

# Saikosaponin-D improves fear memory deficits in ovariectomized rats via the action of estrogen receptor- $\alpha$ in the hippocampus

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**Abstract.** Saikosaponin-D (SSD), which is the main bioactive component in the traditional Chinese medicine Chai Hu (*Bupleurum falcatum* L), possesses estrogen-like properties and is widely used in treating estrogen-related neurological disorders. The current study aimed to investigate the protective effects of SSD on the fear memory deficit in ovariectomized (OVX) rats and the potential underlying mechanism. SSD treatment significantly prolonged freezing time in OVX rats in a manner similar to that of estradiol (E2), whereas this effect was markedly suppressed by co-administration of ICI182780, a non-selective estrogen receptor (ER) inhibitor. The expression of ER $\alpha$  in the hippocampus of OVX rats was significantly elevated by SSD; however, ER $\beta$  expression and E2 synthesis were not markedly affected by SSD treatment. Collectively, this study demonstrated that SSD-mediated fear memory improvement in OVX rats may be attributed not to E2 levels or ER $\beta$  activity, but to ER $\alpha$  activation in the hippocampus.

## Introduction

Epidemiological studies have revealed that menopausal women have a greater risk of developing dysmnnesia, and the prevalence of

Alzheimer's disease in women is twice as high as that in men (1). This sex difference is correlated with lower levels of ovarian hormones, particularly the potent estrogen hormone estradiol (E2) (2). The specific mechanism underlying E2-mediated dementia is not clearly understood, although substantial evidence indicates that an estrogen deficit leads to glutamate toxicity, causes amyloid- $\beta$  deposition, affects neural plasticity, perturbs neurotrophic activities, evokes neuroinflammation, and accelerates neuronal apoptosis in the brain in menopausal women and ovariectomized (OVX) animals (3-6). Notably, estrogen replacement therapy has been frequently used in young women with primary ovarian insufficiency and in postmenopausal women for preventing dementia or cognitive disorders (7-9).

Phytoestrogens are plant-derived estrogen compounds, which are effective substitutes for clinical estrogen usage (10,11). The traditional Chinese medicine, Xiaoyao San, contains Chai Hu (*Bupleurum falcatum* L), which is widely used to ameliorate the postmenopausal syndrome and memory impairment under chronic psychological stress (12-14). The principal bioactive ingredients of Chai Hu are saikosaponins, which comprise three major subtypes: Saikosaponin-A, saikosaponin-C and saikosaponin-D (SSD) aglycones (15,16). Among these subtypes, SSD (Fig. 1) has attracted interest in medical research and clinical trials, due to its potent bioavailability (17). For example, SSD has been reported to significantly ameliorate postmenopausal syndrome and depressive behaviors (18,19).

The hippocampus is pivotal for memory acquisition, consolidation and extinction. In addition, estrogen action in the hippocampus, particularly in the CA1 region, has important roles in the memory process (20). The present study aimed to determine the modulatory role of SSD in fear memory deficit in OVX rats, as well as the underlying mechanism relating to estrogen action in the hippocampal CA1 region. The present findings may provide a promising therapeutic strategy for the treatment of memory deficits, particularly in postmenopausal women.

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## Materials and methods

**Animals.** In total, 84 adult female Sprague-Dawley rats (weight, 180–220 g; age, 6–7 weeks) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. All rats were individually housed in cages under a 12-h light/dark cycle (lights on at 07:00 a.m.) at a temperature of  $22\pm 2^{\circ}\text{C}$  and a humidity of 50–65%. The rats had free access to water and rodent chow. The experimental procedures were approved by The Institutional Committee on The Care and Use of Animals of Nanjing University of Chinese Medicine. Every effort was made to minimize the number of animals used and their suffering. The experimental procedure is presented in Fig. 2.

**Implantation of the infusion cannula.** The rats were anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneal injection; cat. no. 20021216; Sinopharm Chemical Reagent Co., Ltd.) and were then mounted on a stereotaxic frame (RWD Life Science Co., Ltd.) for implantation of the infusion cannula [guide cannula: Inner diameter (ID)=0.38 mm, outer diameter (OD)=0.56 mm; internal cannula: ID=0.36 mm, OD=0.20 mm; RWD Life Science Co., Ltd.]. The cannula was implanted toward the right lateral ventricle (A, -0.8; L, 1.5; H, 4.0) according to a rat brain atlas (21). The cannula was then anchored to the skull with dental cement and skull screws. An osmotic mini pump was implanted subcutaneously (s.c.) on the back and connected by a tube to the infusion cannula. After the surgery, rats were allowed to recover for at least 7 days in their cages.

**Ovariectomy.** Animals were anesthetized with sodium pentobarbital, and the bilateral ovaries were excised through flank incisions (1.5 cm inferior to the palpated rib cage; 1–2 cm from the spine). Sham surgery was performed similarly to the ovariectomy procedure but without ovary removal. After the surgery, rats were placed in their cages and allowed to recover for 7 days.

