8) and the postpartum stress offspring group (PS + MS-F1-M group, n = 8; PS + MS-F1-F group, n = 8). The adolescent period for the pups began on day 28 post-birth.

2.3. Body weight measurements and conception rate

Female mice were weighed weekly throughout the study to monitor changes in their weight over time. The number of successfully conceived female mice in each group was recorded. In addition, the weight of the pups on the 22nd day after delivery was recorded.

2.4. Behavioral test

Both dams and their male and female pups were subjected to the open field test (OFT). The sucrose preference test (SPT) and forced swimming test (FST) were conducted only on the dams, while the tail suspension test (TST) was conducted only on the pups. Due to the low weight of the pups and considerations for animal welfare, the pups were not subjected to the FST. Immobility in the FST and TST was scored by three blind experimenters.

2.4.1. Open field test

The open field apparatus was divided into sixteen equal zones at the bottom. For the test, mice were placed in a fixed corner of the peripheral zone and allowed to acclimate for 2 min. Their movements were then tracked for 4 min using ANY-maze 5.1 software, which recorded their immobility time, total distance traveled, number of entrances to the central area, and time spent in the central area [24].

2.4.2. Sucrose preference test

Dams underwent a sugar-water adaptation period before the SPT. They were housed individually and provided two bottles of 1 % sugar water for the first 24 h, followed by one bottle of pure water and one bottle of 1 % sugar water for the next 24 h. The positions of the bottles were switched after 12 h to avoid position bias. After acclimatization, dams were water-fasted for 12 h, and the consumption of 1 % sugar water and pure water was measured over the next 12 h, with bottle positions switched at hour 6) [25]. The sucrose preference rate was calculated as the ratio of sugar water consumption to total fluid consumption.

2.4.3. Forced swimming test

Dams were placed in a glass beaker filled with water to a depth of 15 cm (maintained at 23 ± 2 °C). Their behavior was observed for 6 min, divided into a 2-min pretest and a 4-min test period. Immobility time was recorded during the last 4 min. Dams were considered immobile when they ceased struggling and floated upright with only minimal limb movements [26].

2.4.4. Tail suspension test

Pups were suspended by 1/3 of their tail tips using a non-sticky adhesive tape attached to a round hook on an iron stand. Opaque baffles separated adjacent stands to prevent visual contact between pups. The test lasted 6 min, with the first 2 min for adaptation. Immobility duration was recorded during the remaining 4 min [27]. Pups were considered immobile when they stopped active struggling and hung without twisting.

2.5. Concentrations of neurotransmitters, stress hormones, and brain-derived neurotrophic factor (BDNF) in the serum

After completing all behavioral tests, blood samples were collected from the orbital plexus of the mice. Samples were centrifuged (3500 r/min, 15 min) to separate the serum, which was then stored at −80 °C. Concentrations of 5-serotonin (5-HT), γ-aminobutyric acid (GABA), norepinephrine (NE), corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and corticosterone (CORT) in the serum of female mice were measured using enzyme-linked immunosorbent assay (ELISA) kits (HePeng Biotechnology Co., Ltd., Shanghai, China). Serum concentrations of 5-HT and BDNF in the pups were also measured using the same method.

2.6. Tissue samples collection and histopathologic analysis

Female mice were euthanized via cervical dislocation after blood sample collection. The liver, kidney, spleen, and uterus were quickly removed and weighed to calculate the organ coefficient (%): organ coefficient (%) = organ mass (g)/body mass (g) × 100 %. The uterus was fixed in formalin for 48 h, and 4-μm-thick paraffin sections were prepared and stained with hematoxylin-eosin (HE) to observe pathologic changes and measure endometrial thickness. Measurements were taken at the widest, narrowest, widest symmetry, and narrowest symmetry points of the endometrium, and the average value was calculated [28]. The brain was divided into two parts: the right hemisphere hippocampus was used for western blotting (WB), and the left hemisphere was dehydrated, embedded, and sectioned sagittally with a rotary slicer (Leica RM2245, Leica Microsystems Trading Co., Ltd, Shanghai, China). These sections were used for HE and Nissl staining [28]. Histopathology of different hippocampal subregions in each group was observed under an optical microscope [29]. Similarly, the right hemispheres of the pups' brains were used for WB, and the left hemispheres for HE staining.

2.7. Immunohistochemistry

Tissue sections (5 μm) stored at −20 °C were dewaxed and repaired by heating with an improved citrate antigen retrieval solution (50 ×, pH 6.0) (P0083, Beyotime Biotechnology Co., Ltd., Shanghai, China). Primary antibodies, including anti-interleukin 6 (anti-IL-6) (Wuhan Boster biological technology Ltd., Wuhan, China, BA4339, 1:100) and anti-tumor necrosis factor-α (anti-TNF-α) (Proteintech Group, Inc., 17590-1-AP, 1:200), were applied to the sections and incubated overnight at 4 °C in a wet box. After three washes with phosphate-buffered saline (PBS, G0002-2L, Wuhan Servicebio Technology Co., Ltd., Wuhan, China), secondary antibodies were added for 2 h at 25 ± 1 °C. Then, 3,3′-diaminobenzidine (DAB, W026-1, Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) was applied dropwise, and the reaction was monitored under a microscope to achieve the desired color intensity. The reaction was terminated by washing the tissue sections with PBS [30]. Image J 1.53 software was used to measure the integrated optical density (IOD) and positive area, allowing calculation of the average optical density (AOD). The expression levels of IL-6 and TNF-α were compared.

2.8. Western blotting

WB was performed according to previously described protocols [31]. The hippocampal tissues of female mice were lysed using a protocol from earlier studies [29]. Supernatants were collected, and sample volumes were recorded. Protein content was determined using a bicinchoninic acid protein assay kit (BCA Protein Assay Kit, P0012, Beyotime Biotechnology Co., Ltd., Shanghai, China).

Protein samples were separated on a 6%–12 % SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Blots were incubated with primary antibodies at 4 °C for 14–16 h. Primary antibodies targeted glucocorticoid receptor (GR) (Proteintech Group, Inc., 66904-1-lg, 1:60000), BDNF (Proteintech Group, Inc., 28205-1-AP, 1:1000), serotonin transporter (SERT) (Proteintech Group, Inc., 19559-1-AP, 1:750), tyrosine kinase B receptor (Trk B) (Wuhan Boster biological technology Ltd., Wuhan, China, M01388-3, 1:1000), phospho-tyrosine kinase B receptor (*p*-Trk B) (Wuhan Boster biological technology Ltd., Wuhan, China, BM4437, 1:250) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Proteintech Group, Inc., 10494-1-AP, 1:8000). After three PBS washes, membranes were incubated with secondary antibodies at 25 ± 1 °C for 2 h. Membranes were then developed using the Chemiscope series (96043, Clinx Science Instruments Co., Ltd, Shanghai, China). The expression levels of target proteins were normalized the GAPDH. Related proteins (BDNF, SERT, and GAPDH) in the pups' brain tissue were determined using the same procedure.

2.9. Statistical analysis

SPSS 26.0 software was used to analyze the experimental data, and GraphPad Prism 9.0 software was used to create the graphs. Data were presented as mean ± SEM ($\overline{x}$ ±S). One-way ANOVA was used to compare the groups. If equal variance was assumed, the Bonferroni test was applied; otherwise, the rank sum test was used. Statistical significance was determined with a *P-value* < 0.05.

3. Results

Only 4 out of 10 female mice in the PGS + MS group were successfully mated (i.e., n = 4), resulting in an insufficient sample size. Consequently, the experimental results of this group do not hold reference value and were not included in this section of the study.

3.1. Studies on animal models of PPD

3.1.1. Conception rate, body weight and organ coefficient

The conception rate was 40 % (4/10) in the female mice subjected to CUMS during pre-pregnancy, whereas all the remaining mouse groups mated successfully (Table 1).

