Molecular Neurobiology (2025) 62:7723–7737
https://doi.org/10.1007/s12035-025-04752-5

RESEARCH

Inhibition of Semaphorin 3A in Hippocampus Alleviates Postpartum Depression-Like Behaviors in Mice

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Received: 28 March 2024 / Accepted: 6 February 2025 / Published online: 11 February 2025

Abstract

Postpartum depression (PPD) is a widespread psychiatric condition affecting up to 20% of postpartum women. Although it is known to be associated with ovarian hormone withdrawal following delivery, current treatments remain limited due to a lack of underlying mechanism. Here, in mice, we identified that semaphorin 3A (sema3A) exhibited a notable increase in expression within the hippocampus of postpartum depression mice, whereas no such upregulation was observed in female mice experiencing depression induced by lipopolysaccharide or chronic restraint stress. The coexpression rate of sema3A and c-Fos was also elevated in the hippocampal CA3 of postpartum depression mice. Importantly, systemic inhibition or genetic knockdown of hippocampal sema3A significantly alleviated the depressive symptoms induced by ovarian hormone withdrawal. Further, overexpression of sema3A in CA3 induced depressive-like behaviors in naïve female mice. In conclusion, our cumulative findings suggest that sema3A in hippocampal CA3 plays a pivotal role in the pathogenesis of postpartum depression, and could serve as a promising treatment target for ameliorating this widespread disorder.

Keywords Postpartum depression · Ovarian hormone withdrawal · Hippocampus · Semaphorin 3A

Introduction

According to the World Health Organization, depression is projected to become the leading global health burden by 2030 [1]. Strikingly, the prevalence of depression is twice as high among females compared to males. Intriguingly, this divergence in gender becomes apparent during adolescence and persists until the onset of menopause. Therefore, a woman's reproductive life-span is a time of heightened vulnerability to depression [2]. Postpartum depression (PPD) stands as a distinct subtype of major depressive disorder (MDD) that exhibits numerous parallels with the aforementioned disorder. However, women with PPD exhibit a higher level of co-morbidity with anxiety and obsessive–compulsive symptoms [3]. What's even more concerning is that even worse, although the mother's well-being is of paramount concern, as suicide is a high risk at this time, there are both short- and long-term implications for the children. Offspring of depressed mothers exhibit a heightened propensity for cognitive and antisocial behavioral difficulties during childhood, subsequently manifesting depression in their adolescent years. Although PPD symptoms affect approximately 20% of childbearing individuals [4], it remains underdiagnosed and undertreated since for the elusive underlying mechanisms [5]. Thus, it is imperative to elucidate the underlying mechanisms of PPD and find effective therapeutic targets, not solely for the wellbeing of women but also for her offspring and family.

Over the past years, quite a few studies have been devoted to identifying the causes of PPD as well as the treatment and prevention, thus several animal models of PPD have been developed for research purposes. For example, similarly to major depression, researchers have exposed pregnant female mice to physical or psychological stress to induce depression after delivery [6]. Additionally,

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given that several biological systems, including the immune system and the hypothalamic–pituitary–adrenal axis, undergo alterations during pregnancy and are implicated in PPD, the dysregulation of these systems during pregnancy could induce PPD in mice [7]. Moreover, it is essential to highlight that the abrupt ovarian hormone withdrawal following childbirth has emerged as a key player in the pathogenesis of PPD, since ovarian hormone markedly rises during pregnancy and sharply declines to early follicular phase level postpartum. Therefore, models involving ovarian hormone withdrawal have garnered increased attention in relevant studies. However, despite the strong association between these hormonal fluctuations and postpartum depression, little is known about its underlying mechanisms as well as possible therapeutic targets. It should be noted that ovarian hormone withdrawal mouse model is widely used in PPD studies to faithfully recapitulate PPD-associated pathological features [7–9], we adopted the same models to explore the underlying mechanisms of PPD.

It has long been put forward that semaphorin 3A (sema3A) has a vital role in the pathophysiology of several psychological diseases. Sema3A is a secreted protein with close contact with estrogen, serum level of sema3A decrease with age or after menopause in humans [10], and ovariectomy reduce the transcription level of sema3A in the hippocampus of middle-aged (13 months old) rats [11], few studies have mentioned the association between the expression level of sema3A in hippocampal CA3 and the level of estrogen or synaptic plasticity. In the adult central nervous system, sema3A is mainly expressed in brain areas that retain plasticity and/or neurogenesis, such as hippocampus, the olfactory bulb and cerebellum [12], and is often implicated in axon guidance [13], cell migration [14], and synapse formation [15]. For instance, the application of sema3A results in a significant decrease in synaptophysin and postsynaptic density 95 puncta [16]. Although studies have mainly centered around its roles in bone metabolism, immunoregulation, angiogenesis, and cancer development [17–19], sema3A has also been implicated in neuropsychiatric diseases, including Schizophrenia [20], Alzheimer's disease (AD) [21], and epilepsy [22]. Otherwise, during normal aging, the expression of sema3A in the hippocampus does not change with increasing age. But compared to normal individuals, AD patients shows a significant upregulation in the expression of sema3A in the hippocampus [23], and accumulated hippocampal sema3A instigates programmed neuron death and induces alterations of synaptic plasticity, causing AD [21]. Elevated cerebellar sema3A downregulates the correlative genes, mediating the formation and maintenance of synapses, thus leading to the development of schizophrenia [20]. Intriguingly, SEMA3A variation is significantly associated with comorbid alcohol dependence and major depression (MD) [24]. However, whether sema3A plays a pathological role in PPD has not been established.

The hippocampus, an underlying brain area in responding to reproductive hormone fluctuations, plays an important role in depression and displays high structural and functional plasticity in adulthood [25, 26]. Mouse hippocampal synaptic plasticity is impaired following postpartum estrogen withdrawal or chronic gestational stress, impairments that have been implicated in PPD-like behaviors [27, 28]. Remarkably, sema3A plays an important role in regulating synaptic plasticity of the adult hippocampus [16]. Based on this, we hypothesized that hippocampal sema3A plays a critical role in the development of PPD. This hypothesis was investigated in vivo using a combination of gain- and loss-of-function genetic approaches, pharmacologic profiling techniques, and behavioral tests in well-established ovarian hormone withdrawal models of PPD.

Methods

Animals

Female C57/BL mice, aged 8–9 weeks, were purchased from Charles River (Shanghai, China). All animals were housed in a humidity (50–60%) and temperature (22–24 °C) controlled room with unrestricted access to food and water. The room was maintained with a standard 12:12 light/dark cycle. The animals were treated in accordance with protocols approved by the Animal Ethics and Welfare Committee of the Zhejiang University School of Medicine (ethics code: ZJU20210216) (Table 1). All experimental procedures were carried out in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals (NIH Publications NO.86–23).

Models

Lipopolysaccharide Depression Model

Administration of lipopolysaccharide (LPS) causes a phase of depressive-like behavior [29]. LPS (L4524, serotype 055: B5, Sigma-Aldrich, USA) was injected intraperitoneally at a dose of 1 mg/kg dissolved in sterile 0.9% saline. Depressive behaviors of female mice were assessed 24 h after injection.

Chronic Restraint Stress Model

Female mice were individually restrained in a well-ventilated 50 ml conical tube for 4–6 h daily for 21 days before behavioral assessments [25].