**Drug preparation and drug treatment.** SSD (purity  $\geq 95\%$ ; cat. no. S9321; Sigma-Aldrich; Merck KGaA) was first dissolved in 12% Tween-80 (cat. no. P4780; Sigma-Aldrich; Merck KGaA) and then diluted with 0.9% saline to a final Tween concentration of 0.5%. E2 (cat. no. E8875; Sigma-Aldrich; Merck KGaA) was dissolved in sesame oil. The non-selective estrogen receptor (ER) inhibitor ICI182780 (cat. no. 1047/1; Tocris Bioscience) was first dissolved in dimethyl sulfoxide (10 mM; cat. no. d103273; Shanghai Aladdin Biochemical Technology Co., Ltd.) and then diluted to 0.1% by volume in artificial cerebrospinal fluid (aCSF). The composition of the aCSF was: NaCl 124.0 mM, KCl 3.0 mM,  $\text{NaHCO}_3$  26.0 mM,  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  1.2 mM,  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  1.2 mM,  $\text{C}_6\text{H}_{12}\text{O}_6$  10.0 mM,  $\text{CaCl}_2$  2.0 mM. All chemical reagents were purchased from Sigma-Aldrich (Merck KGaA).

OVX rats were randomly divided into six subgroups ( $n=12/\text{subgroup}$ ): Saline subgroup, 0.9% NaCl containing 0.5% Tween-80 was administered via intragastric gavage (i.g.) to the rats at the same concentration as in the other groups for 5 weeks; O-E2 subgroup, 2  $\mu\text{g}/\text{kg}$  E2 (s.c.) was administered for 5 weeks; O-SSD 1.8 subgroup, 1.8 mg/kg SSD (i.g.) was administered for 5 weeks; O-SSD 0.9 subgroup, 0.9 mg/kg SSD (i.g.) was administered for 5 weeks; O-SSD 1.8-ICI, 1.8 mg/kg SSD was administered for 5 weeks, with

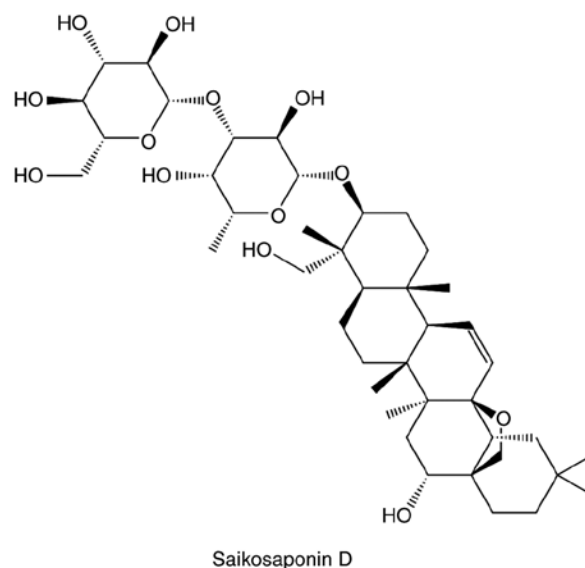


Figure 1. Molecular structure of saikosaponin-D.

500  $\mu\text{g}/\text{day}$  ICI182780 (intracerebroventricular injection) co-administered for the final week; and O-SSD 0.9-ICI subgroup, 0.9 mg/kg SSD was administered for 5 weeks, with ICI182780 co-administered for the final week. The dosages of SSD (0.9 and 1.8 mg/kg) used in the current study were based on previous reports, on the basis of sufficient behavioral discrimination and an absence of significant side effects (19,22,23). Rats in the sham group ( $n=12$ ) underwent no drug treatment. The experimental procedure is shown in Fig. 2. All drugs were administered with a comparable volume once per day at 8:00 a.m.

**Contextual fear conditioning test.** Fear conditioning tests were performed in a chamber composed of a plexiglas box (27x31x36 cm) with stainless steel grids (diameter, 5 mm; pitch, 15.7 mm) on the floor (Coulbourn Instruments). Each rat was placed in the chamber and subjected to a 2-min adaptation period followed by a 2-min preconditioning phase (without any stimulation) in which the freezing time was measured. During the conditioning period, a tone (80 dB) was presented as the conditioned stimulus for 20 sec, and then a foot shock (0.5 mA) was delivered as an unconditioned stimulus during the last 2 sec of the tone stimulus. After 10 tone-shock pairs with 60 sec interstimulus intervals (all animals exhibited  $>12$  sec freezing in a 2-min period), the rats were returned to their cages. Context-dependent tests were performed 1 and 24 h after the conditioning, in which the freezing time was measured in the same contextual conditions, but without any stimulation, for 3 min. Freezing time (%) = freezing time/total measurement time (24).

**ELISA and western blot analysis.** Nine rats randomly selected from each group were sacrificed on the day after behavioral tests under anesthesia at 8:00–9:00 a.m. The skulls were then dissected, and the bilateral hippocampi were quickly removed and placed on ice. The hippocampal CA1 areas were rapidly extracted and cut into  $\sim 1 \times 1 \times 0.5$  mm<sup>3</sup> sections according to the brain atlas (21); these sections were immediately frozen in liquid nitrogen.