The weight changes in female mice throughout the experiment are shown in Fig. 2-A. In the GS + MS group, the body weights of pregnant female mice were not substantially different from those of the normal mice; however, the weight gain of the female mice undergoing MS was significantly less (The first week of MS, $F_{2,21} = 6.784$, $p = 0.004 < 0.005$; The second week of MS, $F_{2,21} = 8.341$, $p = 0.002 < 0.005$; The third week of MS, $F_{2,21} = 4.985$, $p = 0.016 < 0.05$). The PS + MS group mice showed significantly less weight gain compared to the normal mice (The first week of MS, $p = 0.035 < 0.05$; The second week of MS, $p = 0.007 < 0.05$).

The liver, kidney, spleen, and uterus coefficients of female mice in each group are shown in Fig. 2-B. The coefficients of the liver, kidney, and spleen in the two PPD animal model groups were comparable to those in the control group, suggesting that CUMS followed by MS may not harm the liver, kidney, and spleen. However, the uterine coefficients were significantly reduced in the female mice receiving CUMS during gestation or the postnatal period followed by MS compared to the normal mice ($F_{2,21} = 8.711$, vs. Control: GS + MS, $p = 0.019 < 0.05$; PS + MS, $p = 0.002 < 0.005$). This indicates that stress during gestation and the postnatal period may cause damage to the uterus of female mice.

3.1.2. Exposure to CUMS at different stages followed by MS causes depression-like behavior in the female mice

Fig. 3-A shows the track plot reports of female mice recorded during the OFT. The control group exhibited more activity and exploration of the central area than the other groups, suggesting that normal female mice had autonomous exploratory behavior in unfamiliar environments. Conversely, mice in the GS + MS and PS + MS groups moved to the peripheral area and chose to remain there, indicating higher levels of anxiety and tension in the unfamiliar and open environment. The total distance traveled by females exposed to gestational stress followed by MS was significantly reduced compared to normal females ($F_{2,21} = 5.284$, $p = 0.016 < 0.05$) (Fig. 3-C), and the immobility time was significantly longer in females exposed to postnatal stress followed by MS compared to normal females ($F_{2,21} = 5.405$, $p = 0.011 < 0.05$) (Fig. 3-D). Additionally, females exposed to stress during gestation and the postnatal period followed by MS spent less time in the center compared to normal females ($F_{2,21} = 6.318$, vs. Control: GS + MS, $p = 0.015 < 0.05$; PS + MS, $p = 0.019 < 0.05$) (Fig. 3-B). These results indicated high anxiety and poor exercise capacity in these two groups. Only indicators with significant differences were analyzed.

Compared to the control group, the sucrose preference rate was significantly lower in the PS + MS group ($F_{2,21} = 3.980$, $p = 0.049 < 0.05$) (Fig. 3-E), indicating that female mice subjected to double stress (CUMS and MS) during the postnatal period exhibited anhedonia. In contrast, the sucrose preference rates in the GS + MS group was not significantly different from that in the control group.

Table 1
Conception rate of female mice (n = 10).

Groups	The number of pregnant mice	Conception rate (%)
PGS + MS group	4	40 %
GS + MS group	10	100 %
PS + MS group	10	100 %
Control group	10	100 %

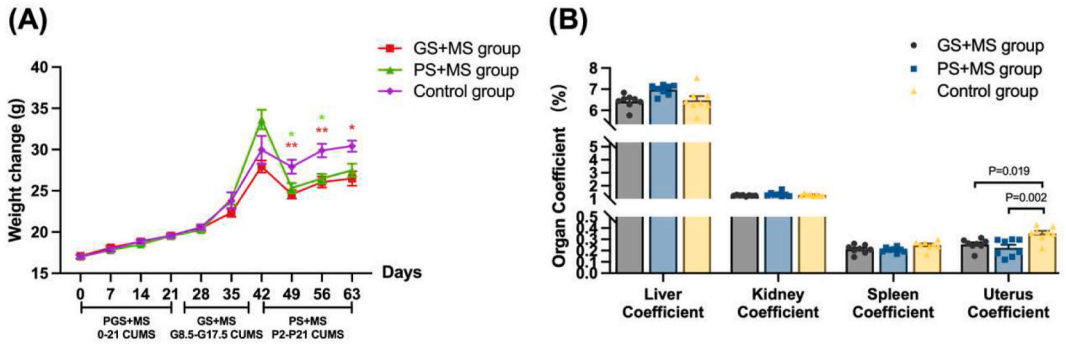

Fig. 2. Body weight changes and organ coefficients of female mice in different PPD models (n = 8). (A) Changes in body weight of female mice during the modeling period; (B) Liver, kidney, spleen and uterus coefficients of female mice in each group at the end of the modeling. In the analysis of this study, $*p < 0.05$, $**p < 0.005$ vs. control group.

Fig. 3-F shows the cumulative immobility time of female mice in the last 4 min of the FST. The cumulative immobility time was longer in the female mice subjected to gestational or postnatal stress followed by MS compared to normal mice ($F_{2,21} = 6.921$, vs. Control: GS + MS, $p = 0.011 < 0.05$; PS + MS, $p = 0.013 < 0.05$), suggesting that the female mice in these two groups had a significantly lower desire to survive and exhibited depression-like behavior.

3.1.3. Effects of CUMS at different stages followed by MS on the concentrations of serum neurotransmitters and stress hormones in female mice

As shown in Fig. 4 (A-C), the concentrations of serum 5-HT, GABA, and NE were significantly reduced in the female mice subjected to CUMS during the gestation or postnatal period followed by MS compared to the normal mice (vs. Control: GS + MS: 5-HT, $F_{2,21} = 17.928$, $p = 0.013 < 0.05$, GABA, $F_{2,21} = 6.490$, $p = 0.020 < 0.05$, and NE, $F_{2,21} = 6.559$, $p = 0.029 < 0.05$; PS + MS: 5-HT, $p = 0.000 < 0.001$, GABA, $p = 0.012 < 0.05$, and NE, $p = 0.009 < 0.05$).

Fig. 4 (D-F) shows the hormonal changes associated with the hypothalamic-pituitary-adrenal (HPA) axis in the serum samples of female mice. The results indicated that female mice subjected to gestational stress followed by MS had significantly higher levels of CRH, ACTH, and CORT in their serum compared to normal mice (CRH, $F_{2,21} = 6.709$, $p = 0.007 < 0.05$, CORT, $F_{2,21} = 3.971$, $p = 0.041 < 0.05$, and ACTH, $F_{2,21} = 235.405$, $p = 0.000 < 0.001$). Similarly, the serum CRH concentration was higher in the PS + MS group compared to the control group ($p = 0.038 < 0.05$). Overall, the most significant changes in the HPA axis were observed in the GS + MS group, indicating severe disruption in female mice exposed to CUMS during gestation followed by MS.

3.1.4. Exposure to CUMS at different stages followed by MS causes uterine inflammation in the female mice

Female mice in the GS + MS group exhibited a significantly higher mean endometrial thickness than those in the control group ($F_{2,21} = 9.773$, $p = 0.002 < 0.005$), as shown in Fig. 5-A and B. In addition, the expression levels of IL-6 and TNF-α in the uterus of female mice exposed to stress during gestation followed by MS were significantly higher than those in normal females (IL-6, $F_{2,21} = 5.769$, $p = 0.008 < 0.05$; TNF-α, $F_{2,21} = 7.234$, $p = 0.013 < 0.05$) (Fig. 5-D and E). Furthermore, the presence of brown plaques in the uterus was significantly increased in the GS + MS group (Fig. 5-C). Interestingly, although the PS + MS group also exhibited brown plaques in the uterus, the expression levels of IL-6 and TNF-α were comparable to those in the control group, suggesting that uterine inflammation may be specifically associated with gestational stress in female mice.

3.1.5. Exposure to CUMS at different stages followed by MS causes hippocampal damage in the female mice

Fig. 6-A shows the HE staining images of the hippocampus of female mice. In the hippocampi of normal mice, the cone cell layers in the CA1, CA3, and dentate gyrus (DG) regions were closely and regularly arranged, neuronal cell morphology was intact and clear, nuclei were round or oval, cytoplasm was evenly distributed, and nucleoli were visible. Compared to the normal mice, the hippocampal neurons in the PPD model mice exhibited reduced cell numbers, increased cell spacing, abnormal cell morphology, atrophy of cell bodies, and densely stained cell nuclei. These findings indicated varying degrees of damage to the neuronal cells in the hippocampi of the PPD model groups.