Table 1 Summary of experimental groups of C57BL/6 J female mice

Figure	Experimental groups			Number of mice	
Figure1	FST	Saline	LPS	13	13
		Control	CRS	14	15
		Sham	PPD	10	10
	TST	Saline	LPS	13	14
		Control	CRS	13	15
		Sham	PPD	10	10
	mRNA of sema3a	Saline	LPS	7	7
		Control	CRS	7	7
		Sham	PPD	6	6
	Sema3A/β-actin	Saline	LPS	4	4
		Control	CRS	4	4
		Sham	PPD	6	6
Figure2	Fluorescence intensity	Sham	PPD	3	3
	c-Fos	Sham	PPD	3	3
	Sema3A and c-Fos	Sham	PPD	3	3
Figure3	mRNA of sema3a	Sham+Saline	Sham+EGCG	5	5
		PPD+Saline	PPD+EGCG	5	5
	Sema3A/β-actin	Sham+Saline	Sham+EGCG	5	5
		PPD+Saline	PPD+EGCG	5	5
	FST	Sham+Saline	Sham+EGCG	7	8
		PPD+Saline	PPD+EGCG	7	8
	TST	Sham+Saline	Sham+EGCG	8	7
		PPD+Saline	PPD+EGCG	7	7
	Number of c-Fos in CA3	PPD+Saline	PPD+EGCG	3	4
Figure4	mRNA of sema3a	Scramble	shRNA(sema3a)	3	3
	FST and TST	Sham+Scramble		8	
		PPD+Scramble		11	
		PPD+ shRNA		12	
	OFT	Sham+Scramble		6	
		PPD+Scramble		6	
		PPD+ shRNA		6	
	% of c-Fos$^+$ cells	PPD+Scramble		4	
		PPD+ shRNA		4	
Figure5	mRNA of sema3a	EGFP	Sema3A	4	4
	FST and TST	EGFP	Sema3A	13	13
	OFT	EGFP	Sema3A	6	6

Postpartum Depression Model

The ovarian hormone withdrawal model of PPD was used as previously described [8, 30]. Briefly, female mice were bilaterally ovariectomized (OVX) under 1.5–2% isoflurane anesthetic. The sham operation was the same procedure as OVX excepting ovarian excision. After 7 recovery days, the OVX mice were subcutaneously injected with estradiol (E2, 0.5 ug, E8875, Sigma-Aldrich, USA) and progesterone (P4, 8 mg, P0130, Sigma-Aldrich, USA) dissolved in 0.1 ml sesame oil (A34192, Xiya Reagent, China) daily for 16 days, and then treated with E2 (10 ug/0.1 ml) daily for 7 consecutive days, this phase was referred to as the hormone-simulated pregnancy (HSP) stage. After the final E2 injection, the process transitioned into the estradiol withdrawal following HSP (HSP-EW) stage, which corresponded to the postpartum depression phase. Control mice received sesame oil injections following the same schedule. Behavioral tests were performed on the third day after drug withdrawal.

Behavioral Tests

Forced Swim Test

Mice were cup handling twice a day for 14 consecutive days before behavioral tests, each handling lasts 30 s as described before [31, 32]. A single cohort of animals was used for the following test sequence: open field test (OFT), light–dark box test, tail suspension test (TST) and then forced swim test (FST) as previously described [9]. The forced swim test (FST) was conducted as previously described [29]. Briefly, mice were placed into the center of a transparent glass cylinder (30 cm in height × 15 cm in diameter) of 23 °C water for 6 min. Cumulative immobility (i.e., no active movement except that needed to keep the animal from drowning) time was recorded during the final 4 min.

Tail Suspension Test

The tail suspension test (TST) was conducted as previously described [29]. Briefly, mice were individually suspended for 6 min by fixing their tails with adhesive tape to the ceiling of a rectangular box, leaving them 50 cm above the table surface. The cumulative immobility time (i.e., when the mouse ceased moving its limbs and body, except to breath) was recorded.

Open Field Test

The open field test (OFT) was conducted as previously described [33]. Briefly, mice were individually placed in the center of the box (50 cm × 50 cm × 50 cm) in a room with dim light and were allowed to explore the area for 10 min. The total distance traveled, entries into the center zone, and time spent in the center zone were recorded and analyzed with AnyMaze software (Stoelting, USA).

Light/Dark Box Test

The light/dark box test was conducted as previously described [34]. Briefly, the box (44 cm × 21 cm × 21 cm) is divided into two regions, the smaller area is a dark box and the larger is a brightly lit box. Animals were individually placed in the middle of the light chamber for the 10-min test. Entries and time spent in the light chamber were recorded and analyzed with AnyMaze software (Stoelting, USA).

Real-Time Polymerase Chain Reaction

Mouse hippocampus were rapidly collected and frozen in liquid nitrogen. RNA was extracted by standard procedures using FastPure Cell/Tissue Total RNA Isolation Kit (RC112-01, Vazyme, China). Then, 500 ng of total RNA from each sample was reverse-transcribed with HiScript III RT SuperMix (R223-01, Vazyme, China) for qPCR. Expression of each mRNA was quantified using SYBR qPCR Master Mix (Q711-02, Vazyme, China). Bio-Rad CFX Manager 3.1 was used to analyze qPCR data.

Western Blot

Mouse hippocampus were extracted and homogenized in RIPA buffer (P0013B, Beyotime, USA) containing proteinase inhibitor and phosphatase inhibitor (05892970001 and 04906845001, Sigma-Aldrich, USA). Then, the homogenates were centrifuged and supernatant was collected to determine the protein concentration. Next, 30 ug protein was loaded on SDS-PAGE gel and transferred to a PVDF membrane (#1620184, Bio-Rad, USA). Membranes were blocked in 5% nonfat dry milk for 2 h at room temperature, then treated with specific antibodies for sema3A (1:1000, ab23393, Abcam, USA) and β-actin (1:1000, #4967, Cell Signaling Technology, USA) overnight at 4 °C. After washing, the membranes were incubated with a secondary antibody for 1 h at room temperature. All samples within each experiment were run on the same gel and transferred to the same membrane to ensure consistency. Protein bands were detected with an enhanced chemiluminescent substrate and analyzed with Image J (National Institutes of Health, USA). The values of each sample were normalized with the corresponding amount of anti-β-actin.

Immunofluorescence

Mice were deeply anesthetized using 3–5% isoflurane in oxygen and perfused intracardially with phosphate buffered saline followed by 4% paraformaldehyde fix solution. Whole brains were dissected out, then post-fixed in 4% paraformaldehyde at 4 °C overnight before being dehydrated in 30% sucrose solution for at least 48 h. The brains were frozen in optimal cutting temperature compound (Sakura Finetek, USA) and cut into 30 um sections (Thermo Fisher Scientific, USA). Then, brain slices were blocked for 2.5 h and incubated with primary antibodies: c-Fos (1:1000, 226004, Synaptic Systems, Germany); sema3A (1:100, PAB7888, Abnova, USA); or DAPI (1:3000, ab104139, Abcam, USA) at 4 °C overnight. Sections were then washed and incubated at room temperature for 1 h with secondary antibodies. The corresponding sections were imaged by virtual microscope slide scanning system VS120 (Olympus, Japan) and confocal laser scanning microscope (Nikon, Japan), then analyzed with Image J.

Drug Treatment

Starting from the day before the termination of the hormone-simulated pregnancy, mice were administered an intraperitoneal injection of vehicle or EGCG (50 mg/kg) once daily for 5 days. Behavioral tests were performed 1 h after the last administration.