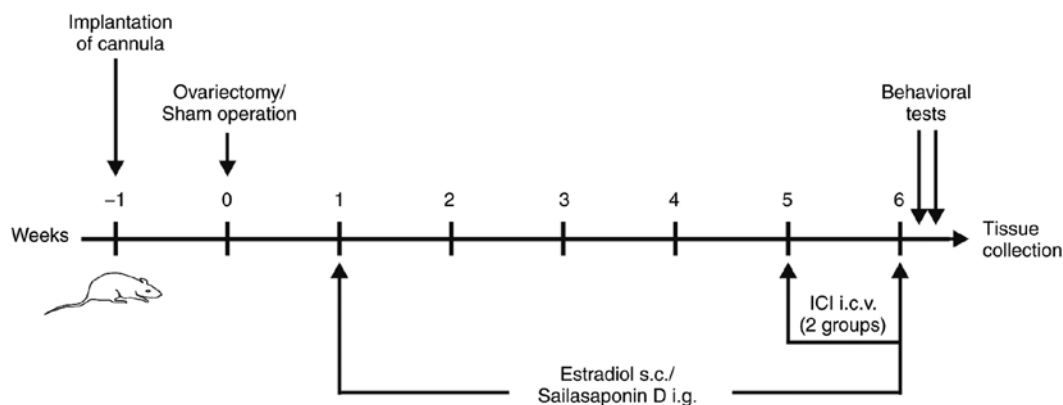


Figure 2. Experimental protocol. Behavioral tests were performed 1 and 24 h after the last drug administration, and tissues were collected the next day. i.c.v., intracerebroventricular; ICI, ICI182780; i.g., intragastric gavage; s.c., subcutaneous.

Samples from six rats in each group were analyzed by ELISA (cat. no. KGE014; R&D Systems, Inc.), in order to determine the content of E2 in the hippocampus, according to the manufacturer's protocol. Absorbance was measured at 450 nm with a microplate reader.

Samples from the remaining three rats in each group were used for western blot analysis. Tissue proteins were extracted with 5% RIPA lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology), and centrifuged at 13,500 x g for 15 min at 4°C, and the protein concentration was determined using a Pierce bicinchoninic protein assay kit (cat. no. 23223; Thermo Fisher Scientific Inc.). Equal amounts of protein (30 µg/lane) were separated by 7.5-12% SDS-PAGE (cat. nos. 1610171TA and 1610175TA; Bio-Rad Laboratories, Inc.) and transferred to PVDF membranes (cat. no. IPVH00010; EMD Millipore). The membranes were incubated with TBS containing 0.05% Tween-20 (TBS-T; cat. no. SRE0031; Sigma-Aldrich; Merck KGaA) and 5% non-fat dry milk for 2 h at room temperature to block nonspecific binding and were immunoblotted at 4°C overnight with the following primary antibodies: Rabbit anti-ERα (1:1,000, cat. no. 21244-1-AP; Proteintech Group, Inc.), rabbit anti-ERβ (1:1,000, cat. no. 14007-1-AP; Proteintech Group, Inc.), rabbit anti-GAPDH (1:1,000, cat. no. AP0063; Bioworld Technology, Inc.). After being washed by TBS-T, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody goat anti-rabbit immunoglobulin G (1:5,000, cat. no. BS13278; Bioworld Technology, Inc.) at room temperature for 2 h. Subsequently, the membranes were washed in TBS-T, and were developed with an enhanced chemiluminescence kit (cat. no. NEL104001EA; PerkinElmer, Inc.). Each experiment was performed independently at least three times, and the integrated intensity was measured using Image-Pro-Plus 6.0 (Media Cybernetics, Inc.).

**Immunohistochemistry and Nissl staining.** In each group, tissues from the remaining three rats were processed for immunohistochemistry, according to our previously described procedure (25). Briefly, the rats were deeply anesthetized and transcardially perfused with 4% paraformaldehyde (cat. no. AR1068; Wuhan Boster Biological Technology, Ltd.). The brains were extracted, postfixed in 4% paraformaldehyde at 4°C for 24 h and embedded in paraffin; subsequently,

samples were cut into 5-µm sections. The three sections containing the hippocampal CA1 region were incubated with 3% hydrogen peroxide (cat. no. AR1108; Wuhan Boster Biological Technology, Ltd.) at room temperature for 10 min, 10% goat serum (cat. no. ab7481; Abcam) at 37°C for 30 min. Subsequently, sections were incubated with the primary antibodies rabbit anti-ERα (1:200; cat. no. 21244-1-AP; Proteintech Group, Inc.) or rabbit anti-ERβ (1:200, cat. no. 14007-1-AP; Proteintech Group, Inc.) at 4°C overnight and the biotinylated secondary antibody goat anti-rabbit IgG (1:500, cat. no. BS13278; Bioworld Technology, Inc.) were incubated at room temperature for 30 min. The sections were subsequently counterstained with hematoxylin (0.5%; cat. no. H104302; Shanghai Aladdin Biochemical Technology Co., Ltd.) at room temperature for 3 min.