Nissl bodies are characteristic structures of neurons and are sites for the synthesis of structural and functional proteins. Their number and distribution are closely related to the functional state of neurons [32]. Nissl bodies were abundant and evenly distributed in the control group. However, in the PPD model groups, the number of Nissl bodies in the cytoplasm was significantly reduced due to disintegration, and protein synthesis ability was significantly weaker (Fig. 6-B).

3.1.6. Western blotting analysis

HPA axis dysfunction, decreased levels of BDNF, and abnormalities in the serotonergic system are closely associated with the development of PPD [2,6,33]. WB was performed to quantify the relative abundance of GR, BDNF, SERT, Trk B, and *p*-Trk B in the mouse hippocampi.

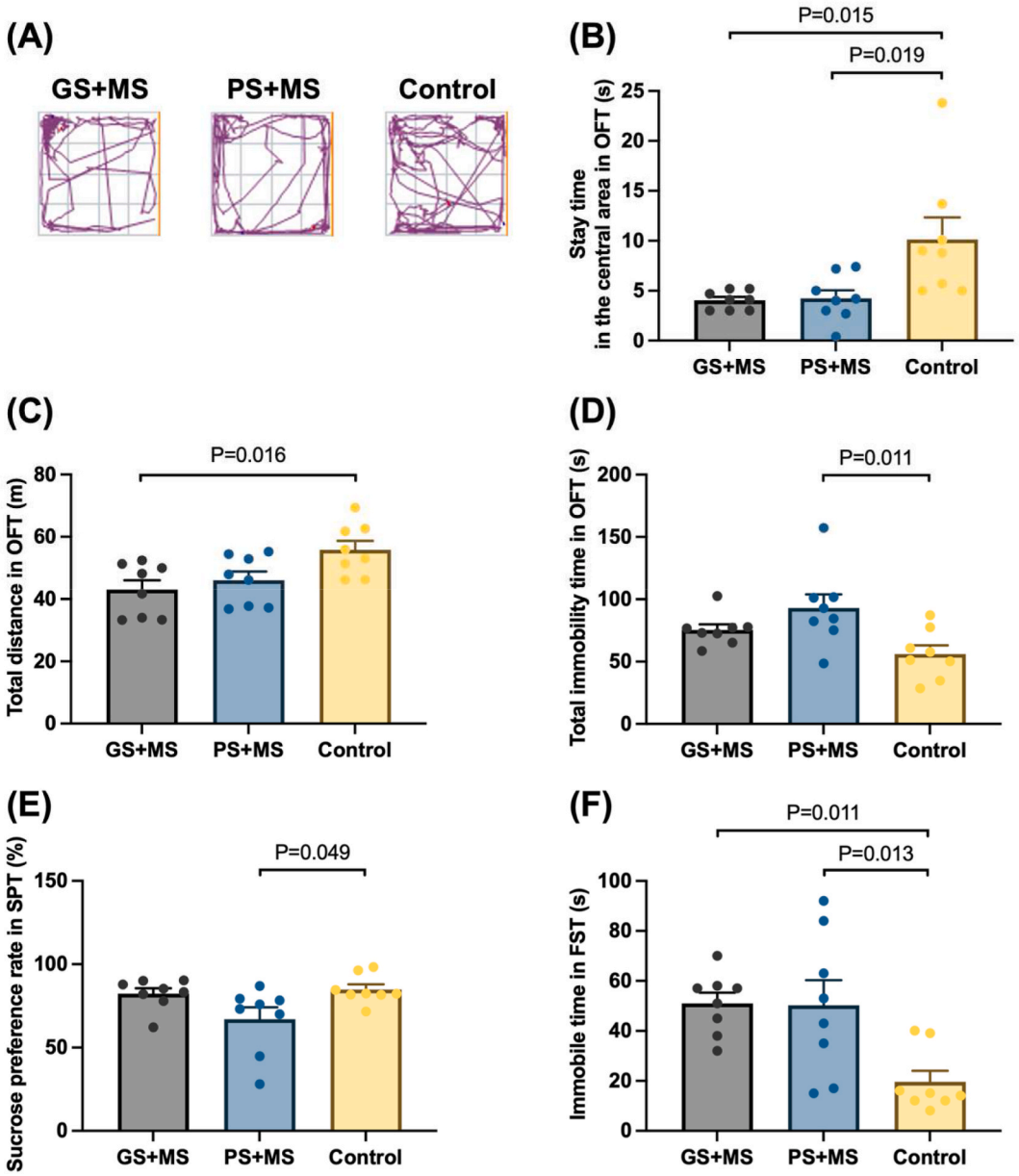

Fig. 3. Depression-like behavior of female mice in different PPD models (n = 8). (A) Track plot reports of female mice; (B) Time staying in the central area, (C)total distance and (D) total immobility time in the open field test (OFT); (E) Sucrose preference rate in the sucrose preference test (SPT); (F) Immobility time during the last 4 min of the forced swimming test (FST).

The protein expressions in the hippocampal tissues of the female mice are depicted in Fig. 7-A. Compared to normal mice, the relative expression of GR was significantly lower in both PPD model groups ($F_{2,6} = 9.376$, vs. Control: GS + MS, $p = 0.029 < 0.05$; PS + MS, $p = 0.028 < 0.05$) (Fig. 7-A and B) (Supplementary Figs. 1–1 and Figs. 1–4). However, the relative gray value of SERT did not show any significant differences between the normal mice and PPD model females (Fig. 7-A and C) (Supplementary Figs. 1–1 and Figs. 1–3). BDNF, a neurotrophic protein produced in the nervous system that binds to Trk B, showed a reduction in relative expression to varying degrees in both model groups of female mice compared to normal mice ($F_{2,6} = 7.828$, vs. Control: GS + MS, $p = 0.047 < 0.05$; PS + MS, $p = 0.038 < 0.05$) (Fig. 7-A and D) (Supplementary Figs. 1–1 and Figs. 1–2). Furthermore, the *p*-Trk B/Trk B ratio was significantly lower in the female mice subjected to stress during gestation and the postnatal period followed by MS compared to normal mice ($F_{2,6} = 8.584$, vs. Control: GS + MS, $p = 0.031 < 0.05$; PS + MS, $p = 0.038 < 0.05$) (Fig. 7-A and E) (Supplementary Figs. 1–1, Figs. 1–5 and Figs. 1–6).