Viral Vectors and Injections

AVV2/9-shRNA(scramble)-CMV-EGFP and AVV2/9-shRNA(sema3a)-EGFP (Viral: 5×10^{12} vector genomes/ml), AVV2/9-CMV-EGFP, and AVV2/9-CMV-sema3a-EGFP (Viral: 5×10^{12} vector genomes/ml) were purchased from BrainTVA Co., Ltd (Wuhan, China). Sema3a-EGFP is expressed as a fusion protein. The interference sequence for shRNA(sema3a) is GCTAGAATAGGTCAGATATGC, and the sequence for the empty vector is CCTAAGGTT AAGTCGCCCTCG. The primer sequences: qSema3a F: 5' GGGCTTTCCATCCAATCTGCACCTA 3', qSema3a R: 5'AGCGAAGTCCC GTCCCATGAAGTC3'; qh GAPDH F 5' ACGGGAAGCTCACTGGCATGG 3', qh GAPDH R: 5' CGCCTGCTTCACCACCTTCTTG3'. The mice were anesthetized by 1.5–2% isoflurane and head-fixed in a stereotaxic frame (RWD, China). After the dura was exposed, a 25-gauge needle, with calibrated glass microelectrodes, connected to a 10-μL microinjection syringe (Gaoge, China) was used to infuse virus into the CA3 (AP − 1.96 mm; ML ± 2.04 mm; DV − 2.1 mm; relative to bregma). The virus was injected at 50 nL/min by a microsyringe pump (#78–8710 KD Scientific, USA). We injected 150 nl of AAVs into the CA3 for one side. The mice were allowed to recover for 4 days. After behavioral tests, mice were sacrificed and examined for the position of virus by immunofluorescence. To be specific, among 12 mice subjected with injection of AVV2/9-shRNA(sema3a)-EGFP, 9 mice were confirmed with the position of virus by immunofluorescence and 3 mice were confirmed the effectiveness of virus by PCR. Among 13 mice subjected with injection of AVV2/9-CMV-sema3a-EGFP, 9 mice were confirmed with the position of virus by immunofluorescence and 4 mice were confirmed the effectiveness of virus by PCR.

Statistical Analysis

All experiments were randomized. Animals were randomly chosen from multiple cages. For behavior experiments, measurements were taken blinded to condition. Statistical analyses were conducted using GraphPad Prism TM 8.0. All data are presented as mean ± standard error (SEM). Normal distribution was assessed using the Shapiro–Wilk test, the homogeneity of variance was assessed by Levene's test. Two-sample comparisons were performed using Student's *t*-tests when the data followed a normal distribution. Multiple comparisons were performed using one-way ANOVAs followed by Tukey's post hoc tests. Two-way ANOVA followed by Bonferroni's multiple comparisons test was used to compare data from different groups with different treatment. $P<0.05$ was considered statistically significant (Table 2).

Results

Ovarian Hormone Withdrawal, but not Lipopolysaccharide or Chronic Restraint Stress, Increase Expression of Hippocampal Sema3A in Female Mice

To investigate the role of hippocampal sema3A in different depression states, we employed LPS or chronic restraint stress (CRS) was adopted to induce MDD, and ovarian hormone withdrawal was used to induce PPD. The experimental timelines for the model establishment and behavioral tests are shown in Fig. 1a. Immediately after the behavioral experiments, all mice were euthanized and hippocampal tissues were extracted and collected. LPS-treated mice exhibited increased immobility times on the FST (Fig. 1b; $t_{(24)}=3.449$, $P<0.01$) and the TST (Fig. 1c; $t_{(25)}=4.188$, $P<0.001$) and CRS-treated mice exhibited increased immobility times on the FST (Fig. 1f; $t_{(27)}=3.819$, $P<0.001$) and the TST (Fig. 1g; $t_{(26)}=6.444$, $P<0.0001$), but there was no significant change in the level of hippocampal sema3A in LPS-treated mice (Fig. 1d, e; $t_{(12)}=1.333$, $P=0.2073$; $t_{(6)}=0.7788$, $P=0.4657$, respectively) or CRS-treated mice (Fig. 1h, i; $t_{(12)}=2.006$, $P=0.0680$; $t_{(6)}=1.106$, $P=0.3113$, respectively). Interestingly, mice subjected to ovarian hormone withdrawal also exhibited increased immobility times in the FST (Fig. 1j; $t_{(18)}=7.419$, $P<0.0001$) and TST (Fig. 1k; $t_{(18)}=4.999$, $P<0.0001$); Simultaneously, ovarian hormone withdrawal upregulated the expression of hippocampal sema3A (Fig. 1l, m; $t_{(10)}=2.717$, $P<0.05$, $t_{(10)}=6.335$, $P<0.0001$, respectively).

Given that PPD co-occurs with anxiety, anxiety-like behaviors were also evaluated in PPD mice. For the OFT, Fig. S1a shows representative movement tracks for individual mice from each group. Compared with control mice, ovarian hormone withdrawal mice exhibited less total distance, time in the center area, and center zone entrances (Fig. S1b, c and d; $t_{(16)}=2.708$, $P<0.05$; $t_{(18)}=3.932$, $P<0.001$; $t_{(18)}=4.968$, $P<0.0001$, respectively). In the light/dark box test, PPD mice showed a decreased time in the light side and transition to the light side (Fig. S1e, f; $t_{(17)}=4.957$, $P<0.001$; $t_{(17)}=3.624$, $P<0.01$, respectively).

Table 2 Statistical analysis

Figure		Experimental groups		Statistical analysis
Figure1	FST and TST	Saline	LPS	Unpaired t-test
		Control	CRS	Unpaired t-test
		Sham	PPD	Unpaired t-test
	mRNA of sema3a	Saline	LPS	Unpaired t-test
		Control	CRS	Unpaired t-test
		Sham	PPD	Unpaired t-test
	Sema3A/β-actin	Saline	LPS	Unpaired t-test
		Control	CRS	Unpaired t-test
		Sham	PPD	Unpaired t-test
Figure2	Fluorescence intensity	Sham	PPD	Unpaired t-test
	c-Fos	Sham	PPD	Unpaired t-test
	Sema3A and c-Fos	Sham	PPD	Unpaired t-test
Figure3	mRNA of sema3a	Sham+Saline	Sham+EGCG	Two-way ANOVA
		PPD+Saline	PPD+EGCG	
	Sema3A/β-actin	Sham+Saline	Sham+EGCG	Two-way ANOVA
		PPD+Saline	PPD+EGCG	
	FST and TST	Sham+Saline	Sham+EGCG	Two-way ANOVA
		PPD+Saline	PPD+EGCG	
	The number of c-Fos	Sham+Saline		Unpaired t-test
		PPD+Saline		
Figure4	mRNA of sema3a	Scramble	shRNA(sema3a)	Unpaired t-test
	FST, TST and OFT	Sham+Scramble		Ordinary one-way ANOVA
		PPD+Scramble		
		PPD+ shRNA		
	% of c-Fos$^+$ cells	PPD+Scramble		Unpaired t-test
		PPD+ shRNA		
Figure5	mRNA of sema3a	EGFP	Sema3A	Unpaired t-test
	FST, TST and OFT	EGFP	Sema3A	Unpaired t-test

Sema3A is Highly Upregulated in Hippocampal CA3 of PPD Mice and Promotes Activation of Hippocampal Neurons

Immunofluorescence analysis revealed that sema3A expression in ovarian hormone withdrawal mice was dramatically upregulated in the hippocampal CA3 compared with control mice (Fig. 2a–c; $t_{(27)} = 7.073$, $P < 0.0001$). To further assess the role of CA3 in postpartum depressive-like behaviors, all mice were euthanized 1.5 h after the FST and immunofluorescence staining was conducted to examine c-Fos expression in the CA3. Compared with the control group, the PPD group exhibited a higher density of c-Fos-positive nuclei in the CA3 (Fig. 2d, e; $t_{(24)} = 3.034$, $P < 0.01$). Additionally, a significant increase in the number of sema3A and c-Fos coexpressed cells was found in the CA3 of the PPD group compared with the control group (Fig. 2f, g; $t_{(24)} = 4.837$, $P < 0.0001$).

