

Low-dose S-ketamine exerts antidepressant-like effects via enhanced hippocampal synaptic plasticity in postpartum depression rats

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ABSTRACT

Rapid antidepressant effects of S-ketamine have repeatedly been confirmed in patients with depression, as well as in chronic unpredictable mild stress (CUMS) animal models. However, the pharmacological study of S-ketamine for anti-postpartum depression has not been considered. In this study, the classical method of reproductive hormone withdrawal was used to construct a rat model of postpartum depression (PPD). Subsequently, the study evaluated the effects of low-dose S-ketamine on behavior and synaptic plasticity, which is related to depression, in the hippocampus of PPD rats. Multiple behavioral tests were used to evaluate depression-like behaviors in PPD models. Synaptic plasticity of the hippocampus can be demonstrated by Western blot, Golgi staining, transmission electron microscopy, and electrophysiological recording. Our study provides insight into the role of low-dose S-ketamine in antidepressant as well as antianxiety and indicates that maintaining synaptic plasticity is a key target for S-ketamine therapy for postpartum depression induced by reproductive hormone withdrawal.

1. Introduction

Postpartum depression (PPD) is a common condition that was estimated to affect nearly 10–15% of new mothers and that can have a devastating effect on the mother as well as the infant (Liu et al., 2019). PPD negatively affects family relationships, including marital discord, persistent doubt of the ability to take care of the infant, impaired mother-infant contact, and even thoughts of self-harm or harm of the infant (Ko et al., 2017; O'connor et al., 2019). However, until recently, there were no approved medications that effectively alleviated postpartum depression (PPD). The US Food and Drug Administration approved brexanolone and intranasal S-ketamine in February 2019, brexanolone for PPD and treatment resistant major depressive disorder (MDD) (Kanes et al., 2017; Kim et al., 2019). Since this time, there has been a growing interest in developing additional therapeutics for PPD and extending the therapeutic indications of intranasal S-ketamine, including for the treatment of PPD. This research was the pioneer to conduct a preclinical study of S-ketamine as a prophylactic treatment for postpartum depression and we hope to promote consideration of

S-ketamine as a clinical intervention for PPD in the near future.

Numerous underlying neurobiological mechanisms are implicated in PPD, including genetic and epigenetic factors, biochemical factors, neuroinflammatory changes, as well as bioelectric circuit-level changes (Payne and Maguire, 2019). These risk factors mainly include, but are not limited to, dramatic fluctuation of ovarian hormones and the release of stress hormones caused by severe labor pain. Although estrogen deficiency is associated with the development of postpartum depression, the mechanisms by which reproductive hormones modulate depression-like behaviors, to a large extent, remain unknown. We established a model of postpartum depression induced by hormone withdrawal, and showed that the reduction of synaptic plasticity in the hippocampus contributes to depression-like behavior and neurobiological changes in PPD rats.

What's more, ketamine has rapid and lasting anti-depressive effects through influencing synaptic plasticity in previous studies (Kavalali and Monteggia, 2012; Williams and Schatzberg, 2016). Ketamine stimulates the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, resulting in the activity-dependent release of BDNF (Duman

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et al., 2012; Autry et al., 2011). The changes described above increase synaptic plasticity and contribute to the rapid antidepressant effect of ketamine. Maintenance of synaptic plasticity by ketamine can be demonstrated by increasing the number of mature mushroom-shaped spines and increasing excitatory postsynaptic currents (Niciu et al., 2014). Meanwhile, ketamine can increase levels of postsynaptic density proteins, including glutamate-AMPA receptor-1 (GluR1), postsynaptic density protein-95 (PSD95), and synapsin-1 and activity-regulated cytoskeletal protein (Arc) (Li et al., 2010). Hence, synaptic plasticity, keystone in the pathophysiology of depression and postpartum depression, is a key target for developing novel antidepressant drugs with fast onset of action and broader efficacy (Covvey et al., 2012; Murrough, 2012; Hasselmann, 2014; Ardalan et al., 2017a; Ohgi et al., 2015). S-ketamine is the S-enantiomer of racemic ketamine. It is two times more potent compared to the racemic mixture. This contributes to the favoring of S-ketamine in therapeutic roles in depression models (Kaur et al., 2021). Furthermore, previous study showed that S-ketamine may promote microvascular elongation to increase synaptic plasticity and neuronal activity in CUMS models (Ardalan et al., 2017a; Castrén and Hen, 2013). Nonetheless, it is unclear whether postpartum depression induced by hormone withdrawal and associated with synaptic plasticity damage can be reversed by S-ketamine. Therefore, this study testing the above hypothesis.

There are some clinical studies that report effective prevention of postpartum depression in women undergoing cesarean section with prophylactic ketamine (Ma et al., 2019; Yao et al., 2020), but additional multicenter double blind crossover studies are required to confirm this. There is also an ongoing clinical trial (NCT03927378) investigating S-ketamine for PPD, no results have been published to date. Hence, there are no other published works that have investigated the potential utility of S-ketamine for the indication of postpartum depression in preclinical studies. In this study, we are committed to exploring whether the S-ketamine of lower dose, compared with the S-ketamine of a conventional subanesthetic dose (10–20 mg/kg (Ardalan et al., 2020a; Pereira et al., 2019; Treccani et al., 2019a; Ardalan et al., 2017b; Aikawa et al., 2020)) used in rodent model, possesses antidepressant effects through improving synaptic plasticity in PPD rodent models. We established rat models of PPD and examined a group of indicators related to synaptic plasticity, including expression level of BDNF, PSD-95 and Synapsin-1, spine density, as well as change of ultra-microstructure and electrophysiology between synapse. What's more, all animals underwent behavioral tests to assess depressive and anxiety-like behavior prior to testing indicators above.

2. Materials and methods

2.1. Subjects

Two-month-old female Sprague–Dawley rats (Jinan Peng Yue Laboratory Animal Breeding Co. Ltd., Jinan, China.) were settled three per in the amber polycarbonate cages in this study. All animals were maintained under a 12 h light/dark cycle (lights on at 07:00 h) and the room temperature at $(22 \pm 2^\circ\text{C})$ with ad libitum access to maintenance rat chow and sterile water. Animals were accustomed to the housing conditions for at least 7 days before the beginning of the experimental procedures. During this period, we establish baseline sucrose intake values for the sucrose preference test. In line with the rule of making every effort to minimize animal suffering and reduce the number of animals used. All animal procedures were conducted following the ARRIVE guidelines and National Institutes of Health (NIH) guidelines for the care and use of laboratories and were approved by the Animal Care Committee of Weifang Medical University.

2.2. Experimental design

As shown in Fig. 1, after all animals were acclimated to their new environment, baseline levels of sucrose preference and open field performance were evaluated. Rats were excluded if they exhibited any abnormal behavioral indicators (exceed the range of mean ± 3 * standard deviation). As a result, a total of seven rats were excluded and twelve eligible rats per group were selected for testing. The groups were based on a combination of drug treatment and ovariectomy (OVX) surgery and are as follows: non-OVX–(1) sham; OVX–(2) PPD; (3) L: low dose S-ketamine (2.5 mg/kg); (4) H: high dose S-ketamine (conventional subanesthetic dose, 10 mg/kg). Then, animals were subjected to the corresponding surgical procedure as per grouping. The animal has subsequently performed hormone administration for 23 days. Next, an open field test and sucrose preference test were performed again on all animals to verify the success of pseudopregnancy models. Eventually, after drug treatment and subsequent behavioral tests were performed, the rats were killed for subsequent studies.