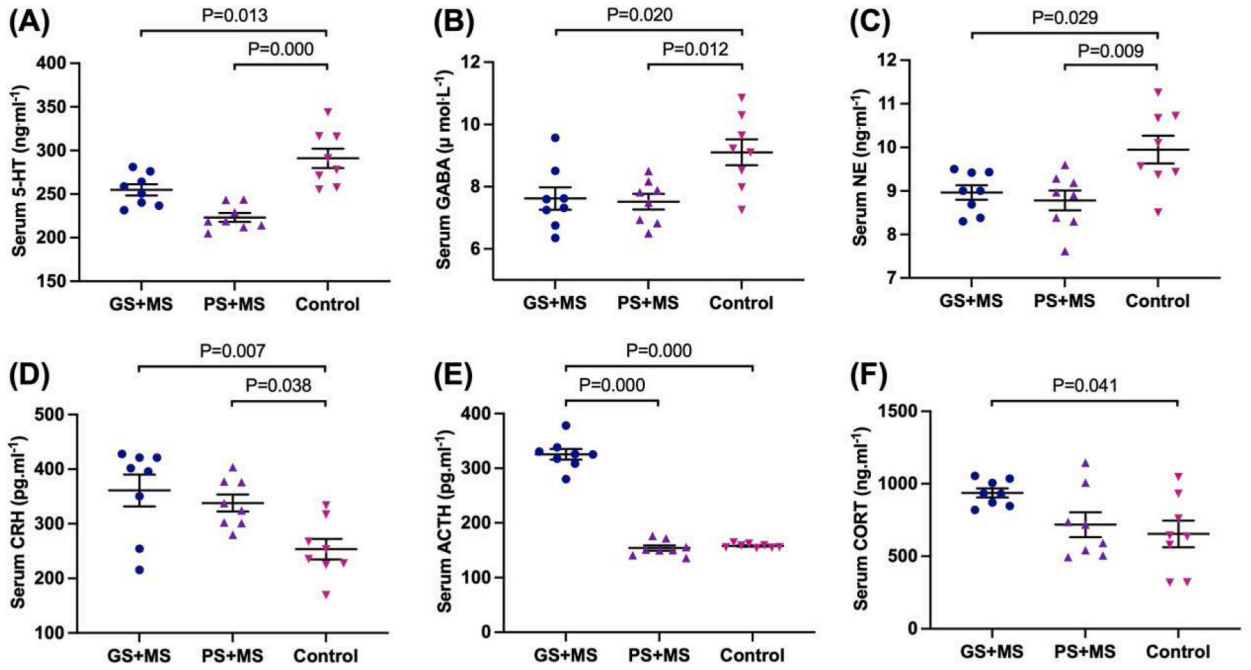

Fig. 4. Validation of PPD animal models by evaluating serum neurotransmitter concentrations and HPA axis-related hormones (n = 8). Serum (A) 5-HT, (B) GABA and (C) NE concentrations; Serum (D) CRH, (E) ACTH and (F) CORT concentrations.

3.2. Offspring studies

3.2.1. Mean body weight of pups

The mean body weight of the pups on the 22nd day after delivery in the PS + MS group was considerably lower than that of normal pups (vs. Ctl-F1: PS + MS-F1-F, $F_{2,21} = 4.563$, $p = 0.040 < 0.05$; PS + MS-F1-M, $F_{2,21} = 10.404$, $p = 0.003 < 0.005$), and there were no sex differences. This may be attributed to the stress during the postnatal period, leading to insufficient lactation in females, lack of care for the pups, and negative rearing performance, resulting in lower body weight. The mean body weight of the pups in the GS + MS group was not significantly different from that in the control group (Fig. 8).

3.2.2. PPD in mothers can cause depression-like behavior in adolescent pups

Fig. 9-A shows the track plot reports of the pups recorded during the OFT on the 28th day after delivery. The pups in the control group were active, moved around curiously, showed more excitement in the new environment, and were able to move independently. In contrast, the pups in the GS + MS and PS + MS groups exhibited a preference for locomotion in the peripheral area and were less curious and active in the new environment. These behaviors did not significantly differ between female and male pups. The pups in the PS + MS group rested more frequently than normal pups (vs. Ctl-F1: PS + MS-F1-F, $F_{2,21} = 8.718$, $p = 0.011 < 0.05$; PS + MS-F1-M, $F_{2,21} = 4.730$, $p = 0.036 < 0.05$) (Fig. 9-B). The male pups of the GS + MS group and the pups of the PS + MS group entered the central area less frequently than normal pups (vs. Ctl-F1: PS + MS-F1-F, $F_{2,21} = 6.250$, $p = 0.013 < 0.05$; GS + MS-F1-M, $F_{2,21} = 5.230$, $p = 0.043 < 0.05$; PS + MS-F1-M, $p = 0.025 < 0.05$) (Fig. 9-C). The female pups in the PS + MS group and the male pups in the GS + MS group spent less time in the center than normal pups (vs. Ctl-F1: PS + MS-F1-F, $F_{2,21} = 10.975$, $p=0.006 < 0.05$; GS + MS-F1-M, $F_{2,21} = 4.085$, $p = 0.033 < 0.05$) (Fig. 9-D). Only indicators with significant differences were analyzed.

Fig. 9-E shows the total immobility time of the pups in the last 4 min of the TST. Compared to the normal pups, the pups in the PS + MS group and the male pups in the GS + MS group had longer total immobility time (vs. Ctl-F1: PS + MS-F1-F, $F_{2,21} = 19.654$, $p = 0.006 < 0.05$; GS + MS-F1-M, $F_{2,21} = 17.020$, $p = 0.004 < 0.005$; PS + MS-F1-M, $p = 0.001 < 0.005$) and showed intermittent immobility, indicating a state of "behavioral despair". Interestingly, the analysis of immobility time in the pups of the two PPD model groups showed that the male offspring were more likely to show a state of "despair".

3.2.3. Effects of PPD on serum 5-HT and BDNF concentrations in the adolescent pups

Serum 5-HT concentrations were significantly reduced in the pups born to PPD mothers in both groups compared to normal pups (vs. Ctl-F1-F: GS + MS-F1-F, $F_{2,21} = 16.613$, $p = 0.008 < 0.05$; PS + MS-F1-F, $p = 0.000 < 0.001$; vs. Ctl-F1-M: GS + MS-F1-M and PS + MS-F1-M, $F_{2,21} = 27.942$, $p = 0.000 < 0.001$) (Fig. 10-A). Additionally, compared to normal pups, serum BDNF concentrations were significantly lower in the male pups of the GS + MS and PS + MS groups (vs. Ctl-F1-M: GS + MS-F1-M, $F_{2,21} = 7.633$, $p = 0.005 < 0.05$; PS + MS-F1-M, $p = 0.017 < 0.05$) (Fig. 10-B). These findings suggest that exposure to CUMS in female mice at different stages, followed by MS, can alter the serum 5-HT and BDNF concentrations in their offspring. Interestingly, male pups were found to be more

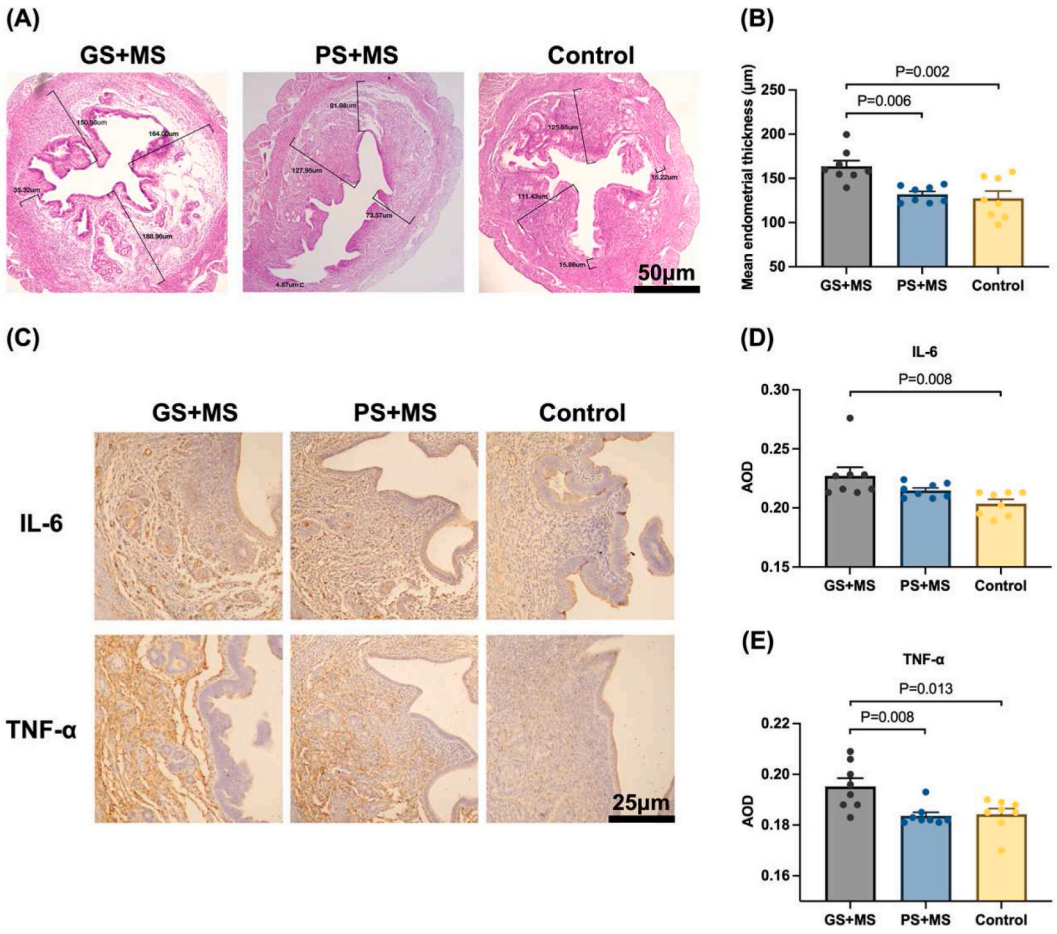

Fig. 5. Validation of PPD animal models by evaluating uterine inflammation (n = 8). (A) HE staining of the uterus from the female mice (200 ×); (B) Mean endometrial thickness; (C) Immunohistochemical staining of the uterus (400 ×); AOD values of (D) IL-6 and (E) TNF-α in the uterus.