Systemic Inhibition of Sema3A Alleviates Ovarian Hormone Withdrawal-Induced Depression-Like Behaviors

We conducted further investigations to determine whether the systemic inhibition of sema3A by its specific antagonist, epigallocatechin-3-gallate (EGCG), would reverse depressed behaviors. The experimental timelines and flowcharts of drug administration and behavioral tests are shown in Fig. 3a. For the expression of sema3A in the hippocampus, EGCG administration led to a downregulation of the mRNA and protein levels of Sema3A (Fig. 3b, c; $P < 0.01$, $P < 0.01$, respectively). For FST, the results of the two-way ANOVA showed that there were significant differences between the groups for the model treatment ($F(1, 26) = 8.368$, $P < 0.01$), but no significant differences for EGCG administration ($F(1, 26) = 4.185$, $P = 0.0.0570$), and there was a significant interaction between the two factors

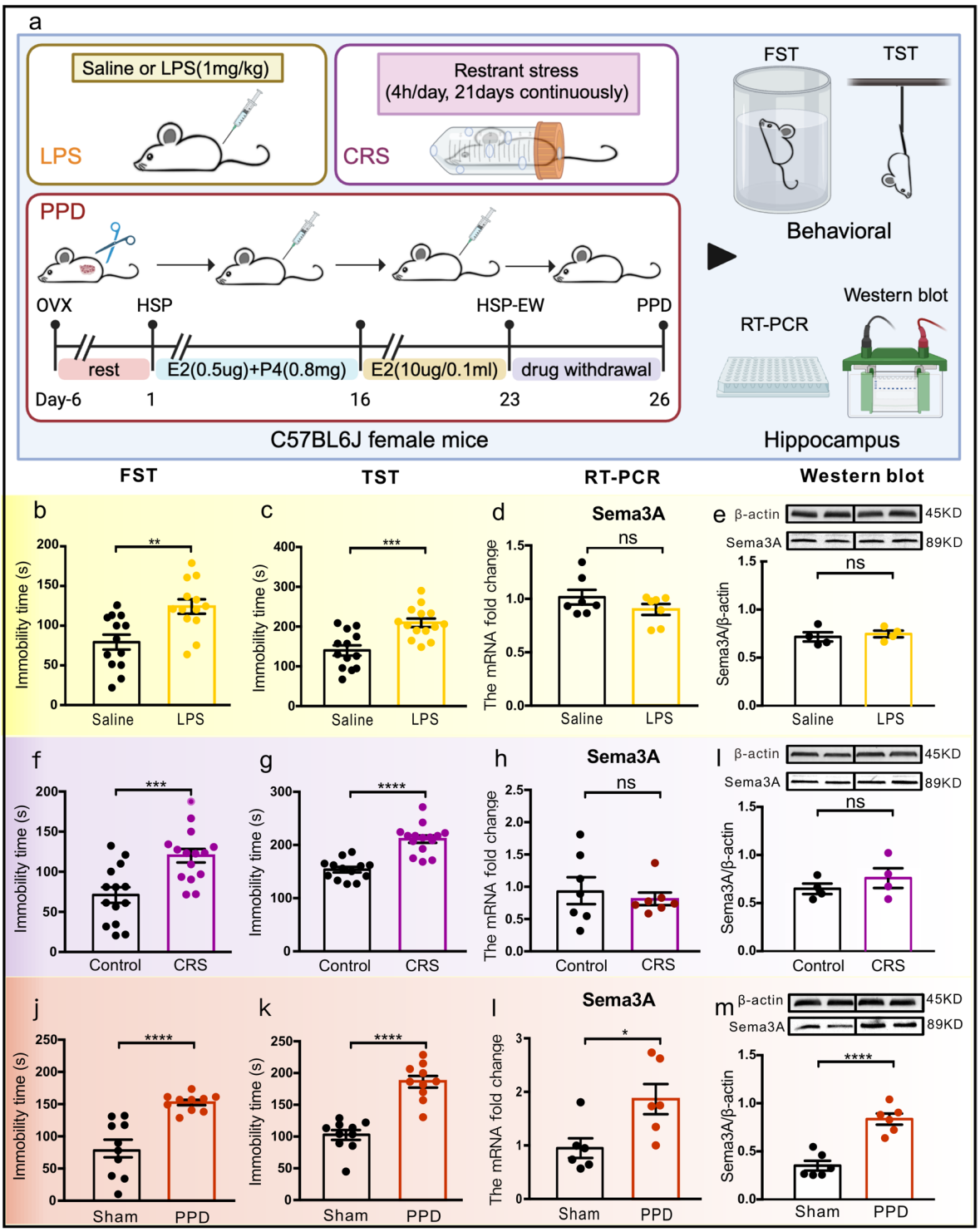

Fig. 1 Postpartum estrogen withdrawal induced depression-like behaviors and upregulated hippocampal sema3A in female mice. **a** Schematic diagram of experimental design. **b** Immobility times on the forced swimming test (FST) and (**c**) tail suspension test (TST) among mice administered lipopolysaccharide (LPS) or saline, $n = 13–14$ mice per group. **d** mRNA and (**e**) protein expression of hippocampal sema3A in mice administered LPS or saline, $n = 4$ or 7 mice per group. **f** Immobility times on the FST and (**g**) TST of chronic restraint stress (CRS) and control mice, $n = 13–15$ mice per group. **h** mRNA and (**i**) protein expression of hippocampal sema3A in CRS and control mice, $n = 4$ or 7 mice per group. **j** Immobility times on FST and (**k**) TST of postpartum depression (PPD) and sham mice, $n = 10$ mice per group. **l** mRNA and (**m**) protein expression of hippocampal sema3A in PPD and sham mice, $n = 6$ mice per group. In the representative images of the Western blot, the two bands for each group are derived from two different samples. All data are expressed as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$