The adjacent slices were selected for Nissl staining (0.5% thionine; cat. no. 78338-22-4; Sigma-Aldrich; Merck KGaA; 37°C, 10 min), and micrographs of the hippocampal CA1 region were captured under a light microscope (Axio Vert A1; Carl Zeiss AG). Nissl-labeled neurons were calculated from two or three random fields per slice, and technicians blinded to the samples manually made the measurements. Briefly, in each Nissl-stained slide (magnification, x400) the number of neurons was counted in a calibrator (125x125 µm) and the density (cells/mm<sup>2</sup>) was subsequently calculated. The criterion for acceptance as a neuron was clear staining of a soma and a nucleus, which were distinctly differentiated from their backgrounds (25).

**Statistical analysis.** Data are expressed as the means ± SEM. The significance of the differences between groups was evaluated by one-way analysis of variance (ANOVA) followed by Fisher's least significant difference post hoc test using SPSS version 16.0 (SPSS Inc.). P<0.05 was considered to indicate a statistically significant difference.

## Results

**SSD markedly rescues ovariectomy-induced fear memory deficit.** Among all groups (sham, O-saline, O-E2, O-SSD 1.8, O-SSD 0.9, O-SSD 1.8-ICI and O-SSD 0.9-ICI), the mean freezing fraction prior to conditioning exhibited no significant differences ( $F_{6,77}=0.47$ ,  $P=0.65$ , one-way ANOVA;

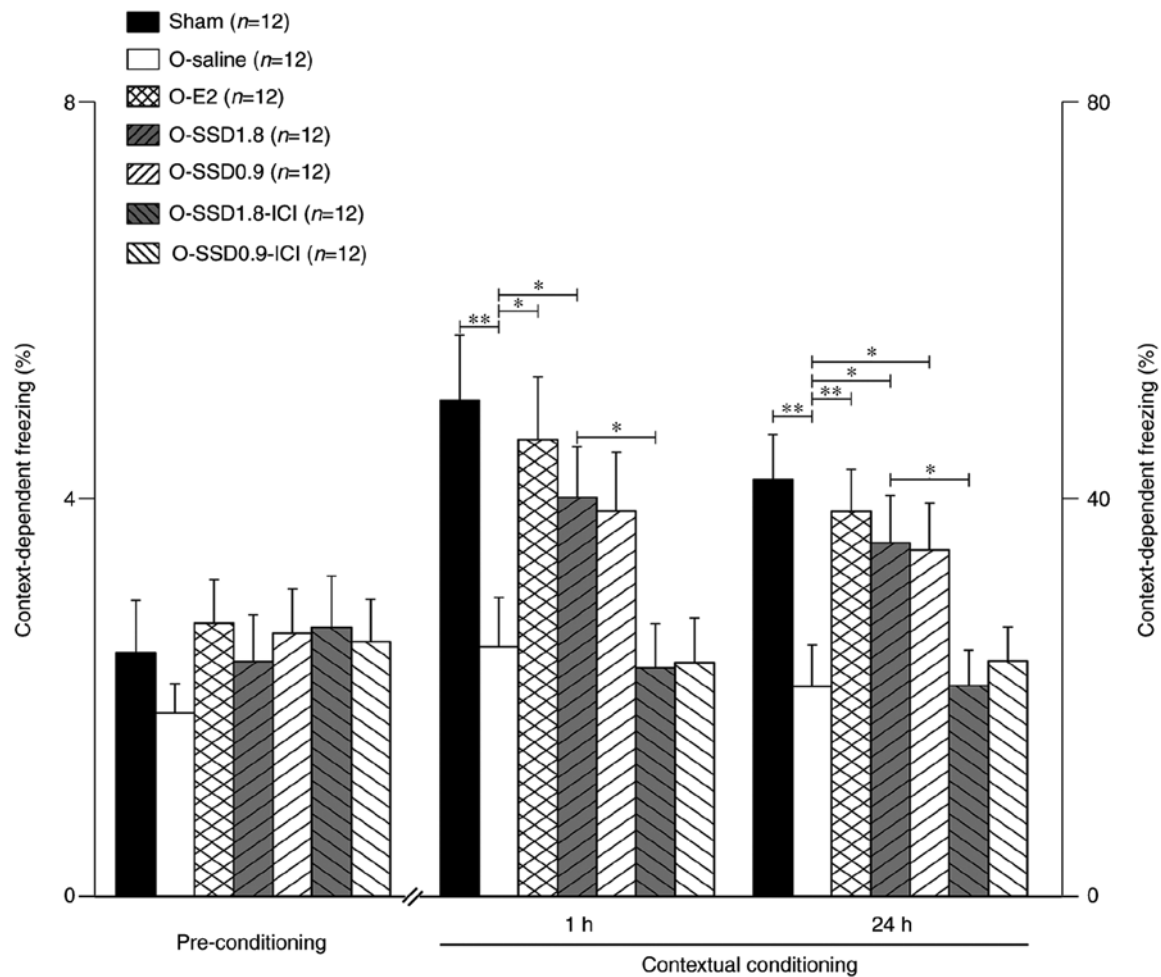


Figure 3. Context-dependent freezing time (%) among groups. Data are presented as the means  $\pm$  SEM. \* $P$ <0.05; \*\* $P$ <0.01, one-way analysis of variance and Fisher's least significant difference post hoc test. E2, estradiol; ICI, ICI182780; O, ovariectomized; SSD, saikosaponin-D.