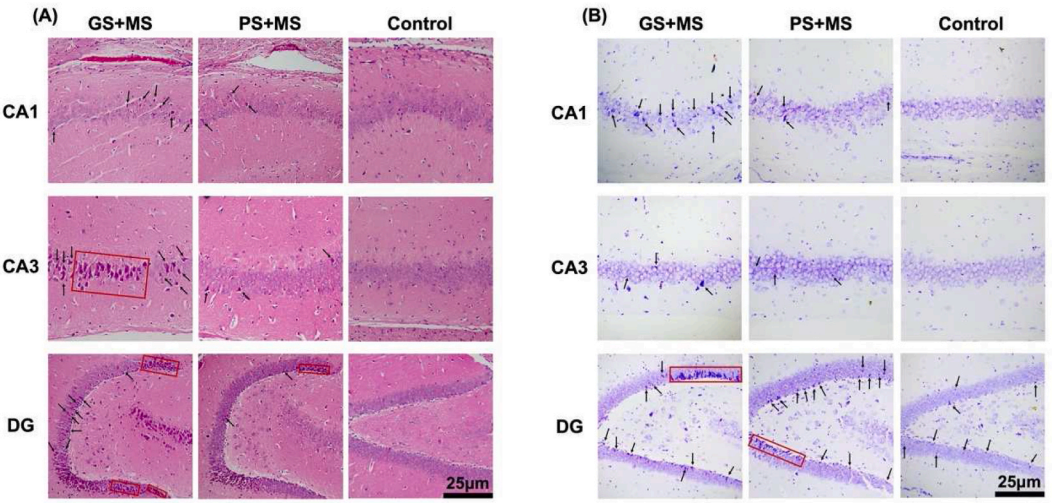

Fig. 6. Validation of PPD animal models by evaluating brain histopathologic changes (n = 8). (A) HE staining of hippocampal tissues from the female mice (400 ×); (B) Nissl staining of hippocampal tissues from the female mice (400 ×). Red box represents extensive neuronal cell necrosis, reduced cell numbers, and aberrant cell morphology. Black arrows indicate necrosis of individual neuronal cells as assessed by deep cell staining and nuclear fixation.

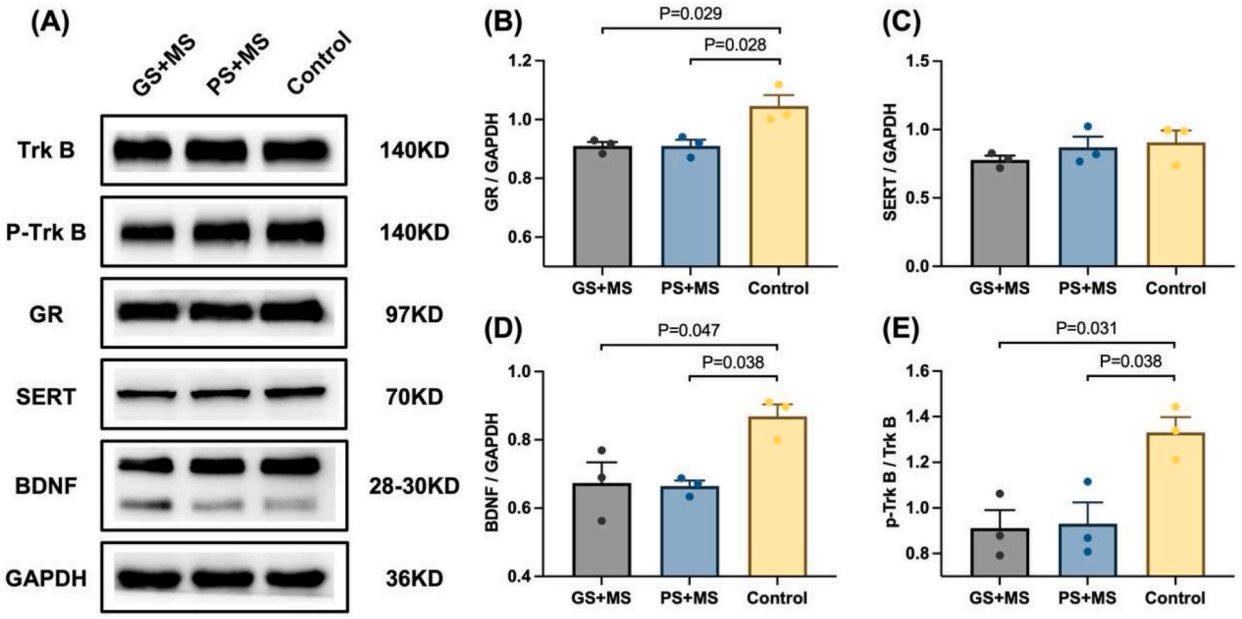

Fig. 7. Results of western blotting (n = 3). (A) Relative expressions of GR, SERT, BDNF, Trk B, and *p*-Trk B in hippocampal tissues of GS + MS and PS + MS groups; Relative gray values of (B) GR, (C) SERT, and (D) BDNF and (E) the ratio of *p*-Trk B to Trk B in hippocampal tissues of female mice.

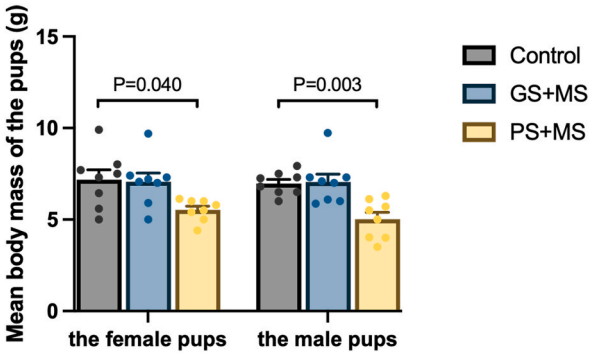

Fig. 8. Mean body weight of pups in each group at day 22 after delivery (n = 8).

susceptible to maternal influence during learning and memory formation, as evidenced by the pronounced alteration in their serum BDNF concentrations.

3.2.4. Effects of PPD on the hippocampal tissue of the adolescent pups

Fig. 11-A and B display HE staining images of the hippocampus in male and female pups, respectively. The hippocampal neuronal cells of normal male and female pups were morphologically intact, tightly arranged, and had clear intercellular layers. No neuronal necrosis was observed, and the nuclei were round or oval, full, and complete. However, the structure of neuronal cells in the pups of the PPD model groups was damaged, with numerous neuronal cells showing degeneration, necrosis, elongation with markedly shrunken cell bodies, enhanced cytoplasmic eosinophilia, and densely stained solid nuclei. Moreover, the male pups in the GS + PS group had increased spacing and a loosely disorganized arrangement of neuronal cells in the CA3 region, with multiple obvious cell breaks, reduced number of normal cells, deeply stained cells, and nuclear sequestration. Overall, PPD caused more hippocampal tissue damage in adolescent male offspring than in female offspring.