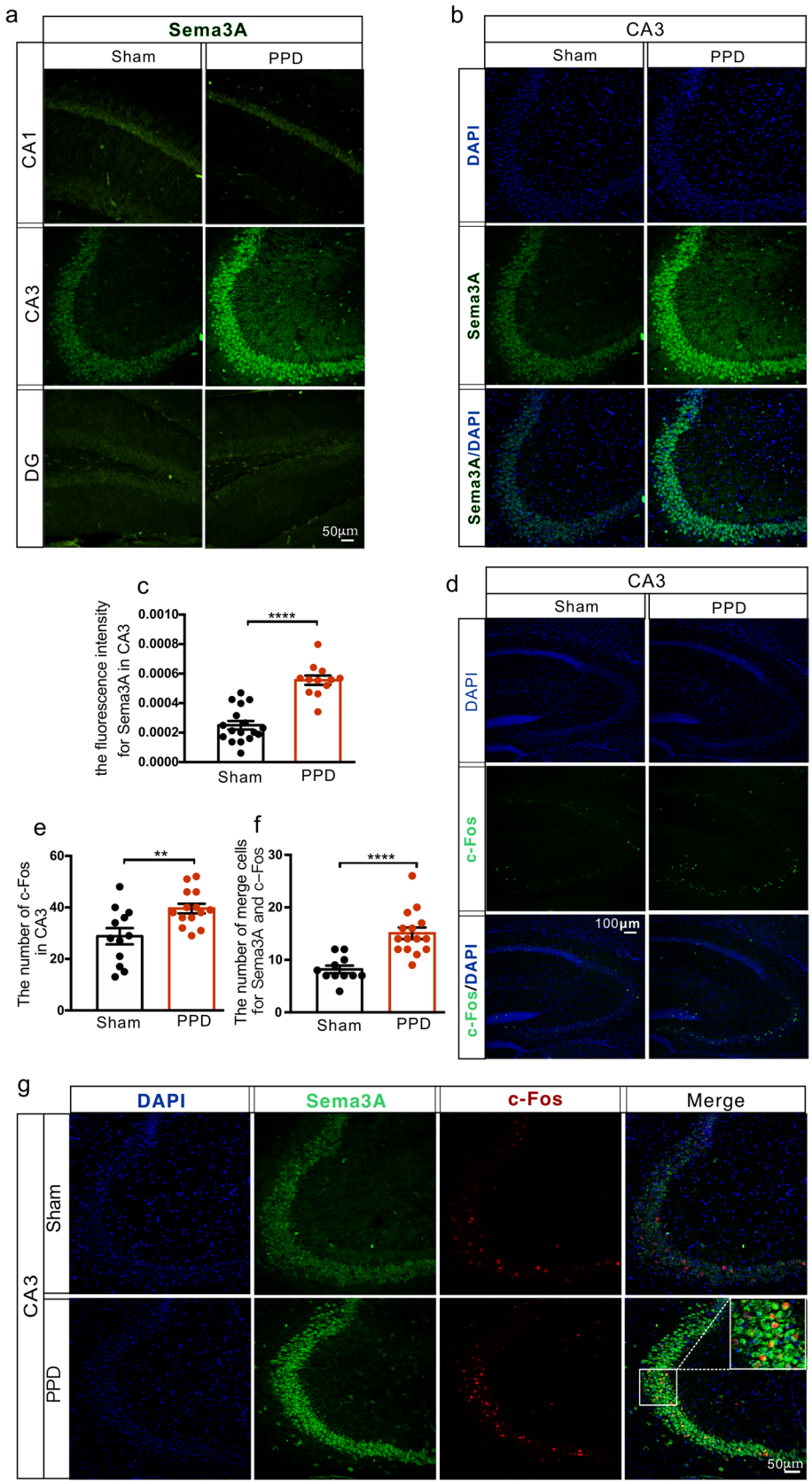

Fig. 2 Postpartum estrogen withdrawal upregulated the expression of sema3A, and coexpression of sema3A and c-Fos, in hippocampal CA3. **a** Expression of sema3A in hippocampal subregions (CA1, CA3, and DG between the sham and PPD groups, scale bars = 50 μm. **b** Expression of sema3A in CA3 from sham and PPD groups, scale bars = 50 μm. **c** Fluorescence intensity of sema3A in CA3 between the sham and PPD groups. **d** Expression of c-Fos in CA3, scale bar = 100 μm. **e** Number of c-Fos positive cells in CA3 between the sham and PPD groups. **f**, **g** Coexpression of sema3A and c-Fos in CA3, scale bar = 50 μm. n = 3 mice per group. All data are expressed as the mean ± SEM. $^{**}P < 0.01$, $^{****}P < 0.0001$

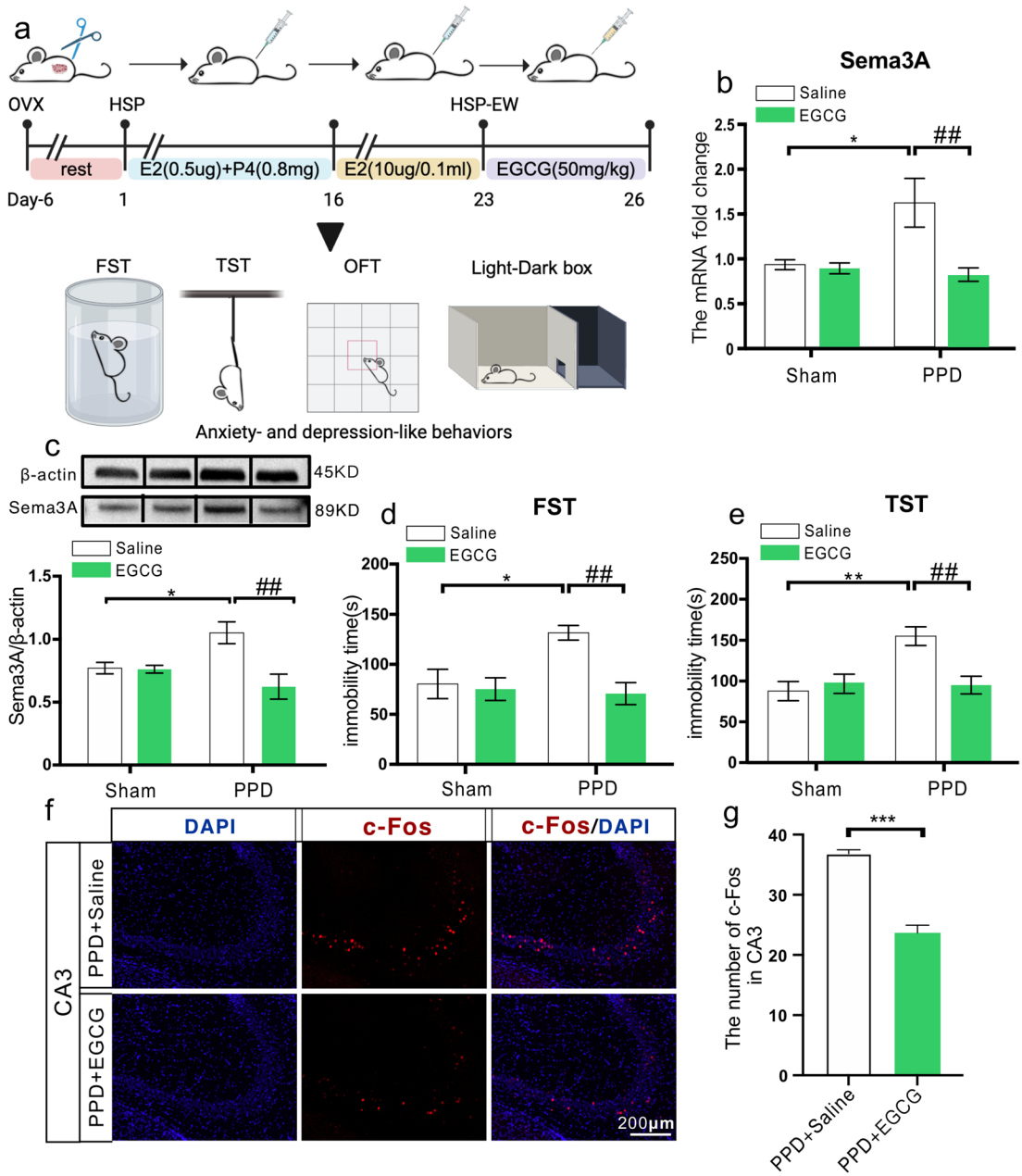

Fig. 3 Systemic inhibition of sema3A by EGCG alleviated postpartum estrogen withdrawal-induced depression-like behaviors. **a** Schematic diagram of model establishment, drug administration, and behavioral tests. **b** mRNA and (**c**) protein expressions of hippocampal sema3A, $n = 5$ mice per group. **d** Immobility times on the FST and (**e**) on the TST, $n = 7$–8 mice per group. **f** Representative images of c-Fos positive neurons in CA3 of PPD + Saline and PPD + EGCG group, scale bar = 200 μm. **g** Statistical analysis of the number of c-Fos positive neurons in CA3. $n = 3$–4 mice per group. All data are expressed as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.01$, $^{\#\#}P < 0.01$