Fig. 3). Compared with the sham operation group, ovariectomy significantly shortened the freezing time ( $P$ <0.01, O-saline vs. sham; Fig. 3); this finding is in agreement with results from a previous study (26). However, the freezing time was markedly prolonged in OVX animals after E2 administration at 1 or 24 h ( $P$ <0.05 or  $P$ <0.01, O-E2 vs. O-saline; Fig. 3), thus indicating that the memory loss in OVX rats was due to an estrogen deficit. Notably, treatment with SSD (SSD 1.8 and SSD 0.9) exerted a similar effect to E2 administration, which lasted for at least 24 h despite slight attenuation (Fig. 3).

Intracerebroventricular administration of ICI182780 markedly blocked the increase in freezing time in the O-SSD 1.8 group at 1 h ( $P$ <0.05 O-SSD 1.8-ICI vs. O-SSD 1.8) and 24 h ( $P$ <0.05, O-SSD 1.8-ICI vs. O-SSD 1.8; Fig. 3), and there was also a clear tendency toward reduced freezing in the O-SSD 0.9-ICI group (Fig. 3). These results indicated that ERs may be involved in SSD-mediated effects.

*SSD does not affect E2 levels in the hippocampus of OVX rats.* The E2 levels in the hippocampus were significantly lower in OVX rats compared with in sham-operated animals ( $P$ <0.01, O-saline vs. sham; Fig. 4), whereas this decrease was reversed following E2 administration ( $P$ <0.05, O-E2 vs. O-saline; Fig. 4). However, SSD treatment (O-SSD 1.8 or O-SSD 0.9) exhibited no influence on E2 levels ( $P$ =0.46

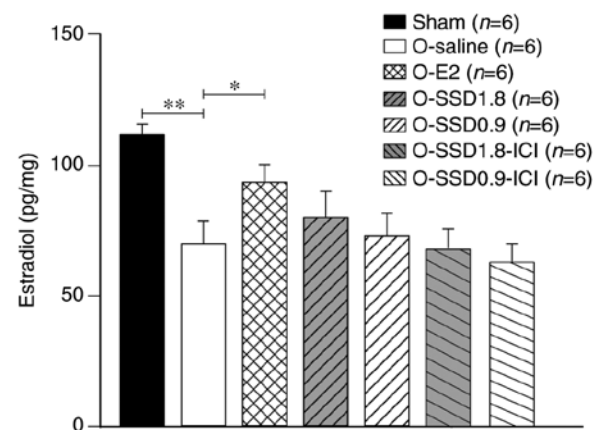


Figure 4. E2 levels in the hippocampal CA1 region among the groups. E2 levels in the hippocampus were measured with an E2 ELISA kit. Data are presented as the means  $\pm$  SEM. \* $P$ <0.05; \*\* $P$ <0.01, one-way analysis of variance and Fisher's least significant difference post hoc test. E2, estradiol; ICI, ICI182780; O, ovariectomized; SSD, saikosaponin-D.

and  $P$ =0.82, respectively, for O-SSD 1.8 or O-SSD 0.9 vs. O-saline; Fig. 4). In addition, the ER inhibitor ICI182780 did not influence E2 levels ( $P$ =0.22, O-SSD 1.8-ICI vs. O-SSD 1.8;  $P$ =0.59, O-SSD 0.9-ICI vs. O-SSD 0.9; Fig. 4). These