3.2.5. Western blotting analysis

SERT is a membrane protein that transports 5-HT from the synaptic gap to presynaptic neurons; it plays a key role in antidepressant and anxiolytic disorders [34]. Additionally, dysregulated neuroplasticity is involved in the pathogenesis of depression, and neurotrophic factors are important indicators for evaluating neuroplasticity [35]. WB was used to quantify the relative expressions of BDNF and SERT in the hemispheres of the pups to investigate the effects of PPD in mothers—who were exposed to stress at different stages of

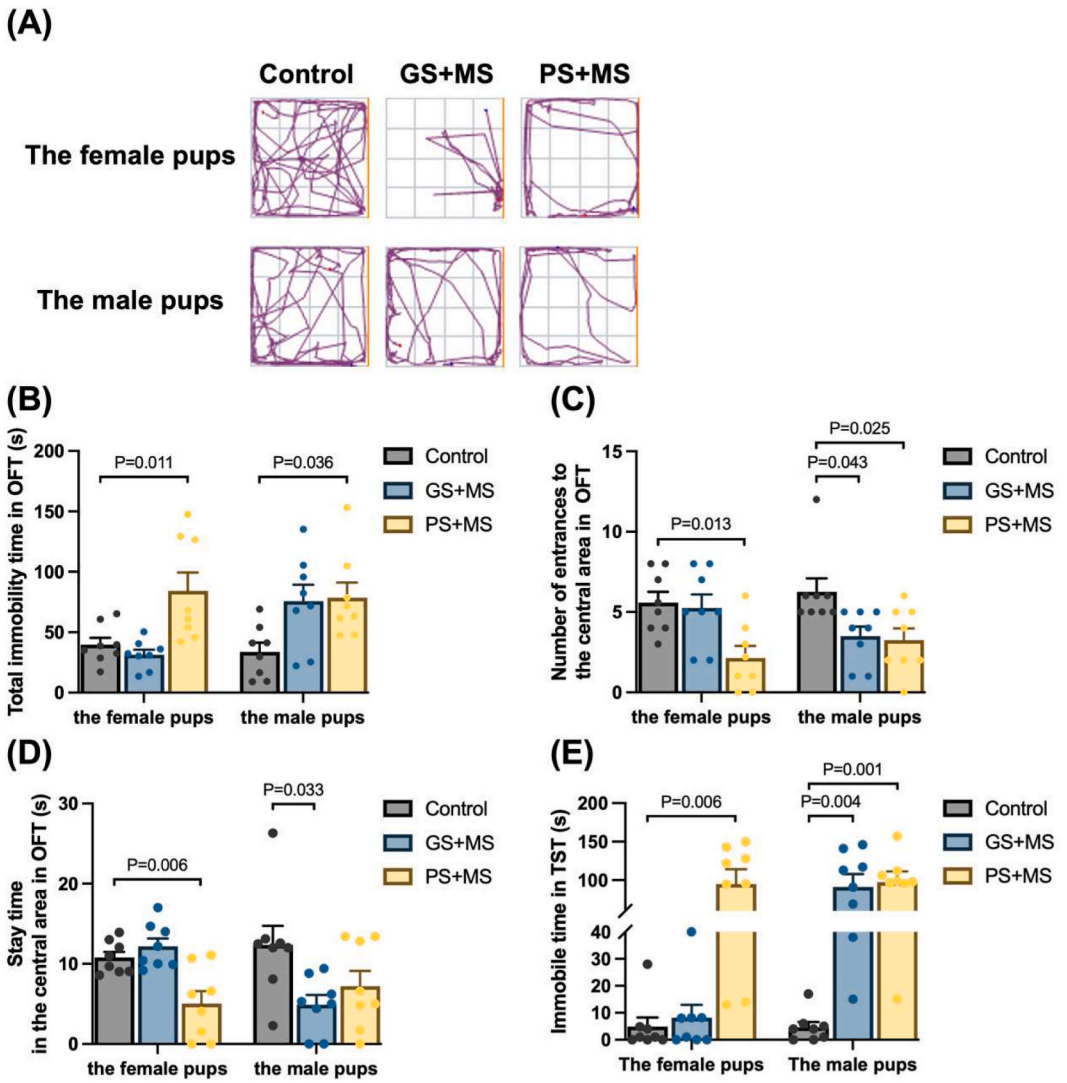

Fig. 9. Effects of PPD mothers on the behavioral of their adolescent offspring (n = 8). (A) Track plot reports of pups; (B) Total immobility time, (C) number of entrances to the central area, and (D) time staying in the central area in the open field test (OFT); (E) Cumulative immobility time in the last 4 min in the tail suspension test (TST).

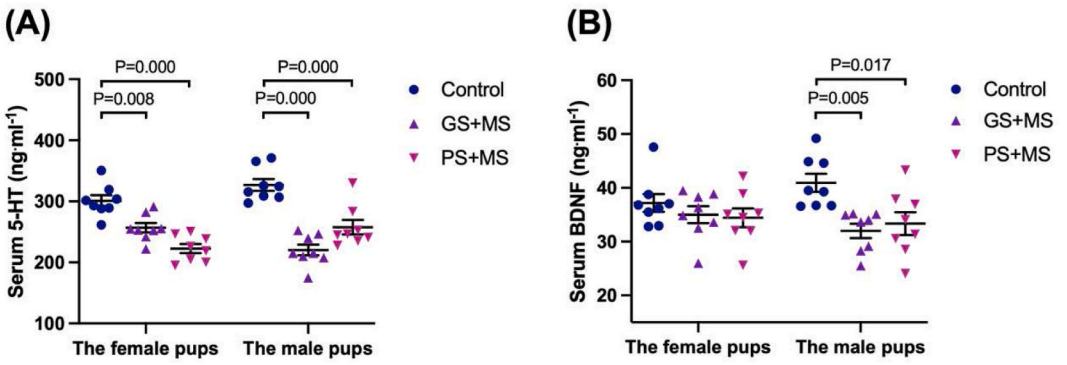

Fig. 10. Effects of PPD mothers on the neurodevelopment of their adolescent offspring (n = 8). Serum (A) 5-HT and (B) BDNF concentrations.

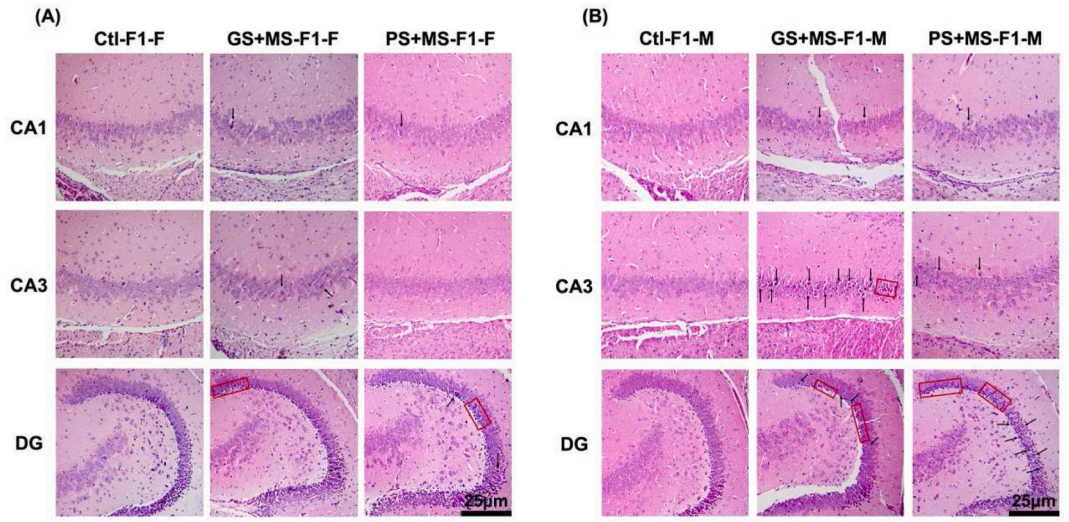

Fig. 11. Effects of PPD mothers on the neurodevelopment of their adolescent offspring (n = 8). HE staining of the hippocampal tissues from the (A) female and (B) male pups, respectively (400 ×). Red box represents extensive neuronal cell necrosis, reduced cell numbers, and aberrant cell morphology. Black arrows indicate necrosis of individual neuronal cells as assessed by deep cell staining and nuclear fixation.