(F (1, 26) = 5.968, $P < 0.05$). For TST, there were significant differences between the groups for both the model treatment (F (1, 25) = 4.476, $P < 0.05$) and EGCG administration (F (1, 25) = 7.508, $P < 0.05$), and there was a significant interaction between the two factors (F (1, 25) = 9.111, $P < 0.01$). Multiple comparisons revealed that inhibiting sema3A with EGCG resulted in the decreased immobility times on the FST (Fig. 3d; $P < 0.01$) and TST (Fig. 3e; $P < 0.01$) of PPD mice. To examine the effects of EGCG on neuronal activity, we analyzed the expression of c-Fos in CA3 and found that the administration of EGCG significantly decreased the expression of c-Fos in PPD mice (Fig. 3f, g; $t_{(5)} = 8.309$, $P < 0.001$).

We also measured anxiety-like behaviors after EGCG administration. There were not significant changes in the OFT (Fig. S2b–d; $P = 0.7991$, $P = 0.9140$, $P = 0.0662$,

respectively) and light/dark box test (Fig. S2e, f; $P = 0.9812$, $P = 0.1873$, respectively) between the PPD + saline group and the PPD + EGCG group.

Knockdown of Sema3A in Hippocampal CA3 Alleviates Ovarian Hormone Withdrawal-Induced Depression-Like Behaviors

To test whether local reduction of sema3A expression in the hippocampus could reverse PPD-like behaviors, sema3A-shRNA was injected into bilateral hippocampal CA3 of OVX mice following the ovarian hormone withdrawal procedure. The timeline of virus injection, model establishment, and behavioral testing were showed in Fig. 4a. The injection sites were confirmed by EGFP fluorescence (Fig. 4b). The efficiency of sema3A interference was validated through real-time polymerase chain reaction (PCR), which revealed a significant decrease in Sema3A mRNA in the PPD + shRNA group compared with the PPD + scramble group (Fig. 4c; $t_{(6)} = 5.237$, $P < 0.001$). To examine the effects of knockdown of Sema3A on neuronal activity, we analyzed the expression of c-Fos in CA3 and found that the knockdown of Sema3A in CA3 significantly inhibited the expression of c-Fos in PPD mice (Fig. 4d, e; $t_{(14)} = 13.25$, $P < 0.0001$).

In postpartum depressive-like behaviors, there was a significant difference in the immobility time in FST ($F(2, 28) = 6.398$, $p < 0.01$, Fig. 4f) and TST ($F(2, 28) = 7.976$, $p < 0.01$, Fig. 4g) among the four groups. In OFT, there were significant differences among the four groups in total distance ($F(2, 15) = 10.71$, $p < 0.01$, Fig. 4h), the time in center area ($F(2, 15) = 5.478$, $p < 0.05$, Fig. 4i) and center zone entrances ($F(2, 15) = 8.790$, $p < 0.01$, Fig. 4j). Multiple comparisons revealed that the knockdown of hippocampal sema3A expression led to decreased immobility times in the FST (Fig. 4f; $P < 0.05$) and TST (Fig. 4g; $P < 0.05$) of PPD mice, with no significant changes in behaviors observed in the OFT (Fig. 4h–j; $P = 0.9074$, $P = 0.9947$, $P = 0.9176$, respectively).

Overexpression of Sema3A in Hippocampal CA3 Induces Postpartum Depression-Like Behaviors in Naïve Female Mice

To further confirm the role of sema3A in promoting in CA3 in PPD, AAV-sema3a or AAV-control was infused into the bilateral CA3 through stereotaxic injection. The timeline of virus injection, model establishment, and behavioral testing were showed in Fig. 5a. EGFP-positive cells in the CA3 were observed (Fig. 5b, c), and an increased sema3A mRNA level was detected through real-time PCR (Fig. 5d; $t_{(6)} = 5.237$, $P < 0.01$). The overexpression of sema3A significantly increased immobility in naïve mice on both the FST (Fig. 5e; $t_{(24)} = 4.095$, $P < 0.001$) and TST (Fig. 5f; $t_{(24)} = 2.189$, $P < 0.05$) without affecting their locomotor activities (Fig. 5g–i; $t_{(10)} = 0.3563$, $P = 0.7297$; $t_{(10)} = 0.1462$, $P = 0.8867$; $t_{(10)} = 0.08173$, $P = 0.9365$, respectively).

Discussion

The neural mechanisms that underlie the depressive effects specifically caused by fluctuations in reproductive hormone levels are the focus of research in the field of PPD. Although initial progress has been made to identify the causes of PPD, the specific cerebral structures vulnerable to hormonal fluctuations and the mechanisms through which their aberrant alterations contribute to depressive-like symptoms remain largely uninvestigated. To date, few have reported the role of sema3A in the pathogenesis of PPD, but we hypothesized that sema3A might be a possible therapeutic target of PPD. Herein, our results provide new insights into the role of sema3A in hippocampus CA3 to elucidate the cause of PPD. Our study showed that sema3A in the hippocampus is mainly upregulated in PPD mice, but not in LPS or CRS female mice. Through pharmacological and genetic interventions, we establish that the regulation of sema3A in the CA3 of the hippocampus can significantly change postpartum depressant-like behaviors. This phenomenon may elucidate the vulnerability of the hippocampus CA3 to hormonal fluctuations, while also establishing a robust basis for future investigations into neural circuit mechanisms.

Structural and functional imaging studies, both clinical and experimental, have pinpointed that several brain regions, including the hippocampus, medial prefrontal cortex, nucleus accumbens, and amygdala, as central players implicated in the pathophysiology of PPD [30, 35–39]. Considerable attention has been devoted to investigating the role of the hippocampus in postpartum depression (PPD) due to its high susceptibility to gonadal hormones, as well as its crucial involvement in synaptic regulation and structural reorganization. Alterations in adult hippocampal neurogenesis and synaptic plasticity may thus be important mechanisms underlying PPD [40].

Hippocampal sema3A is recognized for its importance in synaptic and structural plasticity [41]. Increased sema3A in the hippocampus of AD patients may induce the apoptosis cascade, promoting programmed neuronal death [21]. Upregulated hippocampal sema3A may also be involved in rapid eye movement sleep deprivation-induced spatial learning and memory deficits via neuron damage [42]. Presented here, hippocampal sema3A expression was significantly increased in PPD mice but did not differ in other mouse depression models compared with control mice. This divergence might be attributed to the relatively dramatic fluctuations in postpartum reproductive hormones, particularly the supraphysiologic levels of circulating hormones (i.e.,