2.3. Pseudopregnancy modes

Rats were operated using a relative asepsis technique. Rats were anesthetized with 7% chloral hydrate (5 mL/kg) administered intraperitoneal injection and fixed at the prone position. A bilateral 1.5 cm longitudinal dorsal incision was made. Muscle and peritoneum were separated carefully. The ovaries and fallopian tubes were identified and

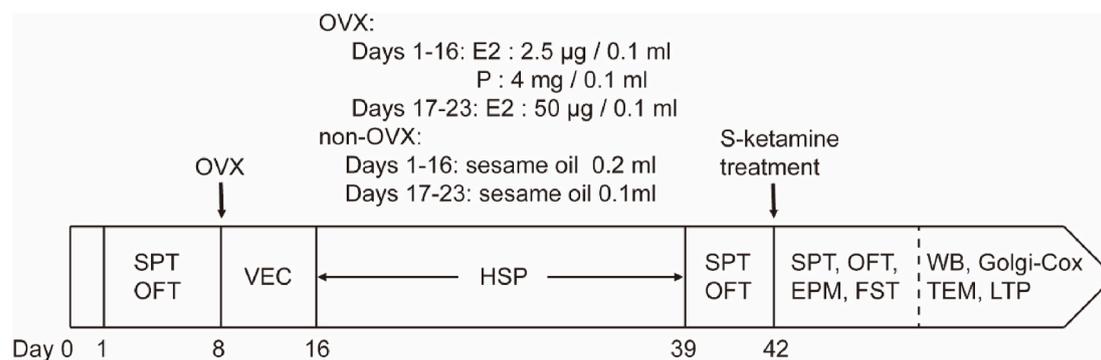


Fig. 1. The experimental timeline. During 7 days of adaptation, the SPT and OFT were carried out for gathering sucrose and spontaneous movement baseline, and rats were assigned to each experimental group after simple randomization. On day 8, OVX and sham surgeries were performed on rats. Then, rats were recovered for one week to eliminate the effects of endogenous reproductive hormones, and cytology of vaginal shedding was performed during this period to determine the effect of ovariectomy. On day 16, reproductive hormones were administered to rats to complete the HSP procedure. Subsequently, SPT and OFT were again implemented to verify the validity of the pseudopregnancy model. Next, rats were administered with drugs, and the behaviors were tested. In the end, rats were decapitated after the behavioral tests, and the brain tissues were collected for subsequent experiments.

ligated, then the ovaries were removed (Fig. 2). The skin was sutured and penicillin was intramuscularly injected to prevent infection (Waynforth and Flecknell, 1992). The control rats were sham-operated: only the surrounding adipose tissue of ovaries was removed. Vaginal exfoliation cytology (Fig. 3) was performed for 5 consecutive days after surgery and allowed 1-week recovery to eliminate endogenous estrogen and progesterone (Goldman et al., 2007). In the light of Galea et al. (2001), rats were administrated hormones (estradiol and progesterone) according to the doses listed in Fig. 1 for 23 days to induce hormone simulated pregnancy (HSP). Both concentrations of estradiol and progesterone were administered in a volume of 0.1 mL/rat. The sham surgery group received sesame oil hypodermic injections on the same schedule.

2.4. Drugs

Estradiol benzoate injection and progesterone injection were purchased from Shanghai Quan Yu Biotechnology Animal Pharmaceutical Co., Ltd. and were dissolved in sesame oil for subcutaneous injection. S-ketamine was obtained from Jiangsu Heng Rui Medicine Co. Ltd. and was diluted in 0.9% saline before usage. S-ketamine was diluted into two different concentrations (12.5mg/mL and 3.125mg/mL) for intraperitoneal injection to ensure that the volume of the therapeutic drug was similar for each rat in L and H groups. S-ketamine injection dosage has been described in Section 2.2.

2.5. Behavioral tests

To ensure consistency of the timing of behavioral testing, each behavioral experiment carries out at 8 a.m. All experimental apparatus was carefully cleaned with 75% alcohol after each rat's test. All trials and datum were tracked and analyzed with Smart version 3.0 (Harvard Apparatus, USA), and videos were recorded by Hikvision 700TVL CCD camera (Hangzhou Hikvision Digital Technology Co. Ltd., China).

2.5.1. Sucrose preference test (SPT)

The SPT is a rodent test that has relevance to the clinical condition of

depression, specifically the endophenotype of anhedonia (Navarre et al., 2010). Animals were single housed in this section. The sucrose preference experiment was carried out three times: (1) when animals adapted to their living conditions; (2) after animals received the HSP procedure; (3) after animals were treated with drugs or saline. The intake of sucrose water and pure water were stable after an initial sucrose training, and all groups had a similar sucrose preference in each group of rats. The SPT was then performed as follows. During the acclimatization phase, two bottles of pure water were put in each cage. After 24 h, two bottles of pure water were replaced with 1% sucrose water. After 24 h, a bottle of pure water and a bottle of 1% sucrose water were replaced in a cage for 24 h. Then, the SPT was performed 24 h after fasting and water cut-off to examine the rats' sucrose consumption for 24 h. During the testing, the water and sucrose bottles were switched to avoid position preference. Sucrose preference was evaluated as the ratio of consumed sucrose solution to total consumption (preference (%) = sucrose solution intake/total intake \times 100).

2.5.2. Locomotor activity

Spontaneous locomotor activity was measured in the open field test (OFT). The open-field apparatus consisted of a black plastic square arena (100 \times 100 \times 50 cm) was placed in a quiet room with dim illumination. The animals were placed in the center of the open space, and then their behavior was recorded by a video tracking system for 5 min. The total distance moved, the number of line crossing, immobile times, the time spent in the center zone (central 50 \times 50 cm area), and the vertical activity (wall climbing, rearing, and grooming activity) were recorded and analyzed.

2.5.3. Elevated plus maze (EPM)

The elevated plus maze (EPM) test, which is a reliable and sensitive method for assessing anxiety in rodents, consists of an elevated plus-shaped platform with two open arms (50 \times 10 cm) and two closed arms (50 \times 10 cm) with 40 cm walls. The lab room is equipped with 40 W incandescent lamps (about 350–470 lumens) with an average light level of about 30 lux. Each rat was placed in the central part of the cross maze and allowed to explore freely for 5 min. Total distance moved,



Fig. 2. Bilateral ovaries (arrow) removed are shown here.

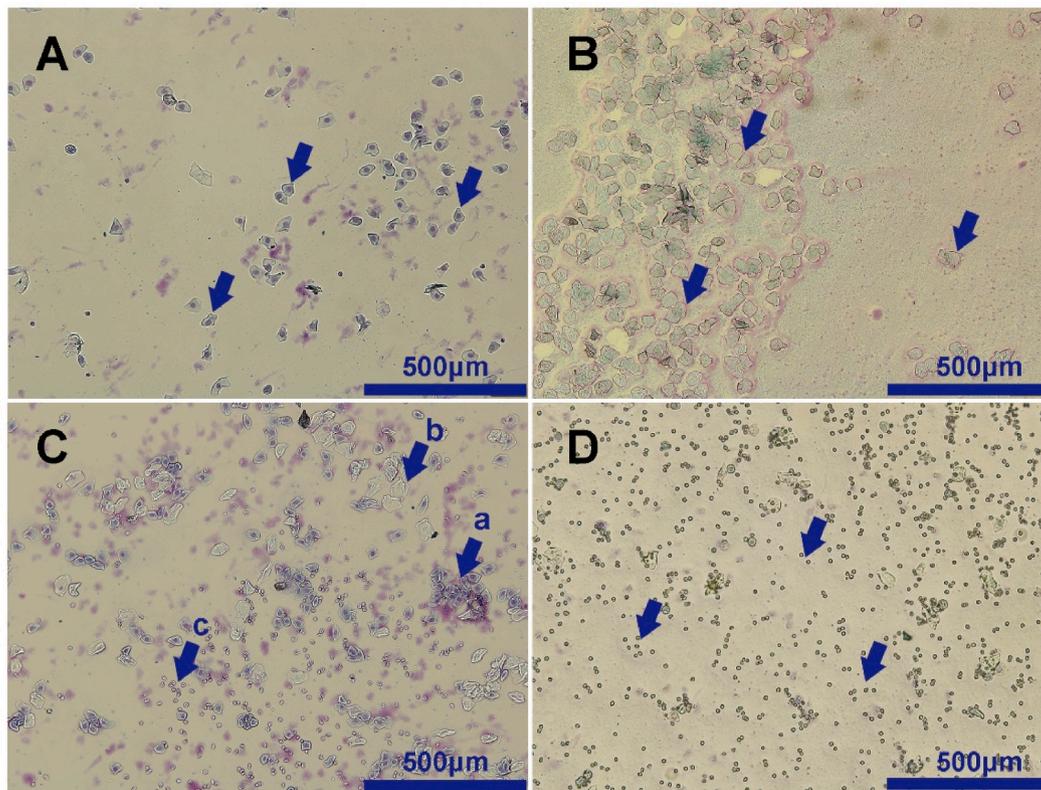


Fig. 3. Wright-Giemsa staining of vaginal exfoliated cells. Rats in the sham group displayed regular changes during 5 consecutive days of smearing, while the ovariectomized rats lost the rhythm. A: Proestrus, with the typical nucleated epithelial cells (arrow) predominating. B: Estrus; the horizon is covered with classic keratinized cells (arrow). C: Metestrus; there is a combination of nucleated epithelial cells (arrow a), keratinized cells (arrow b), and a few smaller leukocytes (arrow c). D: Diestrus; a classic leukocytic smear (arrow) with a few larger round epithelial cells. The scale bar = 500 μm .

number of entry into open arms and time spent in open arms were calculated. Meanwhile, the number of up-right was recorded.