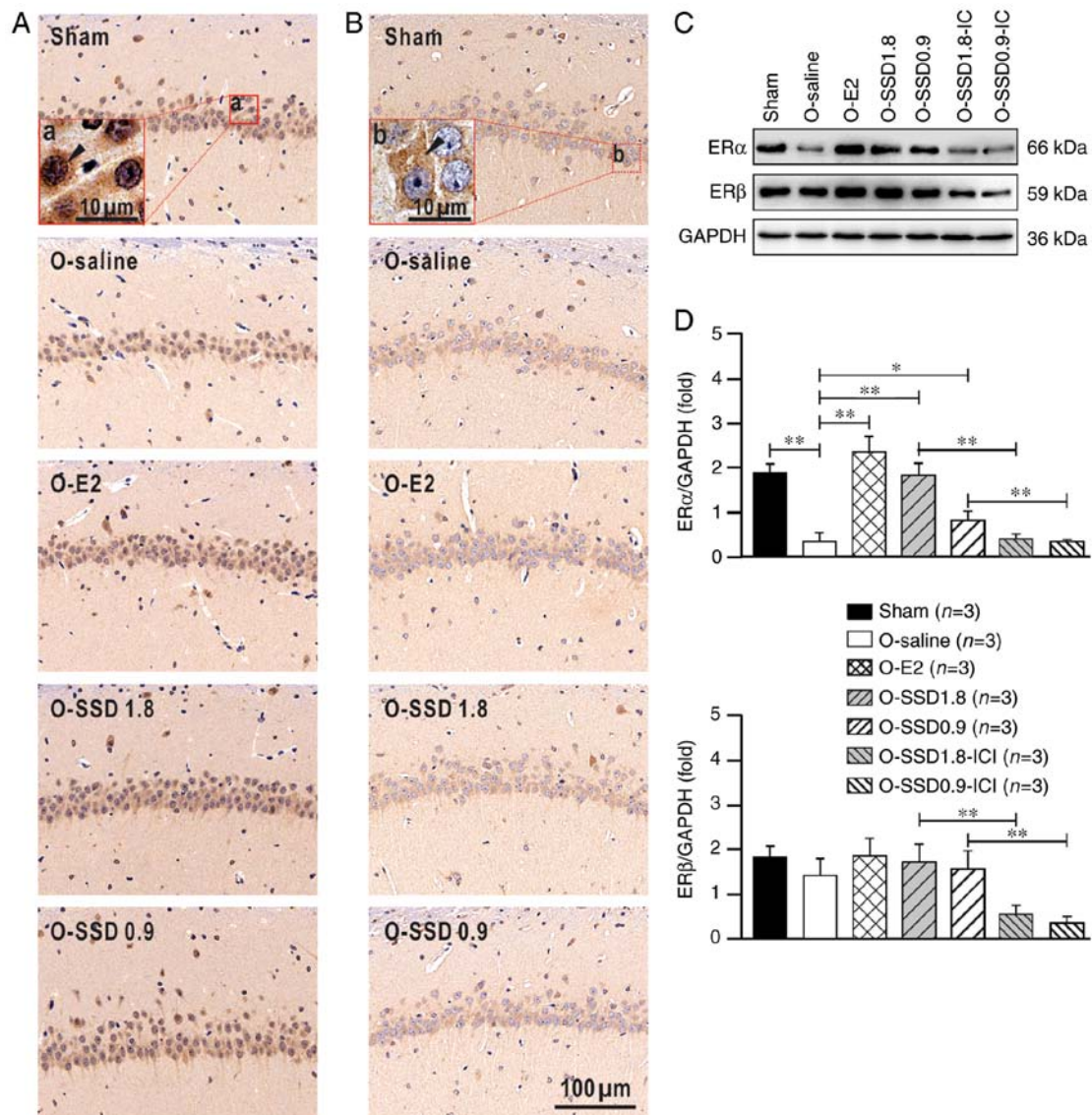


Figure 5. ER $\alpha$  and ER $\beta$  expression in the hippocampal CA1 region among groups. (A and B) Immunohistochemical staining. Sections demonstrated that in the hippocampal CA1 region, ER $\alpha$  was present in extranuclear and nuclear sites, whereas ER $\beta$  was present in extranuclear sites. (a) The red box represents a higher magnification, and the black triangle indicates an ER $\alpha$ -positive neuron; (b) the red box represents a higher magnification, and the black triangle indicates an ER $\beta$  positive neuron. (C and D) Western blot analysis for semi-quantitative analysis of ER $\alpha$  and ER $\beta$  expression, whose bands were 66 and 59 kDa, respectively. The optical density of ER $\alpha$  or ER $\beta$  was normalized to GAPDH. Data are presented as the means  $\pm$  SEM. \* $P$ <0.05; \*\* $P$ <0.01, one-way analysis of variance and Fisher's least significant difference post hoc test. Scale bars: (A and B) 100  $\mu$ m; (a and b) 10  $\mu$ m. E2, estradiol; ER, estrogen receptor; ICI, ICI182780; O, ovariectomized; SSD, saikosaponin-D.

results suggested that the SSD-mediated improvement in memory deficit in OVX rats may not be attributed to E2 alterations in the hippocampus.

**SSD enhances hippocampal expression of ER $\alpha$ , but not ER $\beta$ , in OVX rats.** As shown in Fig. 5, ER $\alpha$  and ER $\beta$  were widely expressed in hippocampal neurons; this has also been reported by previous studies (27-29). ER $\alpha$  was present in both the extranuclear (cytoplasmic regions) and intranuclear sites of the neurons, with prominent extranuclear expression (Fig. 5Aa). In addition, ER $\beta$  was mainly expressed in the extranuclear sites (Fig. 5Bb), which is in line with previous reports (30-32). The staining intensities for ERs exhibited obvious differences among the groups. Western blotting was conducted for semi-quantitative analysis of ER expression (Fig. 5C). ER $\alpha$  exhibited a significant downregulation in the hippocampus of

OVX rats ( $P$ <0.01, O-saline vs. sham; Fig. 5D), whereas E2 supplementation substantially reversed this downregulation ( $P$ <0.01, O-E2 vs. O-saline; Fig. 5D). Notably, ER $\alpha$  expression in the hippocampus was also significantly enhanced by SSD treatment in the O-SSD 1.8 ( $P$ <0.01, O-SSD 1.8 vs. O-saline; Fig. 5D) and O-SSD 0.9 groups ( $P$ <0.05, O-SSD 0.9 vs. O-saline; Fig. 5D) of OVX rats. Conversely, ER $\beta$  expression exhibited no significant differences among these groups (sham, O-saline, O-E2, O-SSD 1.8 and O-SSD 0.9) by one-way ANOVA ( $F_{4,10}=0.75$ ,  $P=0.58$ ; Fig. 5D). These findings indicated that SSD activated ER $\alpha$  expression in the hippocampus of OVX rats. Furthermore, ER $\alpha$  and ER $\beta$  expression in the O-SSD groups was markedly inhibited by ICI182780 intervention ( $P$ <0.01, O-SSD 1.8-ICI vs. O-SSD 1.8 and O-SSD 0.9-ICI vs. O-SSD 0.9; Fig. 5D), confirming that the inhibitor ICI182780 had suppressive effects on ER $\alpha$  and ER $\beta$ .