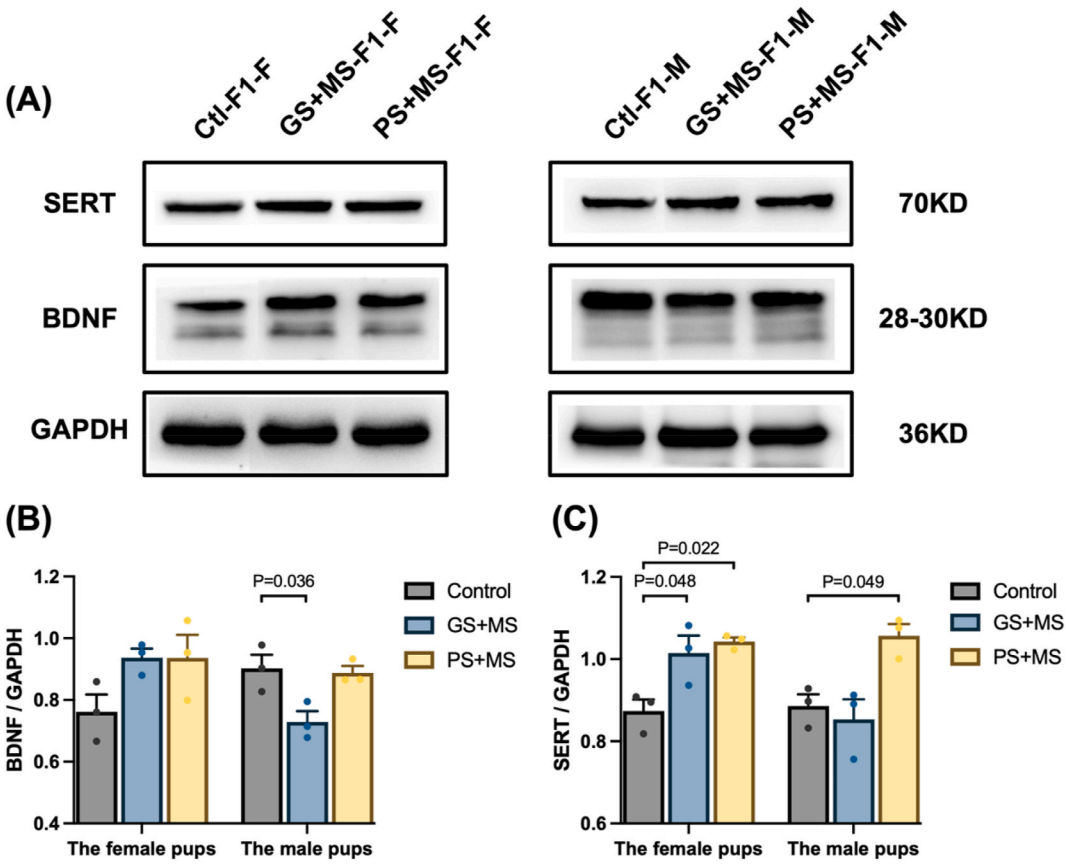

Fig. 12. Effects of PPD mothers on the neurodevelopment of their adolescent offspring (n = 3). (A) Relative expression of SERT and BDNF in pups of GS + MS and PS + MS groups; (B) Relative gray values of BDNF in the half-brain of pups; (C) Relative gray values of SERT in the half-brain of pups.

pregnancy followed by MS—on the neurodevelopment of their adolescent offspring.

Fig. 12-A and B show the relative expression of BDNF determined using WB. Only male pups of the GS + MS group had a lower relative expression of BDNF compared to normal pups (vs. Ctl-F1-M: GS + MS-F1-M, $F_{2,6} = 7.733$, $p = 0.036 < 0.05$) (Supplementary Figs. 2–1, Figs. 2–, Figs. 3–1 and Figs. 3–2). The female pups of the GS + MS group and both male and female pups of the PS + MS group exhibited a higher relative expression of SERT compared to normal pups (vs. Ctl-F1-F: GS + MS-F1-F, $F_{2,6} = 9.070$, $p = 0.048 < 0.05$; PS + MS-F1-F, $p = 0.022 < 0.05$; vs. Ctl-F1-M: PS + MS-F1-M, $F_{2,6} = 8.928$, $p = 0.049 < 0.05$) (Fig. 12-A and C) (Supplementary Figs. 2–1, Figs. 2–3, Figs. 3–1 and Figs. 3–).

4. Discussion

Hormonal changes, including alterations in allopregnanolone, oxytocin, estrogen, and stress hormones, during pregnancy and the postnatal period are primary causes of PPD. Additionally, the risk of PPD increases when women are exposed to various stressors during pregnancy or the postnatal period [6,36]. The overall prevalence of PPD during the COVID-19 pandemic (34 %) was significantly higher than during the non-pandemic period; however, the available data are mostly from developed countries, and maternal stress during the pandemic is considered a risk factor for the development of PPD [37]. The CUMS model, induced by different stressors to simulate social-environmental factors, is a suitable model for exploring the pathogenesis of PPD and new drug development [38,39]. Furthermore, in addition to somatic and social stressors, changes or disruptions in social relationships often cause depression [16]. Female mice exhibit PPD-like behavior after extended (3–4 h or longer) separation from their pups (i.e., maternal separation, MS), and the changes in hormone and neurotransmitter concentrations are similar to those observed in human PPD patients [40,41]. Therefore, CUMS and MS are two feasible approaches for constructing a PPD animal model. The modeling approach of inducing CUMS followed by MS can more accurately simulate the complex social environment and changes in the social relationships of real-life mothers, thereby presenting a more accurate profile of PPD-like symptoms. Although composite modeling is preferred to ensure high-quality animal models, there is typically a single type of stress, and the stress paradigm is not universal. Additionally, the choice of stress modeling periods for PPD animal models has not been thoroughly evaluated. Here, we investigated whether composite models induced by CUMS at different stages of pregnancy can present different PPD-like phenotypes or characteristics by comparing various parameters in PPD mice subjected to CUMS at different stages (pre-pregnancy, pregnancy, or postnatal) followed by MS.