2.5.4. Forced swim test (FST)

The FST is a behavioral test used to screen the efficacy of potential antidepressant compounds. The instrument used in this experiment is a cylindrical transparent vessel, 55 cm in height, 30 cm in diameter, 24 ± 1 °C in water temperature, and 45 cm in water depth. The FST was divided into two stages—pretest and test. In the pretest stage, rats were placed in the water for 15 min, while they were placed in the water for 5 min in the testing stage. The pretest was conducted 24 h prior to testing. Moreover, diving and climbing behaviors at each stage were recorded by video. Before each subject was tested, water was changed and the cylinder was thoroughly wiped with 75% alcohol gauze to eliminate any potential smell left by the previous animal. The above video was later recorded the immobility time by an observer who was blind to the rat's condition. Immobility is defined as floating without any movement or without rapid movement of the forelimb or hindlimb to break the water and to move in a paddling manner.

2.6. Western blot

Isolated hippocampal tissue was bathed in a RIPA buffer with protease inhibitors and phosphatase inhibitors. Subsequently, the tissue was ground into a suspension using an electric homogenizer. After grinding to obtain a homogeneous mixture, the lysate was centrifuged at 12,000 rpm in a cold centrifuge at 4 °C for 15 min. Afterward, the supernatant was carefully transferred into EP tube. Protein concentration was determined by the bicinchoninic acid assay (BCA) method. Before loading the lysate samples onto the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel for protein

separation, each protein lysate was diluted to the same concentration with $5 \times$ loading buffer and boiled for 5 min in a hot-covered metal bath at 100 °C. Then, the proteins in each sample were separated on 12% SDS-PAGE gels and subsequently transferred onto a 0.45 μm polyvinylidene fluoride (PVDF) membrane. Then, after blocking the membrane with 5% skim milk for 3 h. Then, the membranes were incubated with primary antibody against BDNF, GAPDH, SYN-1 and PSD-95 (Wuhan SANYING Biological Technology Co., Ltd., Wuhan, China) at 4 °C overnight. Subsequently, after washing the membrane five times with tris-buffered saline Tween 20 (TBST) for 3 min each time, the membranes were incubated in the secondary antibody of HRP conjugated goat anti-rabbit for 2 h at room temperature. Chemiluminescence HRP substrate was used to visualize the protein bands and the analysis of the protein bands grayscale values was performed by Image J software.

2.7. Golgi-Cox staining

Golgi staining was carried out according to previous research methods (Zhong et al., 2019). The hemispheres of the brain were washed with distilled water and soaked in impregnation solution for 2 weeks. Then transfer it into the cryoprotectant solution at 4 °C for 2–3 days. When the tissue sank into the bottom, the brains were sliced into 100 μm slices with a cryostat (Leica CM1860 UV, Germany) and placed on adhesive slides. Finally, the dried slide specimens were used for staining, dehydration, and mounting step. The image was obtained by optical microscopy (BX53, Olympus Corporation, Tokyo, Japan). Dendrite branches were traced by Fiji software (NIH, Bethesda, MD, USA), and the dendritic length and number of the branches were calculated.

2.8. Transmission electron microscopy (TEM)

Rats were decapitated and the brains were quickly taken out on ice. Then, hippocampi were quickly separated and cut into 1 mm³ pieces with a sharp scalpel, and placed in 2.5% glutaraldehyde. Next these tissues were fixed, dehydrated, embedded, solidified, sectioned, stained, and observed. Ten micrographs were arbitrarily taken from each rat using an HT-7800 transmission electron microscope (TEM) and the synaptic structural parameters including the synaptic cleft width, the postsynaptic density (PSD) thickness, active zones length and the synaptic interface curvature (curvature = arc length/chord length) were quantitatively analyzed by Image-Pro 6.0 software.

2.9. Hippocampus slices preparation and electrophysiological recordings

Hippocampal slice was prepared according to previous description (Zhou et al., 2007; Cui et al., 2016). Rats were anesthetized and the whole brain was taken out on the ice. The brain was quickly dipped in an oxygenated ice-cold cutting solution (pH 7.4) containing (in mM): 2.5 KCl, 1 NaH₂PO₄, 26 NaHCO₃, 1 CaCl₂, 7 MgSO₄, 30 Glucose, 119 choline chloride, 3 sodium pyruvate, 1 kynurenic acid and 1.3 sodium L-ascorbate. In the cutting solution, a 300- μ m-thick coronal slice of the hippocampus were cut out. Then, the slices were immediately transferred to the artificial cerebrospinal fluid (ACSF) (in mM): 85 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 24 NaHCO₃, 0.5 CaCl₂, 4 MgCl₂, 25 glucose and 50 sucrose for incubation. The hippocampal slices were then kept at 26 °C at least 1 h prior to recording. In order to induce LTP, FHC bipolar platinum electrodes and the recording electrode were placed in the Schaffer collateral–commissural fibers and hippocampal CA1 region respectively. During the recording period, ACSF was perfused continuously. In LTP study, all test stimuli and tetanus pulses were 100 μ s in duration and 1/2–2/3 of maximal stimulation strength (100 μ A). A steady baseline was recorded for at least 10 min before the induction of LTP. The field excitatory postsynaptic potential (fEPSP) slope between 10% and 90% was used to indicate the fEPSP magnitude. Slices were considered to demonstrate LTP if the amplitude of the fEPSP was increased by at least 15% compared to baseline. Analog to digital conversion was performed using Digidata 1440A (Molecular Devices) and the signal was acquired using Clampfit 10 (Molecular devices). Data was analyzed using Clampfit 10 (Axon Instruments). All the recording data was acquired at 10 kHz and filtered at 1 or 2 kHz. Data is normalized to the mean baseline value and shown as mean \pm SD.

2.10. Statistical analysis

SPSS 25.0 was used to analyze all data, which is expressed as the mean \pm standard deviation (SD). All charts were produced by the GraphPad Prism, version 7.0 (GraphPad, San Diego, CA, USA) software. Paired-samples *t*-Test was used for comparisons of behavioral effects of rats before and after HSP procedure. The therapeutic effects of S-ketamine in PPD rats were analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett's test or Tamhane T2 test. If the data does not conform to the normal distribution, the nonparametric test (Kruskal Wallis test) is used. Bonferroni corrections was performed where necessary to control for Type I error. For all tests, differences were considered statistically significant at a level of $p < 0.05$.

3. Results

3.1. Effects of hormone withdrawal on pseudopregnancy modes

In this section, all animals had similar sucrose preference and spontaneous activity at baseline (data not shown). The experimental data indicated that sucrose preference and travel distances, compared with before hormone withdrawal, decreased significantly (paired sample *T*-test; sucrose preference: $t_{35} = 29.882$, $p < 0.001$; travel distances:

$t_{35} = 26.603$, $p < 0.001$) after hormone withdrawal (Fig. 4A and B). These results suggest that the pseudo-pregnancy model of postpartum depression is relatively reliable.

3.2. Effects of S-ketamine treatment on depressive behavior in PPD rats

In SPT, all animals had a similar sucrose preference at baseline (Fig. 5A, dotted line). The results of SPT showed that the sucrose preference of rats in the PPD group after ovarian hormone withdrawal was significantly lower than that in the Sham group (one-way ANOVA, $F_{3, 44} = 37.778$, Dunnett's test, $p < 0.001$). And low-dose S-ketamine significantly improved sucrose preference of PPD rats (Dunnett's test, $p < 0.001$, Fig. 5A).

In FST, compared with sham group, the immobile time was significantly increased (one-way ANOVA, $F_{3, 44} = 19.178$, Dunnett's test, $p < 0.001$) in PPD group. Low-dose S-ketamine treatment significantly reversed the prolongation of immobility in FST (Dunnett's test, $p = 0.005$, Fig. 5B). Moreover, we found that low-dose S-ketamine significantly improved the number of climbing (one-way ANOVA, $F_{3, 44} = 12.356$, Tamhane T2 test, $p = 0.047$, Fig. 5C) in FST, but had no effect on the number of diving (Kruskal-Wallis test, $H = 1.831$, Bonferroni calibration, $p = 0.608$).

In the OFT (Fig. 6), one-way ANOVA revealed the total distance ($F_{3,44} = 18.057$, Dunnett's test, $p < 0.001$), the time spent in the central area ($F_{3,44} = 132.165$, Dunnett's test, $p < 0.001$), the number of line crossings ($F_{3,44} = 4.932$, Tamhane T2 test, $p = 0.002$) and the number of rearing ($F_{3,44} = 9.957$, Dunnett's test, $p = 0.007$) in the PPD group were significantly less than that reported in the sham group. Compared with the Sham group, immobility time of rats in PPD group was markedly increased (one-way ANOVA, $F_{3,44} = 40.449$, Dunnett's test, $p < 0.001$). This suggests animals in the PPD group exhibited an increase in depression-like behavior, which could be effectively alleviated by low-dose S-ketamine treatment (the total distance: Dunnett's test, $p = 0.016$; the time spent in the central area: Dunnett's test, $p < 0.001$; immobility time: Dunnett's test, $p < 0.001$; the number of rearing: Dunnett's test, $p = 0.013$). Moreover, the number of rearing was significantly elevated (Dunnett's test, $p = 0.048$) in the H group compared with the L group. Meanwhile, in the OFT, the time spent in the central area of rats in group H was significantly less (Dunnett's test, $p < 0.001$) than that in group L. The number of fecal pellets of rats in group H was significantly more than that in group L (Kruskal-Wallis test, $H = 13.503$, Bonferroni calibration, $p = 0.026$). This results suggest that a high dose of S-ketamine might contribute to more anxiety compared with a low dose of S-ketamine.