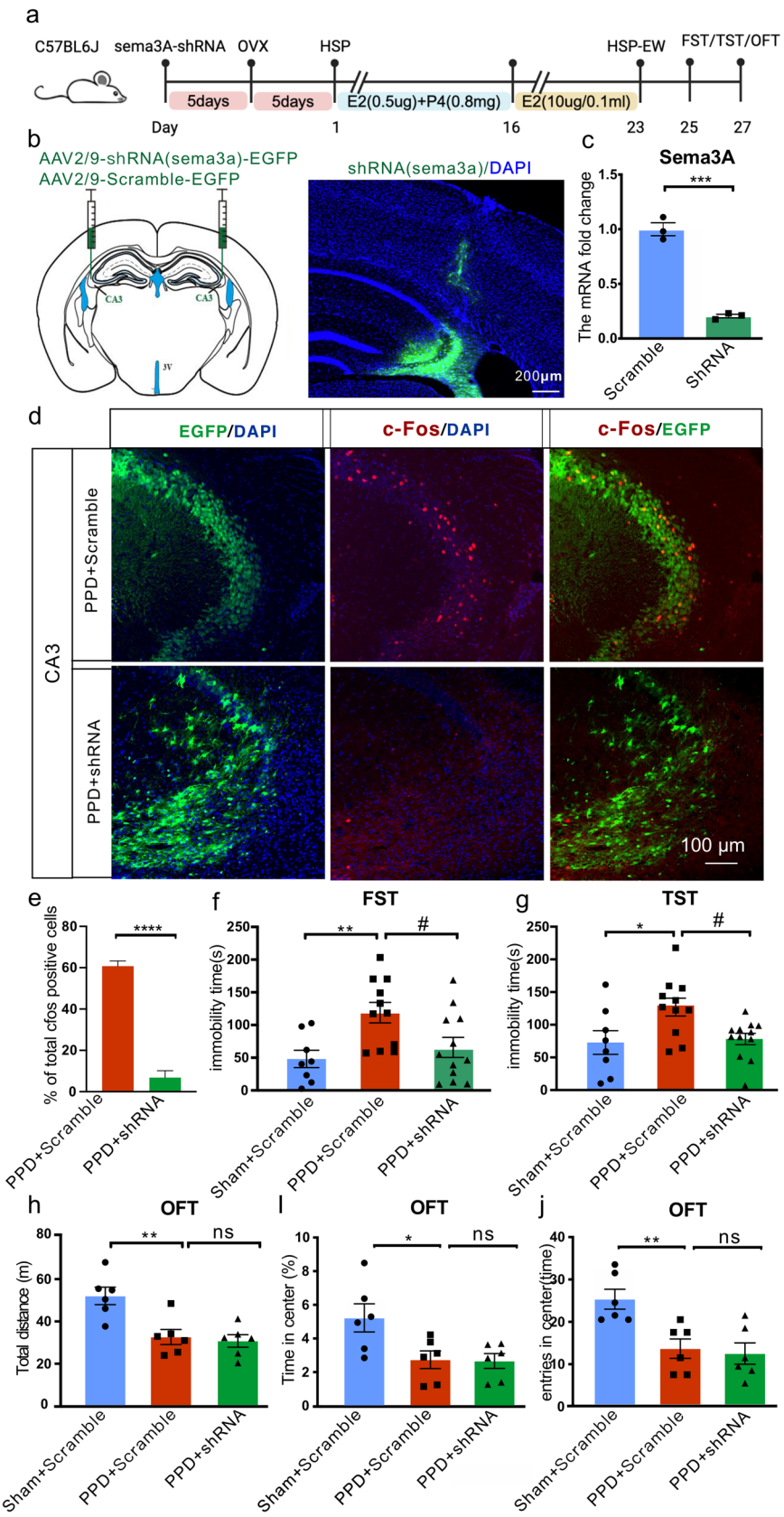

Fig. 4 Sema3A knockdown in hippocampal CA3 facilitated postpartum estrogen withdrawal-induced depression-like behaviors but did not significantly affect anxiety-like behavior of PPD mice. **a** Timeline of virus injection, model establishment, and behavioral testing. **b** rAVV-U6-shRNA (sema3a)-EGFP-SV40pA in CA3 expression, scale bar = 2 mm. **c** mRNA level of sma3A confirmed the efficacy knockdown of shRNA-Sema3A. $n = 3$ mice per group. **d** Representative images of co-expression of sema3A and c-Fos in CA3, scale bar = 100 μm. **e** Statistical analysis of the percentage of double-labeled cells in c-Fos positive cells. $n = 4$ mice per group. **f** Immobility times on the FST and (**g**) TST, n = 8–12 mice per group. **h** total distance, **i** time in center, and (**j**) center zone entrances in the OFT, $n = 6$ mice per group. All data are expressed as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{***}P < 0.001$, $^{\#}P < 0.05$, ns = not significant