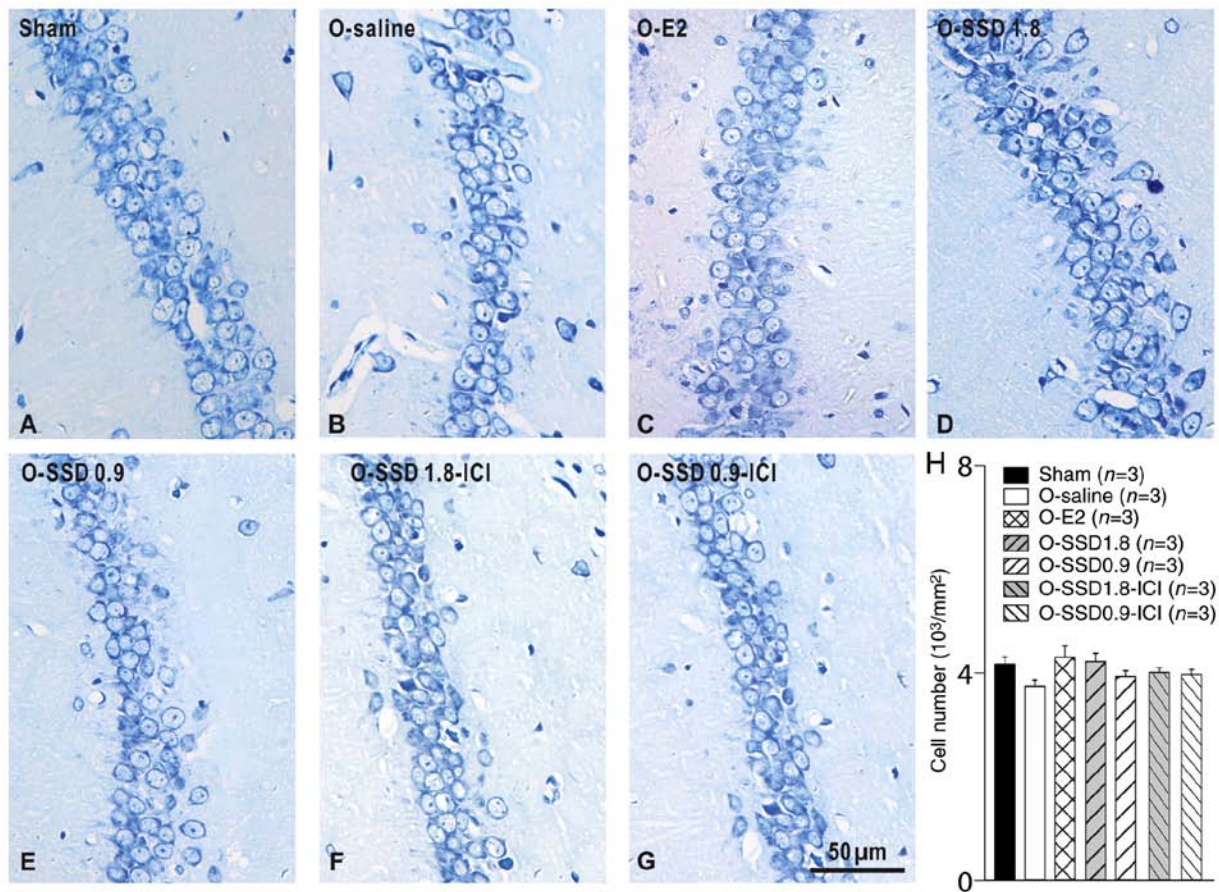


Figure 6. Neuron numbers in the hippocampal CA1 region among the groups. (A-G) Micrographs of Nissl-labeled slices. (H) Histogram of the neuron number. There was no significant difference in neuron number among the groups. Scale bars: 50  $\mu$ m. E2, estradiol; ER, estrogen receptor; ICI, ICI182780; O, ovariectomized; SSD, saikosaponin-D.

*SSD does not affect the number of neurons in the hippocampus of OVX rats.* Given that neuron number conclusively influences neural functioning in the nervous system, this study aimed to determine whether SSD-induced behavioral improvement in OVX rats may be associated with changes in neuron number. As shown in Fig. 6A-H, the number of neurons in the hippocampus exhibited no significant alterations among the groups (sham, O-saline, O-E2, O-SSD 1.8, O-SSD 0.9, O-SSD 1.8-ICI and O-SSD 0.9-ICI;  $F_{6,119}=1.429$ ,  $P=0.209$ ; Fig. 6H), thus suggesting that SSD-mediated improvements in memory deficits in OVX rats were not attributed to changes in the number of neurons.