Hence, the elevated plus-maze was implemented further to explore the changes in anxiety-like behavior in rats (Fig. 7). In the elevated plus maze test, one-way ANOVA revealed the total distance of movement ($F_{3,44} = 33.116$, Dunnett's test, $p < 0.001$), spent time in open arms ($F_{3,44} = 424.582$, Dunnett's test, $p < 0.001$), number of entering the open arms ($F_{3,44} = 33.841$, Dunnett's test, $p < 0.001$) and number of upright movement ($F_{3,44} = 20.743$, Dunnett's test, $p < 0.001$) of rats in PPD group were significantly lower than those in sham group. And low-dose S-ketamine treatment significantly increases exercise and exploration behavior of PPD rats (the total distance: Dunnett's test, $p = 0.005$; spent time in open arms: Dunnett's test, $p < 0.001$; number of entering the open arms: Dunnett's test, $p < 0.001$; number of upright: Dunnett's test, $p = 0.009$). It is noteworthy that, surprisingly, rats in the H group spent less time (Dunnett's test, $p < 0.001$) in open arms and showed more vertical exploration (Tamhane T2 test, $p = 0.003$) compared with rats in the L group. Combine the data of exploration of OTF and data of exploration of open arms of EPM, these results suggested that although high doses of S-ketamine revert depression-like behavior, they may result in more anxiety.

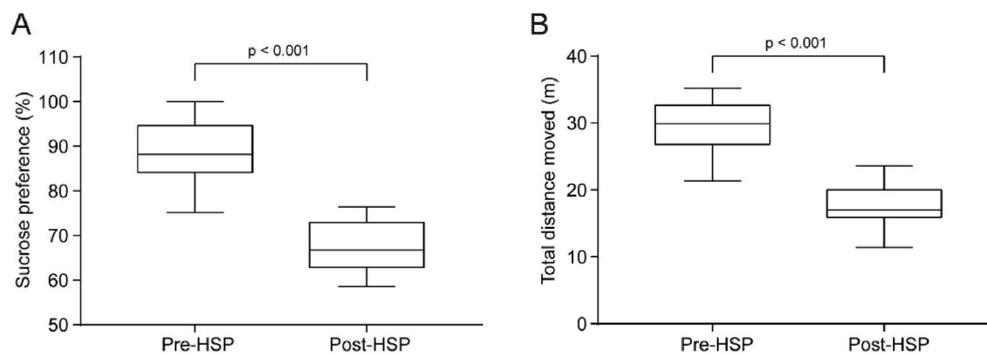


Fig. 4. Effects of hormone withdrawal on pseudopregnancy modes. (A) Paired T-test of sucrose preference before and after HSP. (B) Paired T-test of total distance moved in OFT before and after HSP. Values are expressed as mean \pm SD (n = 36 per group, including model rats in the PPD, L, and H groups).

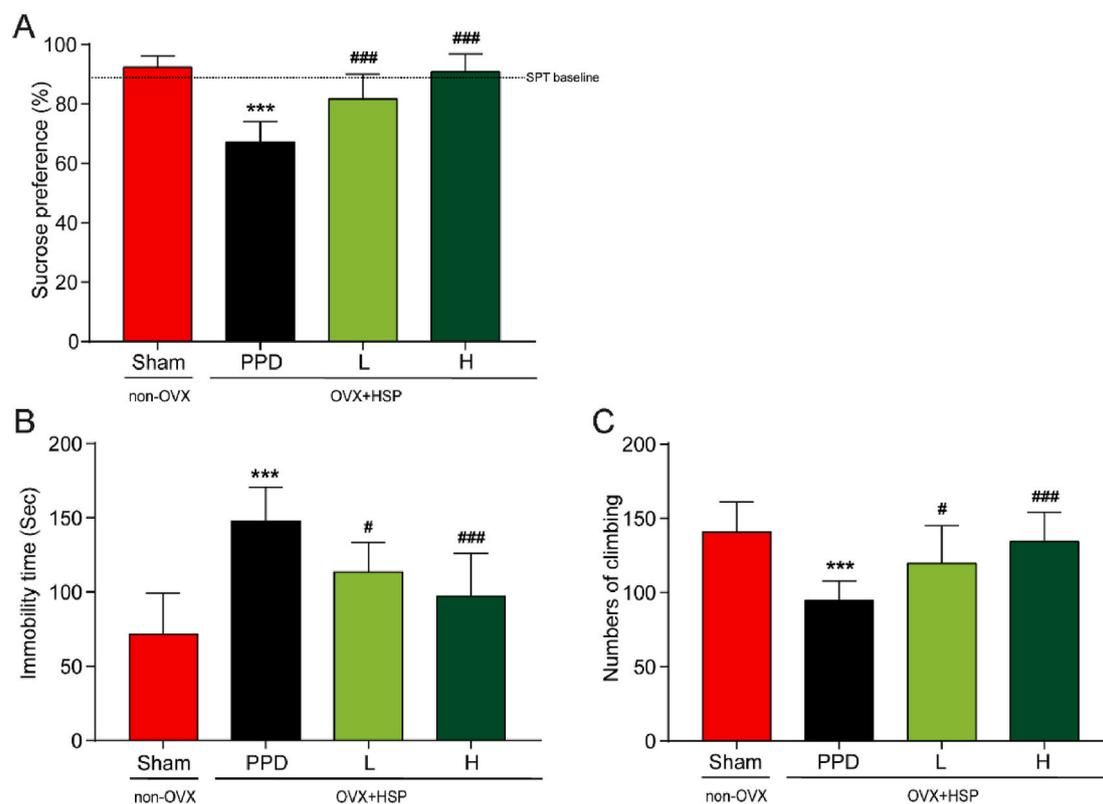


Fig. 5. Effects of S-ketamine on sucrose preference and forced swimming in rats. (A) The proportion of sucrose intake volume to total fluid intake. (B) Total time of immobility was statistically analyzed in the forced swimming test. (C) The number of climbing in the FST. Values are expressed as mean \pm SD (n = 12 per group). *** p < 0.001 vs. Sham group; # p < 0.05, ### p < 0.001 vs. PPD group.

3.3. Effects of S-ketamine treatment on levels of synaptic marker protein in PPD rats

In order to explore the effect of S-ketamine treatment on the expression level of synapse-associated proteins in PPD model, the levels of BDNF, Syn-1 and PSD-95 were examined by Western blot (Fig. 8). Western blot analysis revealed that expression of synapse-associated protein including BDNF (one-way ANOVA, $F_{3,16} = 14.687$, Dunnett's test, $p < 0.001$), Syn-1 (one-way ANOVA, $F_{3,16} = 8.754$, Dunnett's test, $p = 0.001$), and PSD-95 (one-way ANOVA, $F_{3,16} = 19.202$, Dunnett's test, $p < 0.001$) in the hippocampus was significantly reduced in the PPD rats compared to rats in the sham group. However, low-dose S-ketamine therapy significantly reversed the reduction of these proteins of PPD rats (the expression of BDNF: Dunnett's test, $p < 0.001$; the expression of Syn-1: Dunnett's test, $p = 0.006$; the expression of PSD-95: Dunnett's test, $p < 0.001$). This result suggests that low-dose S-ketamine plays a

crucial role in maintaining synaptic plasticity.

3.4. Effects of S-ketamine treatment on the number of dendritic spines in PPD rats by Golgi-Cox staining

The dendritic spines of the pyramidal neurons in the CA1 region were quantified by Golgi-Cox staining (Fig. 9). Data analysis suggested that decreased dendritic spine densities were observed in the PPD group rats compared with the sham group (one-way ANOVA, $F_{3,56} = 23.986$, Dunnett's test, $p < 0.001$), and injection of low-dose S-ketamine increased the density of dendritic spines of PPD rats (one-way ANOVA, Dunnett's test, $p = 0.004$). Spine densities of rats in L and H groups were significantly increased compared with rats in the PPD group, indicating an increase in synaptic plasticity.