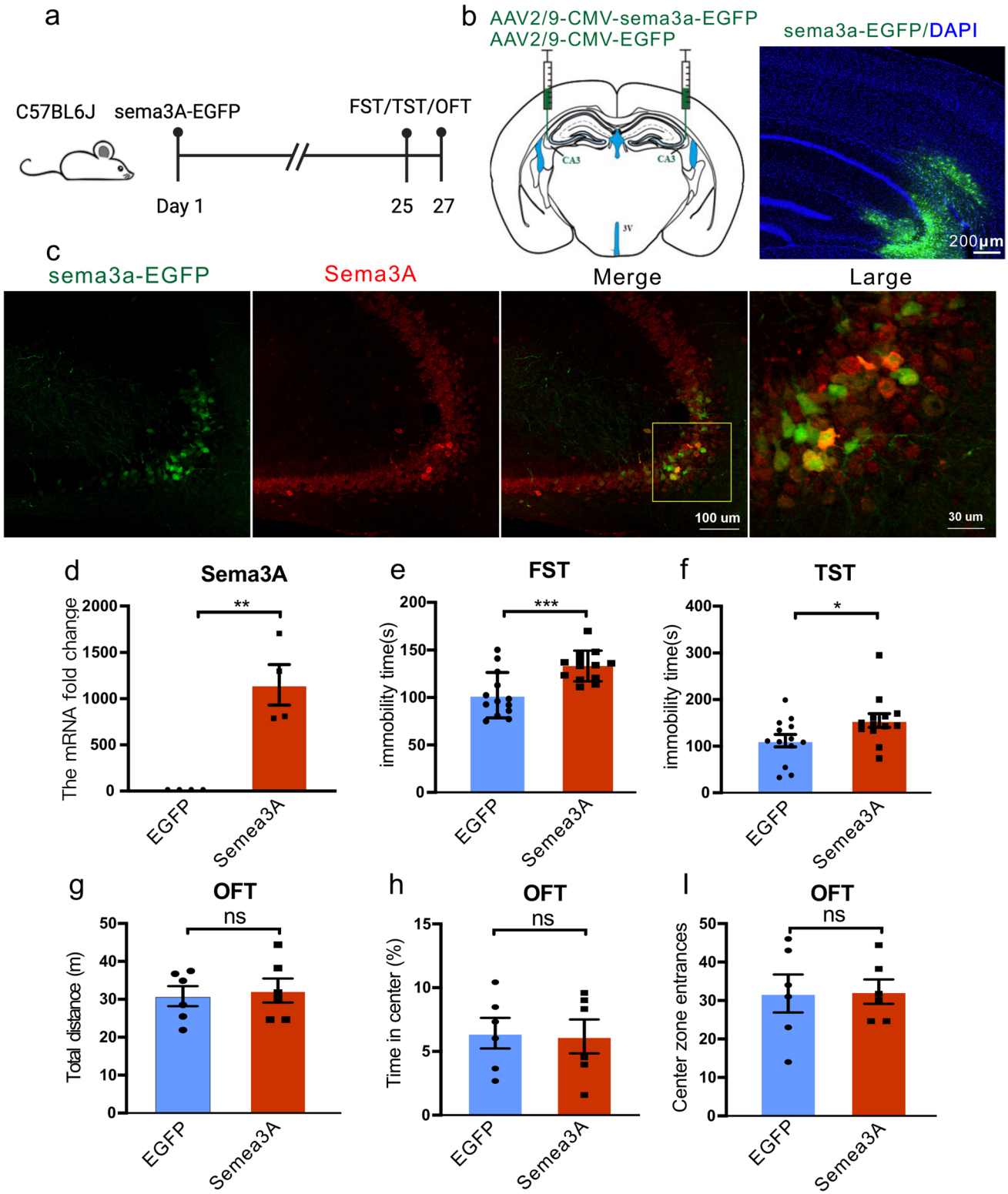

Fig. 5 Overexpression of sema3A in hippocampal CA3 induced postpartum depression-like behaviors but did not significantly affect anxiety-like behavior of naïve female mice. **a** Timeline of virus injection and behavioral testing. **b** AVV2/9-CMV-sema3a-EGFP expression in CA3, scale bar = 200 μm. **c** Co-expression of sema3a-EGFP virus and sema3A antibody in CA3, scale bar = 100 μm (small) and 30 μm (large). **d** mRNA level of sema3A confirmed knockdown efficacy of shRNA-sema3A, $n = 4$ mice per group. **e** Immobility times on the FST and (**f**) TST, $n = 13$ mice per group. **g** Total distance, **h** time in center, and (**i**) center zone entrances in the OFT, $n = 6$ mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns = not significant

estrogen, P4) observed during pregnancy abruptly decline [43]. It is worth noting that sema3A expression in osteoblast lineage cells is regulated by estrogen, and postmenopausal human serum postmenopausal sema3A levels are significantly lower compared to premenopausal levels, further suggesting the regulatory role of estrogen [10].

Sema3A expression is high during the early stages of embryonic development and declines after birth. Yet in adulthood, its expression persists in brain areas that retain plasticity and/or neurogenesis, such as the hippocampus, olfactory bulb, and cerebellum [12]. The hippocampal CA3 region plays a critical role in stress response, which is closely related to depression [25]. The observed CA3 neuronal alterations in different PPD models herein have also been implicated in PPD-like behaviors [44, 45]. Sema3A is expressed in the hippocampal subiculum, CA1, CA3, and dentate gyrus (DG) [41]. In current research, we found that sema3A was highly upregulated in the hippocampal CA3 of PPD mice, that administration of EGCG (the target sema3A antagonist) inhibited its expression in the mouse hippocampus, and that this clearly relieved the depressive behaviors in this PPD model. This aligns with previous research in which EGCG reduced the immobility duration of mice in the FST and alleviated the presentation of depression behaviors [46]. Yet herein, anxious behaviors in PPD mice were not relieved by EGCG administration. Thus, we speculate that sema3A may specifically mediate the depressive aspect of PPD only, without impacting anxiety levels. Furthermore, our knockdown and overexpression of sema3A in the hippocampus CA3 changed PPD-like behaviors suggesting the importance of CA3 sema3A in PPD.

Notably, in our previous study[47], We developed a postpartum depression model using chronic unpredictable stress (CUMS) during gestation days 7 to 16, leading to significant postpartum depression-like behavior and reduced c-Fos expression in the hippocampal CA1 and CA3 regions. Contrastingly, our current study shows that hormone withdrawal after simulated pregnancy causes a significant upregulation of c-Fos expression in the hippocampal CA3 region. The increased c-Fos expression in the hormone withdrawal model may result from abrupt hormonal changes, particularly in estrogen and progesterone, heightening neuronal activity in the CA3 region. [48]. On the other hand, the CUMS model's reduced c-Fos expression might indicate a maladaptive decrease in neuronal activity due to prolonged stress during pregnancy. Chronic stress adversely affects hippocampal function, reducing neuronal excitability and synaptic plasticity[49], which could explain the observed decrease in c-Fos expression. These contrasting findings highlight the necessity of considering the specific nature of stressors when interpreting the molecular underpinnings of PPD. Different types of stress, whether hormonal or environmental, appear to induce distinct alterations in hippocampal activity, which could contribute to the heterogeneous clinical manifestations of PPD. Future studies should aim to further dissect these pathways,thus enhancing our understanding of the differential impacts of various stressors on the brain and may inform the development of more targeted therapeutic strategies for PPD.

Hippocampal connectivity and the corresponding functions within these circuits are abnormal in PPD. Area CA3 is widely recognized as the intermediary station within the trisynaptic pathway, which constitutes the principal conduit for the transmission of information within the hippocampus [50]. Afferent input to CA3 comes from the dental gyrus, the entorhinal cortex and extrahippocampal pathways[51]. Besides, hippocampus CA3 projects to different regions of the brain such as CA1, CA3 and lateral septum (LS), we did not investigate possible projection pathways and focused only on CA3. We are planning to carry out our future work in these directions. Optogenetic, chemogenetic and electrophysiological approaches will be used to study the circuit of hippocampus CA3 involved in PPD and the role of sema3A in the circuit. Sema 3A is a potential molecule that may contribute to the functioning of neuronal circuits. It is suggested that exogenous administration of sema3A has been observed to decrease the effectiveness of synaptic transmission in the CA1 region of hippocampal slices. the application of Sema3A results in a significant decrease in synaptophysin and postsynaptic density 95 puncta[16]. Meanwhile, Sema3A in the hippocampus brain region can induce programmed death of neurons, resulting in defects in axonal guidance and synaptic plasticity[21]. Collectively, the elevated levels of sema3A in the CA3 region may potentially induce postpartum depressive-like behavior by negatively affecting synaptic transmission.

Conclusions

In our current study, we demonstrated for the first time that the expression of sema3A was dramatically upregulated in the hippocampus in PPD mice, and inhibition of the sema3A obviously alleviated the depression-like behaviors. Targeting sema3A in hippocampus may specifically alleviate PPD symptoms. Further research will be needed to explore the specific mechanisms of this system.

Limitations

There are several limitations in our study. First of all, to date, though different kinds of PPD models have been put forward for studies including physical and psychological stress-induced PPD models or immune dysregulation-induced PPD models, we only constructed the PPD models via sudden

ovarian hormone withdrawal. However, it should be noted that the ovarian hormone withdrawal-induced PPD model is the most recognized and mainstream model in research on postpartum depression. At present, there exists a dearth of well-established theoretical inquiries pertaining to the precise mechanisms of PPD, thereby necessitating further comprehensive investigations in subsequent endeavors.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12035-025-04752-5.

Acknowledgements We are grateful to the core facilities of Zhejiang University Institute of Neuroscience for technical assistance, and we wish to thank Pro. Aimin Bao for the experimental guidance.

Author Contributions Xinzhong Chen, Qing Chen and Fang Xu designed and conceptualized the work. Qing Chen, Fang Xu, Hui Wu and Linghua Xie generated the data and drafted the manuscript. Hui Wu, Linghua Xie, Hua Li and Cuicui Jiao contributed to the interpretation of data. Xinzhong Chen, Qing Chen and Honghai Zhang reviewed and edited the manuscript. All authors read and approved the final manuscript.

Funding This study was supported by funding from the National Natural Science Foundation of China (NSFC, No. 82371220).

Data Availability No datasets were generated or analysed during the current study.

Declarations

Ethics Approval The animals were treated in accordance with protocols approved by the Animal Ethic and Welfare Committee of Zhejiang University School of Medicine (Ethic code: ZJU20210216). All experimental procedures were carried out in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals (NIH Publications NO.86–23).

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests

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