Female mice subjected to postpartum stress and MS displayed decreased motor ability, a lack of interest in sugar water (indicative of anhedonia), and more pronounced "behavioral despair". Similarly, female mice subjected to CUMS during gestation followed by MS exhibited poor motor capacity and increased immobility time in the FST. Both groups of female mice demonstrated symptoms similar to those of PPD. Notably, the effect of stress on locomotor activity in mice remains controversial. Some studies suggest that long-term chronic restraint (one of the CUMS stressors) does not affect locomotor activity [42]. However, other studies have shown decreased locomotor activity following CUMS [43,44]. In this study, the two model groups exhibited an increase in total immobility time (PS + MS group), a decrease in total distance (GS + MS group), reduced time in the central area (GS + MS and PS + MS groups) in the OFT, and increased immobility time in the FST (GS + MS and PS + MS groups). These behaviors could either indicate PPD-like behavior following stress or reflect stress-induced changes in locomotor ability. The results of the OFT and FST will be validated by different methods. Histopathological findings indicated that female mice subjected to CUMS during the pregnancy/postnatal period followed by MS had more severely injured hippocampal neurons compared to normal mice. Neurotransmission dysfunction has been identified as potential evidence for diagnosing PPD [45,46]. We measured serum 5-HT, GABA, and NE concentrations in the model mice and found that these concentrations decreased in the GS + MS and PS + MS groups, consistent with clinical findings in PPD patients [47–49]. Serum neurotransmitter concentrations reflect their brain concentrations to some extent. 5-HT is primarily synthesized in the intestinal mucosa and brain. Tryptophan, a precursor for serotonin synthesis, is an essential amino acid obtained through dietary intake. After being absorbed through the intestinal tract, tryptophan crosses the blood-brain barrier and contributes to the synthesis of 5-HT in the brain [50]. PPD disrupts tryptophan metabolism and affects the activity of tryptophan hydroxylase 1, the rate-limiting enzyme for 5-HT synthesis, which is mainly expressed in the gastrointestinal tract. Consequently, this impacts the synthesis and release of 5-HT [51,52]. Dopamine (DA), a precursor for NE biosynthesis, is synthesized in the substantia nigra and ventral tegmental area of the basal ganglia and can be released into the bloodstream. Clinical studies have demonstrated that the density and activity of monoamine oxidase A (MAOA) increase during PPD or the onset of PPD-like symptoms, leading to enhanced catabolism of neurotransmitters such as DA, NE, and serotonin [53]. Although there is no direct evidence that GABA can cross the blood-brain barrier in humans [54], studies have shown that GABA can influence central nervous system GABA concentrations and receptor expression via the vagal pathway in the gut-brain axis [55–57]. Additionally, numerous clinical studies have shown that the concentrations of 5-HT, GABA, and NE in the blood of PPD patients are significantly lower than those in normal women, and measuring serum concentrations of these neurotransmitters can provide objective biological indicators for diagnosing PPD [47–49,58,59]. The lack of BDNF and the down-regulation of its receptor Trk B have been linked to perinatal depression in clinical and preclinical investigations [60]. The relative gray values of BDNF, Trk B, and *p*-Trk B in hippocampal tissues indicated that BDNF expression decreased in the hippocampal tissues of female mice in both model groups after stress exposure. The *p*-Trk B/Trk B ratio was significantly lower in female mice subjected to stress during gestation/postpartum followed by MS than in normal mice. A comprehensive analysis of these indicators suggests that female mice subjected to CUMS during the gestation/postnatal period followed by MS exhibit characteristic signs similar to those observed in patients with PPD.

HPA axis dysfunction is associated with postpartum mood disorders in women [61]. Although there are methodological and temporal variations in measuring HPA axis-related hormones, most authors suggest that the responsiveness of the HPA axis to external stress diminishes in postpartum depressive states [33,61]. We measured serum concentrations of CRH, ACTH, and CORT in two model

groups and found higher levels in the GS + MS group compared to the normal group. Notably, only specific stress hormones, such as CRH, were elevated in the PS + MS group. The hippocampus, a crucial brain region mediating the stress response, is rich in glucocorticoid receptors (GR), which play a negative feedback role in regulating HPA axis activity. The relative expression of GR was significantly lower in both model groups than in normal mice, consistent with previous findings [62,63]. These results indicate that CUMS and MS cause abnormalities in HPA axis functioning in female mice, with the most significant effect observed in PPD mice subjected to CUMS during gestation followed by MS. Several animal and clinical studies suggest that immunologic dysfunction is a major cause of PPD. Cytokines can predict the occurrence of depression during pregnancy [64–66]. Here, IL-6 and TNF-α expressions were significantly increased in the uterus of GS + MS group mice. Compared to female mice subjected to CUMS postpartum followed by MS, PPD mice subjected to CUMS during pregnancy showed more severe impairment of HPA axis functioning and uterine inflammation. Pregnant females are highly vulnerable to stress, with alterations in HPA axis and immune system functioning most associated with stress during pregnancy [67]. Gestational chronic stress can increase serum CORT and pro-inflammatory cytokine concentrations in female mice [67,68]. Neuroinflammation is inextricably linked to HPA axis dysfunction and altered stress hormone levels; similarly, HPA axis function can be activated by immune challenges, leading to altered stress hormone levels. Disruption of HPA axis function and inflammation together contribute to the development of PPD [69–71]. Additionally, the separation of female mice from their pups during the postnatal period further disrupts homeostasis. However, further research is required to determine how dysfunctional HPA axis and inflammation interact to contribute to PPD.

The results of the PGS + MS group were not statistically significant due to the small sample size, necessitating follow-up experiments to evaluate their reliability. Interestingly, when female mice were modeled with CUMS at different stages, pre-pregnancy stress impacted the reproductive process, leading to reduced mating behavior, slower follicular growth, and decreased estrogen production, which may lower the conception rate [72]. Therefore, CUMS modeling during pre-pregnancy may require a larger sample size, increasing costs. Additionally, the duration of pre-pregnancy modeling is longer than that of gestation or postnatal modeling, making it less ideal. In contrast, modeling strategies that induce CUMS during gestation or the postnatal period followed by MS result in stable PPD-like symptoms in mice and may complement existing animal models of PPD.

Preclinical and clinical studies have confirmed that gestational or postpartum depression in mothers adversely affects their offspring, leading to depression, neurodevelopmental delays, and behavioral problems [73,74]. Behavioral tests on the pups showed that the F1 generation of female mice in both model groups exhibited abnormal behavioral responses, with a more pronounced "desperate" state in the pups of the PS + MS group and the male pups of the GS + MS group. Additionally, hippocampal neurons in the offspring of both model groups were damaged to varying degrees. The main causes of depression are an abnormal serotonergic system and a lack of BDNF, both of which play crucial roles in early development [75–77]. Serum 5-HT concentrations decreased in the offspring of both model groups, and serum BDNF concentrations were significantly reduced in the male pups of the GS + MS and PS + MS groups compared with the control group. The relative expressions of SERT in the GS + MS-F1-F and PS + MS-F1 groups were considerably increased, whereas only the relative expression of BDNF in the brains of male pups in the GS + MS group was reduced. In summary, depression-like symptoms in female mice during pregnancy or the postnatal period due to stress can adversely affect the behavior and neurodevelopment of their adolescent offspring. Comparing the effects on the two groups of PPD mothers revealed that the offspring of female mice subjected to CUMS and MS during the postnatal period exhibited more severe behavioral and neurodevelopmental effects, such as impaired motor ability in the OFT, a significant decrease in curiosity about novel environments, and overexpressed SERT proteins in the hemispheres. Double stress (CUMS and MS) experienced by females during the postnatal period resulted in a lack of lactation, affecting the quality of maternal care and attention for pups. Moreover, the behavior and neural development of male pups were more influenced by their mothers than those of female pups [78,79]. Male pups exhibited greater levels of "behavioral despair" in the TST, suffered more severe hippocampal neuron damage, and showed a greater deficiency of BDNF in their serum and half-brain.

5. Conclusion

Our study confirmed the feasibility of composite modeling (increased behavioral despair, lower serum neurotransmitter concentrations, and more severe hippocampal histopathology) during gestation or the postnatal period. The HPA axis was more dysfunctional, and the uterus was more inflamed in female mice subjected to gestational CUMS followed by MS compared with other groups, suggesting that our modeling approach is effective for investigating the pathogenesis of PPD (e.g., HPA axis dysfunction, inflammation) and aiding in the development of new treatments. The animal model of PPD induced by CUMS and MS during the postpartum period showed stable development of PPD-like symptoms in female mice and adverse effects on adolescent offspring. Therefore, this model can be an ideal reference to investigate the correlation between PPD and the behavior and neurodevelopment of offspring and to determine the effect of anti-PPD drugs on mothers and offspring. However, the animal model of PPD induced by pre-pregnancy CUMS followed by MS still needs validation in subsequent studies. We did not compare and evaluate the three composite models of PPD with the exclusive CUMS and MS models. The specific mechanisms by which the interaction of inflammation and HPA axis dysfunction contribute to the development of PPD should be explored in future studies.

Ethics statement

The study protocol was approved by the Animals Care and Use Committee of Zhejiang University of Technology with approval number 20220715067. All animal experimental were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1996).

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Data availability statement

The data associated with this study has not been deposited into a publicly available repository due to the requirements by project funders. However, the data will be made available on request.

CRediT authorship contribution statement

Fei Fei: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation. **Ziwei Chen:** Writing – review & editing, Validation, Conceptualization. **Yi Tao:** Writing – review & editing, Supervision, Conceptualization. **Xinliang Jiang:** Data curation. **Xinyue Xu:** Data curation. **Yifeng Ma:** Validation, Data curation. **Peishi Feng:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Ping Wang:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e35363.

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