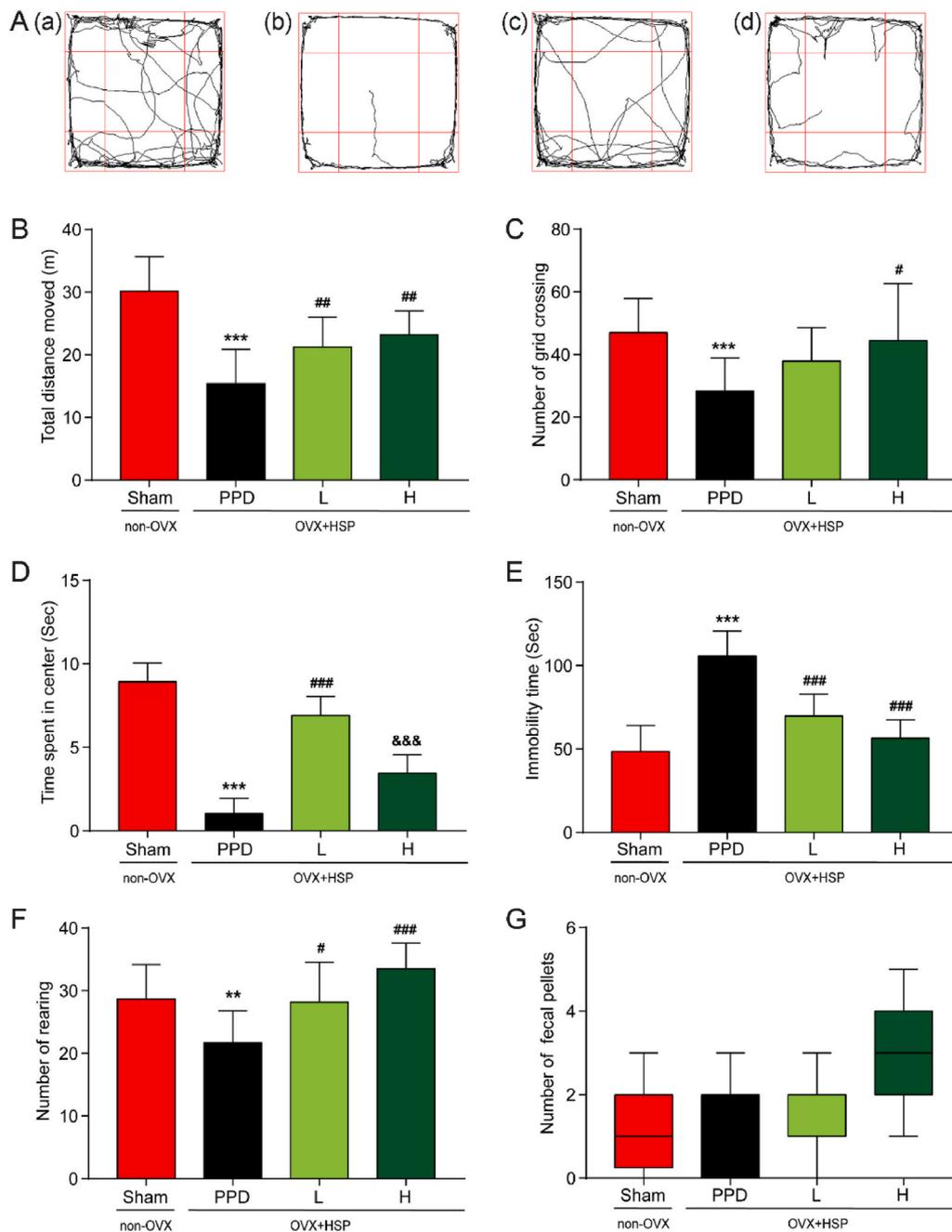


Fig. 6. Effects of S-ketamine on locomotor activity in OFT. (A) The movement trails of rats in each group (a, Sham group; b, PPD group; c, L group; d, H group); (B) The total distance traveled by rats during the open field test; (C) Several line crossings of rats in the open field test; (D) The amount of time spent in the central area; (E) Rats' total immobility time in the open field test; (F) Numbers of rearing in the open field test of rats. (G) The number of fecal particles produced by rats during OFT. Values are expressed as mean \pm SD ($n = 12$ per group). ** $p < 0.01$, *** $p < 0.001$ vs. Sham group; # $p < 0.05$, ### $p < 0.001$ vs. PPD group; &&& $p < 0.001$ vs. L group.

3.5. The treatment of S-ketamine regulated the ultrastructure of synapses observed by transmission electron microscopy

The changes in the synaptic ultrastructure of the pyramidal neurons in the CA1 region in each group were observed under TEM (Fig. 10A–D). Numerous synaptic vesicles were observed in the anterior membrane region, and abundant PSD was detected in the posterior membrane. In the sham group, the synaptic cleft was visible, and the presynaptic membrane was clear and uniform with a complete outline. However, the synaptic space was widened (one-way ANOVA, $F_{3,60} = 10.841$, Dunnett's test, $p < 0.001$) and blurred in the PPD group compared with sham group. And the thickness of postsynaptic densities (one-way ANOVA, $F_{3,60} = 16.118$, Dunnett's test, $p < 0.001$), the curvature of the synaptic interface (one-way ANOVA, $F_{3,60} = 4.205$, Dunnett's test, $p = 0.009$) and the length of active regions (one-way ANOVA, $F_{3,60} = 5.405$,

Dunnett's test, $p = 0.003$) were descended as well. Administration of low-dose S-ketamine restored the indicators of morphological changes of PPD rats to the synapse (synaptic space: Dunnett's test, $p = 0.001$; thickness of postsynaptic densities: Dunnett's test, $p = 0.001$; the length of active regions: Dunnett's test, $p = 0.022$; Fig. 10E–H).

3.6. Effects of S-ketamine on Schaffer-CA1 LTP of hippocampus in PPD rats

To study the effects of S-ketamine on synaptic plasticity in rat hippocampus, LTPs of hippocampus were examined (Fig. 11). In this section, our results showed that the LTP was significantly decreased in rats of the PPD group compared with Sham group (one-way ANOVA, $F_{3,85} = 18.316$, Tamhane T2, $p < 0.001$). LTPs after low-dose S-ketamine therapy were significantly increased compared with rats in the PPD

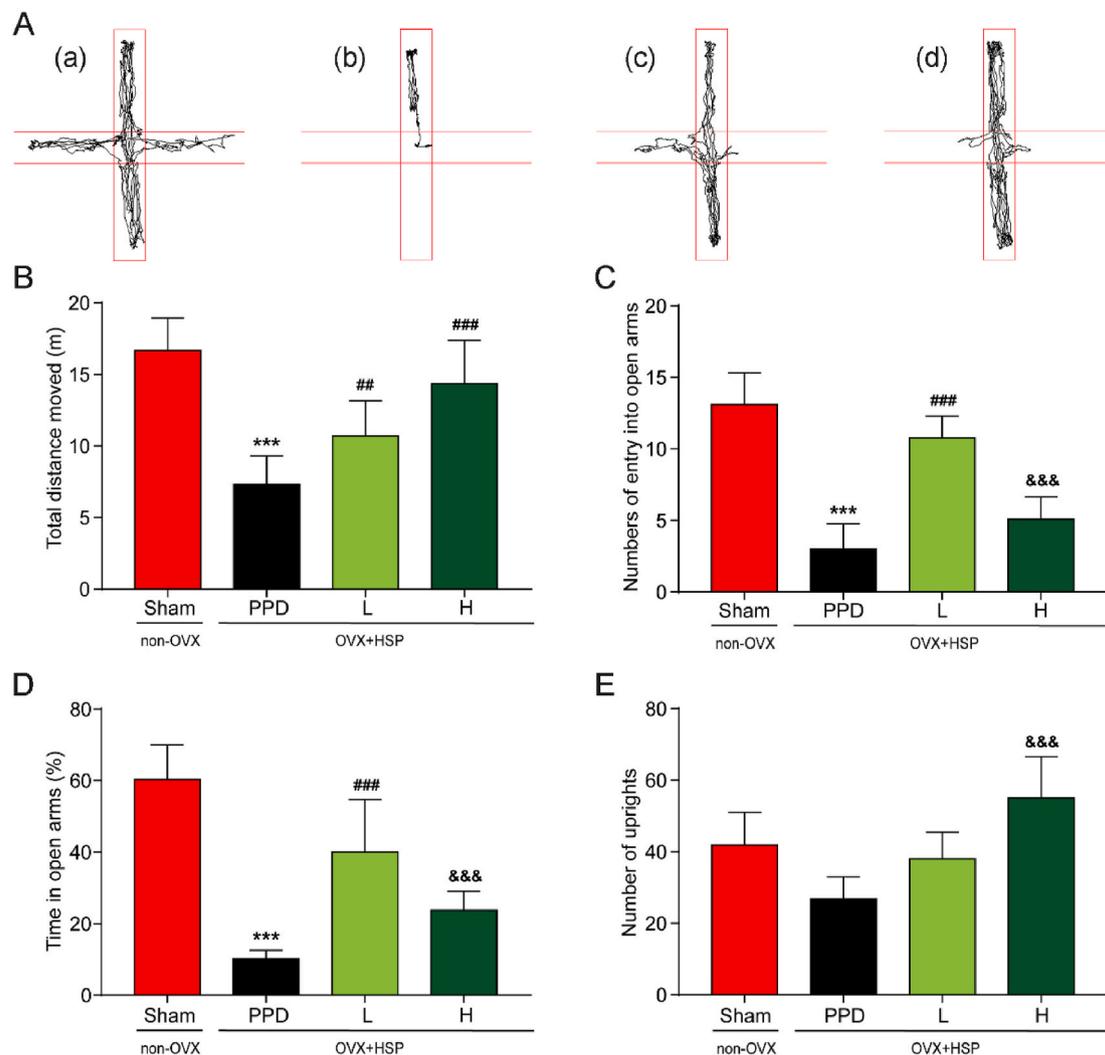


Fig. 7. Effect of S-ketamine on anxiety-like behavior in EPM. (A) The movement traces in the elevated plus maze (a, Sham group; b, PPD group; c, L group; d, H group.); (B) The total distance traveled by the rats during the EPM test; (C) The total number of open-arm entries; (D) The proportion of time spent in open arms; (E) Total number of uprights. Values are expressed as mean \pm SD ($n = 12$ per group). *** $p < 0.001$ vs. Sham group; # $p < 0.01$, ### $p < 0.001$ vs. PPD group; &&& $p < 0.001$ vs. L group.

group (Tamhane T2, $p < 0.001$).

4. Discussion

In this study, the classical method of reproductive hormone withdrawal was used to construct a rat model of postpartum depression, which characterizes depression induced by the pathophysiological process of hormone withdrawal. The study demonstrated that low-dose S-ketamine possesses outstanding antidepressant effects, and it may contribute less to anxiety compared to S-ketamine at a conventional subanesthetic dose. Our results showed that S-ketamine significantly improved the depression-like behaviors induced by hormone withdrawal, which was associated with the reverse of synaptic plasticity from injury.

4.1. Consideration of low-dose S-ketamine in the treatment of postpartum depression

Given the need for new mothers to breastfeed after delivery, the use of as few doses of narcotic drugs as possible should be considered to alleviate the impact on the baby. However, previous preclinical studies of ketamine for depression have mostly used male animals and used a

higher subanesthetic dose (10–20 mg/kg) of ketamine as a therapeutic dose. In fact, this subanesthetic dose may not be appropriate for postpartum depression. Both clinical application of low-dose S-ketamine and pharmacokinetic study of antidepressant dose S-ketamine seem to imply that the usage of a tiny dose of S-ketamine may serve as an amazing prospect in the treatment of postpartum depression (Bornemann-Cimenti et al., 2016; Eckert et al., 2014). Recently work by Samantha et al. revealed a higher sensitivity of female rodents to the antidepressant-like effects of ketamine (Saland et al., 2016; Saland and Kabbaj, 2018). Coincidentally, Nicole et al. investigated the behavioral and molecular effects of ketamine in both male and female rats and demonstrated greater sensitivity in female rats at a low dose (2.5 mg/kg) of ketamine. This dose does not have antidepressant-like effects in male rats (Carrier and Kabbaj, 2013). These exciting studies seem to suggest that low-dose S-ketamine has greater potential to be used in treating female depression than in males. This is in line with previous reports (Ardalan et al., 2020b), demonstrating that S-ketamine induces alteration of structural plasticity of the hippocampus within 1 h after a single administration in a sex-dependent manner, especially at low doses (Franceschelli et al., 2015). Of course, more clinical data and preclinical studies are needed to support this conjecture.

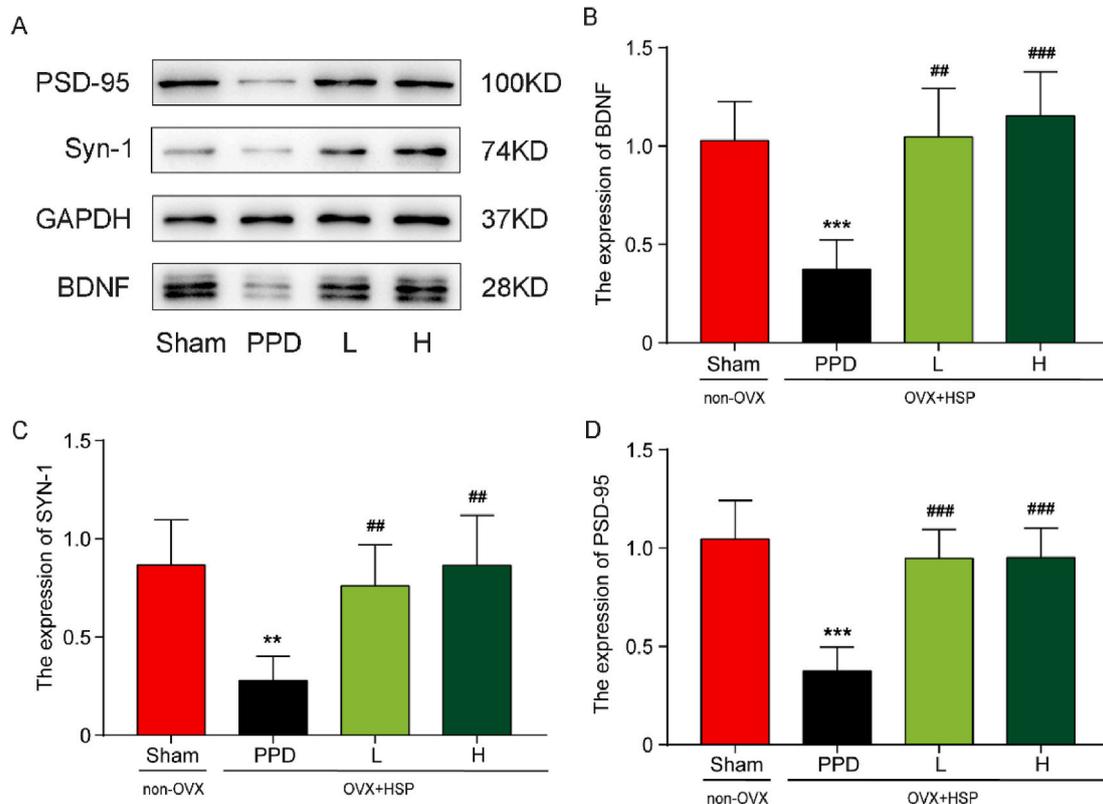


Fig. 8. Effects of S-ketamine on levels of a synaptic marker protein in PPD rats. (A) Immunoblotting bands of BDNF, SYN-1, PSD-95, and GAPDH. (B–D) The expression of BDNF, SYN-1, and PSD-95. All gels were run under the same experimental conditions and blots were processed in parallel. The values of densitometric analysis were normalized to the level of GAPDH. Histograms represent the mean \pm SD (random unilateral hippocampus of 5 rats per group). ** $p < 0.01$, *** $p < 0.001$ vs. sham group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. PPD group.

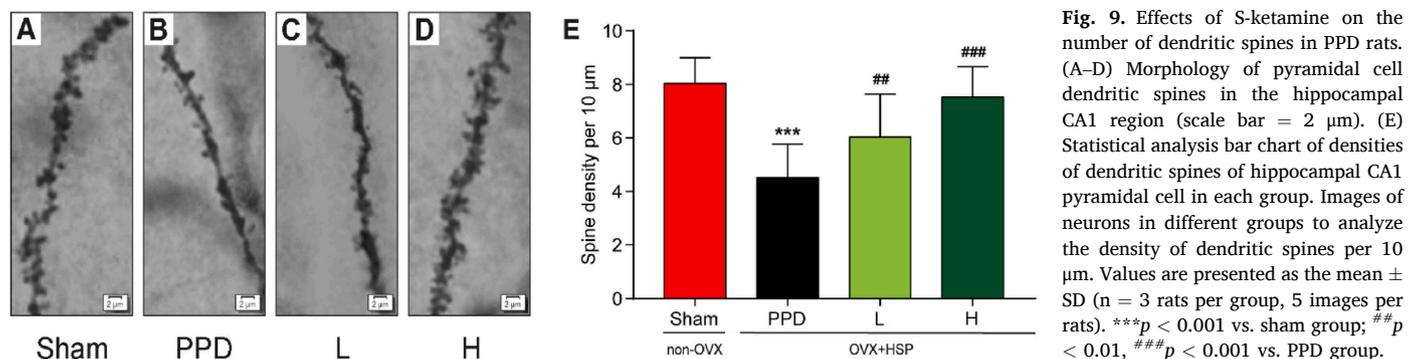


Fig. 9. Effects of S-ketamine on the number of dendritic spines in PPD rats. (A–D) Morphology of pyramidal cell dendritic spines in the hippocampal CA1 region (scale bar = 2 μ m). (E) Statistical analysis bar chart of densities of dendritic spines of hippocampal CA1 pyramidal cell in each group. Images of neurons in different groups to analyze the density of dendritic spines per 10 μ m. Values are presented as the mean \pm SD ($n = 3$ rats per group, 5 images per rat). *** $p < 0.001$ vs. sham group; ## $p < 0.01$, ### $p < 0.001$ vs. PPD group.

4.2. Different S-ketamine doses have different effects on the behavior of PPD rats

In several behavioral tests, we found that low-dose S-ketamine significantly reversed the reduction in sucrose preference induced by PPD and was effective in preventing prolongation of immobility of PPD rats in FST. In addition, in an open field test, our study found that the activity of rats in the H group increased significantly, but they were afraid to enter the central region, and they produced more excrement and showed more rearing. This phenotype exhibited by PPD animals may indicate that high-dose S-ketamine injection could render the female rats more anxious (Ge et al., 2020). Therefore, we further implemented elevated plus maze test, an internationally recognized classical method for assessing rodents' anxiety responses, and found that the exploration of rats in H group in open arms was weakened compared with rats in the L group. Meanwhile, vertical and horizontal movement

in the relatively in-closed and stygian closed arms was significantly increased. Interestingly enough, Nikolaos et al. reported that a single anesthetic ketamine administration apparently induces an anxiety-like state, while largely preserving exploratory behavior in the rat (Pitsikas et al., 2019). This research is consistent with our speculation, although they suggested that these effects were time-dependent. Given previous studies showing that female animals have a higher sensitivity to ketamine, it is reasonable to speculate that subanesthetic ketamine can increase anxiety in PPD rats. Although we did not design an independent study to confirm this hypothesis, the dose-induced behavioral differences at least suggest that low doses of S-ketamine may be beneficial in treating PPD models. When combined with the line of evidence above, these viewpoints intensify our conclusion that low-dose S-ketamine may possess a unique dual effect of anti-depression and anti-anxiety, whereas a high-dose of S-ketamine was absent.

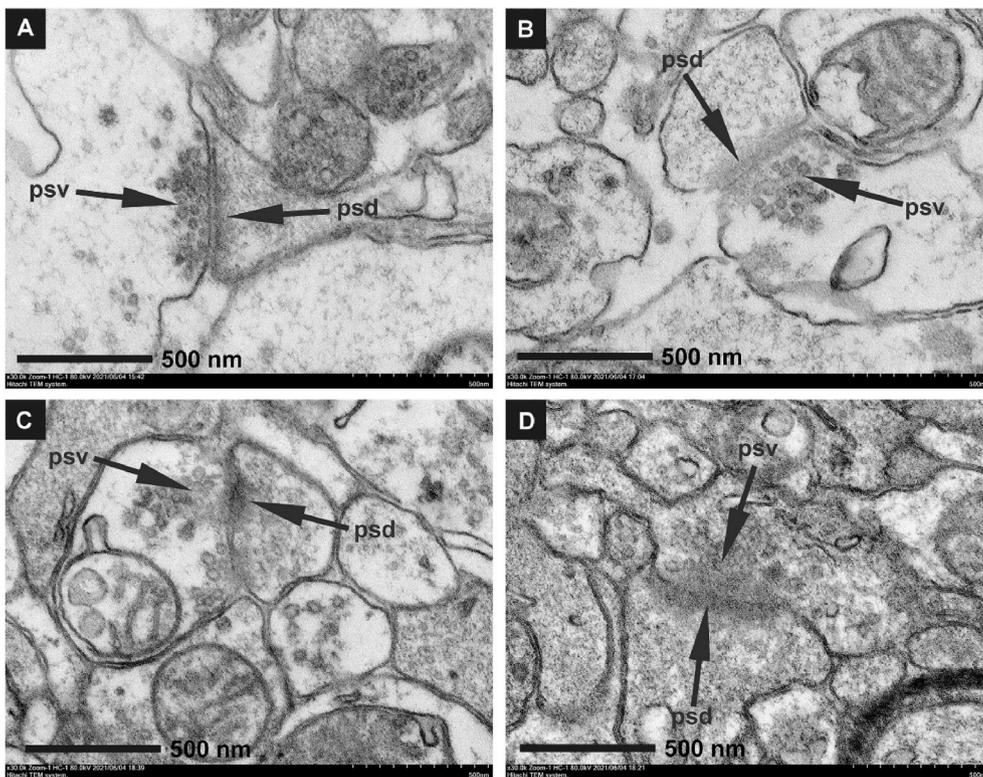
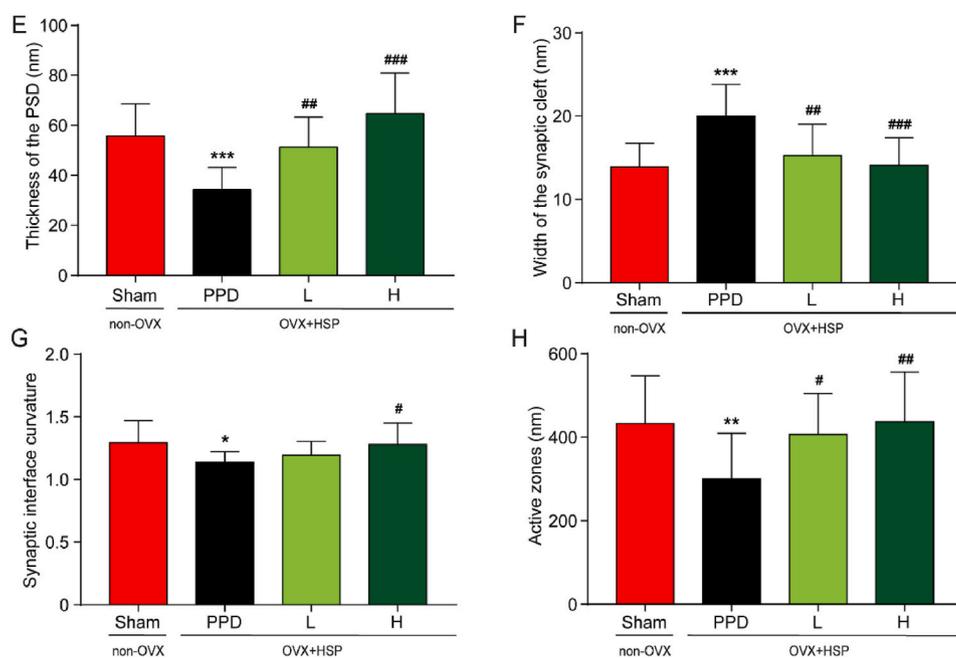


Fig. 10. The influence of S-ketamine on synaptic ultrastructure and morphological parameters of pyramidal cell layer in the hippocampal CA1 region. (A–D) Each group’s synaptic ultrastructure (A, sham group; B, PPD group; C, L group; D, H group; scale bar = 500 nm). Arrowheads point to examples of spine synapses’ structures. Abbreviations: psv, presynaptic vesicles; psd, postsynaptic densities. (E) Thickness of the PSD. (F) Width of the synaptic cleft. (G) Synaptic interface curvature. (H) Active zones. Data are presented as the mean ± SD (n = 3 rats per group, 16 images were arbitrarily taken). **P* < 0.05, ***p* < 0.01 ****p* < 0.001 vs. sham group; #*P* < 0.05, ##*p* < 0.01, ###*p* < 0.001 vs. PPD group.



4.3. A low dose of S-ketamine is sufficient to remedy postpartum depression

Although the dose we employed is lower than that reported by the literature, our study shows that low dose administration of S-ketamine can prevent depressive behaviors without the onset of anxiety compared to conventional high-dose S-ketamine. This provides a valuable insight for the clinical application of S-ketamine in the prevention of depression, as low doses of S-ketamine may mean fewer psychiatric side effects. It is also noteworthy that a recent study (Tizabi et al., 2012) in the Wistar–Kyoto rat model of depression, rapid and lasting antidepressant-like effect of a relatively low ketamine dose (0.5, 2.5, 5

mg/kg) was confirmed. Moreover, a study by Caffino et al. (2016) showed that a single 0.5 mg/kg ketamine dose yielded Cmax values that correspond to those achieved in humans after antidepressant treatment. Thus, these studies demonstrate that low-dose of S-ketamine are sufficient to reverse depression. Surprisingly, a randomized, tri-blind controlled clinical trial showed that minimal-dose (0.015 mg/kg/h) S-ketamine was comparable to the conventional low-dose (0.125 mg/kg/h) regimen in reducing postoperative opioid consumption and hyperalgesia (Bornemann-Cimenti et al., 2016). This dose was nearly equivalent to the 2.5 mg/kg used in this study, based on the ratio of equivalent doses in terms of body surface area between humans and animals. To sum up, this research provides valuable insights into the

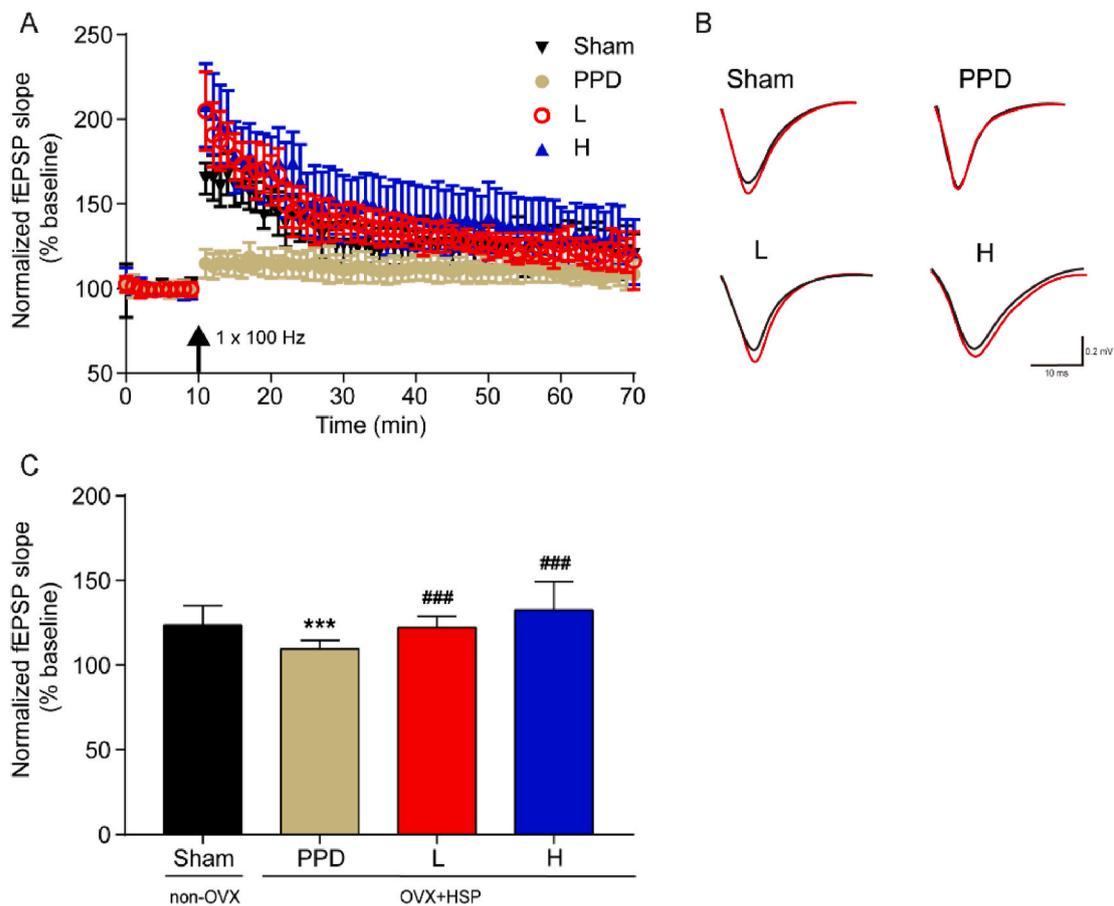


Fig. 11. S-ketamine reversed hippocampal dysfunction induced by ovarian hormone withdrawal. (A) LTP induced by 100 Hz stimulus in hippocampal Schaffer-CA1 synapses. fEPSP slopes normalized to the average baseline response (10 min) before LTP induction (time 0), are plotted in 1-min blocks. (B) Schematic representation of fEPSP before (black) and after (red) tetanic stimulation in the four groups. Calibration bars, 10 ms; vertical bars, 0.2 mV. (C) Averaged percentage change of fEPSP slope after LTP induction (last 20 min of recording) showing S-ketamine administration prevented depression of Schaffer-CA1 LTP in the hippocampus. Data are presented as mean \pm SD ($n = 19\text{--}25$ slices from 4 to 6 rats per group). *** $p < 0.001$ vs. Sham group; ### $p < 0.001$ vs. PPD group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

prevention of postpartum depression through postoperative analgesia of S-ketamine.

4.4. A low-dose of S-ketamine produced rapid anti-depressant effects via increasing synaptic plasticity

Accumulating evidence suggests that S-ketamine can rapidly induce synaptogenesis and reverse synaptic deficits associated with the pathophysiology of depression by acting at the synaptic level to increase the expression of synaptic proteins and promote structural plasticity in the hippocampus (Browne and Lucki, 2013; Zanos and Gould, 2018; Trecani et al., 2019b). And increased translation and release of the neurotrophic factor BDNF in the hippocampus plays a critical role for the rapid antidepressant action of S-ketamine (Autry et al., 2011; Zhou et al., 2014; Garcia et al., 2008). Our study confirmed that the low-dose S-ketamine can rapidly augment BDNF levels in the brain. Meanwhile, presynaptic and postsynaptic synaptic plasticity associated proteins (SYN-1 and PSD-95) increased significantly. This change was further verified by the analysis of synaptic ultrastructural parameters. The result of TEM showed that not only the abundance of PSD was affected by injection of S-ketamine, but synaptic cleft width was as well. These changes apparently contribute to enhanced transmission efficiency of neurotransmitter. Moreover, a single injection of low-dose S-ketamine rescues morphological deficits of pyramidal neurons in the hippocampal CA1 area of PPD rats as early as several hours after administration, which was proved by increased density of dendritic spines. These results

suggest that synaptic structural plasticity is an important basis for the occurrence and prevention of depression, and form the basis of a synaptogenesis hypothesis of depression and treatment response.

In order to investigate the effects of S-ketamine on synaptic plasticity, we examined a hypothesis that depression is caused by disruption of homeostatic mechanisms that control synaptic plasticity, resulting in destabilization and loss of synaptic connections in mood and emotion circuitry. Electrophysiological data from this study showed that S-ketamine can improve synaptic transmission efficiency in the hippocampus of rats. These results suggest that S-ketamine not only increases synaptic protein levels to maintain synaptic connections, but improves synaptic transmission efficiency to withstand depression. This reveals the mechanism by which instability and loss of synaptic connections in the synaptic circuit cause depression. And it also reveals the mechanism of antidepressant-depression of S-ketamine works through enhancing the function of synaptic circuitry.

Taken together, our results confirm that S-ketamine plays an antidepressant role by increasing the number of synaptic connections and the efficiency of transmission between synapses in postpartum depression rats. Certainly, further research is needed in order to elucidate the differences of specific mechanisms between depression induced by hormone withdrawal and by chronic stress. Meanwhile, it is necessary to further study the specific mechanism of the sex-dependent antidepressant effects of S-ketamine, which is conducive to further clarifying the specific mechanism and treatment of postpartum depression.

4.5. Study limitation

Physiological and psychological factors combine to cause postpartum depression, and we did not simulate whether depression caused by environmental stressors can also be sensitive to therapy with S-ketamine. Furthermore, because the rats died immediately after the behavioral tests, we don't know how long the drug's antidepressant effect can last.

What's more, as a psychotropic drug, this may hinder the clinical application of S-ketamine in postpartum depression. Overall, mixed results were reported with respect to the effect of S-ketamine on depression. The most common adverse effects of S-ketamine included nausea, dizziness, dissociation, headache, vertigo, somnolence, and dysgeusia (altered sense of taste) (Sapkota et al., 2021). Fortunately, most were mild-moderate in severity, and these side effects are not enough to cause undue concern about the application of S-ketamine for postpartum depression. However, there are also some side effects that may be unacceptable to new mothers—the neurotoxicity of ketamine to the immature brains of developing fetuses and children (Ikonomidou et al., 1999, 2001; Hansen et al., 2004). This may be why the use of S-ketamine in the treatment of postpartum depression has not been widely accepted. However, as we mentioned above, S-ketamine is more sensitive to female animals. This means that a smaller dose is needed in the treatment of depression, and this dose may not be enough to bring the concentration of ketamine in breast milk to a level that causes neurotoxicity. We will continue to investigate the minimum effective dose of ketamine in a model of postpartum depression and the extent to which the level of exposure to breast milk affects neuronal development in infants and young children.

CRedit authorship contribution statement

Zhuoyu Ren: Investigation, Methodology, Software, Formal analysis, Writing – original draft. **Mingling Wang:** Investigation, Funding acquisition, Supervision. **Mokhtar Aldhabi:** Writing – review & editing. **Rui Zhang:** Supervision. **Yongxin Liu:** Formal analysis, Statistical analysis. **Shaoyan Liu:** Investigation. **Rundong Tang:** Investigation. **Zuolei Chen:** Conceptualization, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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