## Discussion

Estrogen serves important roles in the central nervous system and is involved in various processes, including neuronal differentiation, synaptic formation and neural repair (33-35). Accordingly, estrogen deficits lead to substantial neural malfunction, particularly cognitive impairment (36,37). The hippocampus is a crucial center for cognitive processes, which is extensively modulated by estrogen; furthermore, estrogen disorders often lead to cognitive impairment, including fear memory deficits (38,39). In the present study, contextual fear conditioning was used to reveal the hippocampal action of estrogen involved in fear memory.

E2 levels in the hippocampus are markedly decreased in OVX rats, probably due to a local decrease in estrogenic synthesis (40). The present results demonstrated that decreased E2 in the hippocampus was associated with fear memory impairment in OVX rats. Notably, this impairment was reversed by E2 supplementation, in a manner reminiscent of clinical estrogen replacement therapy for menopausal women or OVX rats (34,36,41,42). However, E2 in the hippocampus exerts distinct roles under different conditions; for example, E2 serves an active role at low doses, whereas higher doses exert a suppressive effect on working memory (43,44). Notably, the phytoestrogen SSD has a similar chemical structure and physiological function to E2, and is frequently used as a substitute for estrogen in clinical trials. In the present study, it was revealed that SSD prolonged freezing time in OVX rats in a manner similar to E2. However, the E2-mediated improvement in memory deficit was associated with elevated hormone levels, whereas the SSD-induced improvement was independent of E2 levels in the hippocampus of OVX rats. In addition, SSD did not exert any influence on serum E2 levels in OVX rats (Liu *et al*, unpublished data), strongly indicating that the SSD-rescued memory deficit in OVX rats does not rely on E2 levels.

Although ERs are expressed in neurons and glial cells, the expression is highly distinct among different brain regions (30). ERs in the hippocampal CA1 area are mainly expressed in



neurons (27-29). In the current study, immunohistochemistry was performed to discern the cytoplasmic and nuclear distribution of ERs, and western blotting was used to semi-quantify protein expression. The results revealed that ER $\alpha$  and ER $\beta$  were expressed in the hippocampus; however, ER $\beta$  expression in the hippocampal CA1 region remained unchanged in OVX rats. This outcome may be associated with elevated serum corticosterone (Liu *et al*, unpublished data), since E2 deficiency stimulates the hypothalamic-pituitary-adrenal axis to release corticosterone, which subsequently counteracts the decrease in ER $\beta$  (45,46). In addition, SSD treatment did not affect ER $\beta$  protein expression. Conversely, significant alterations in ER $\alpha$  expression were detected in the hippocampus of OVX rats following SSD treatment. ER $\alpha$  has frequently been reported to be associated with spine structure and/or post-synaptic functions (47,48); for example, selective activation of ER $\alpha$  in OVX mice results in an increased spine density in the hippocampus, which is inversely correlated with memory dysfunction (49). In particular, ICI182780, a non-selective inhibitor of ERs, markedly blocked the memory improvement induced by SSD in OVX rats, thus corroborating a mechanism of SSD-mediated memory restoration via ERs. Previous studies reported that ovariectomy stimulates ER $\alpha$  promoter methylation, which in turn inhibits ER $\alpha$  expression (47,50). Therefore, it may be hypothesized that SSD prevents ER $\alpha$  promoter methylation and induces ER mRNA expression, thus resulting in upregulation of ER $\alpha$  protein expression in OVX rats. In the present study, ovariectomy did not alter the number of neurons in the hippocampus; this was also the case in a previous study (46). In addition, neither SSD nor E2 supplementation affected neuron number. Therefore, SSD-induced activation of ER $\alpha$  in hippocampal neurons may be a potential mechanism underlying SSD-improved fear deficits in OVX rats.

Despite the novel findings of the present study, several limitations must be solved for further improvement. Firstly, because the amygdala serves a pivotal role in triggering a state of fear, and is also involved in the modulation of memory consolidation (51), a thorough investigation of the neural circuit for SSD-mediated promotion in fear memory deficit is worthy of future consideration. Secondly, although this study revealed that SSD differentially upregulates ER expression in the hippocampus, whether SSD modulates neuronal activities, and whether ER $\alpha$  and ER $\beta$  have different roles in the hippocampus in OVX animals, remains to be determined. Thirdly, the present study, along with various previous reports, suggested that a suitable low dosage of SSD may benefit neurological functions (19,52), whereas higher concentrations of SSD may cause neurotoxicity (22,53); although the different concentrations of SSD activate distinct signal pathways (54), further research is required to clarify the precise mechanisms.

In conclusion, this study demonstrated that the phytoestrogen SSD rescued fear memory deficit in OVX rats. The results suggested that this effect may be mediated through activation of ER $\alpha$ , rather than ER $\beta$  or E2, in the hippocampus.

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## Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

## Authors' contributions

LL and CZ conceived and designed the study. LL, JY and XX performed the experiments. HS, FG, JL SL and YZ analyzed the data and revised the manuscript. LL, JY and CZ wrote the manuscript. All authors read and approved the final version of the manuscript.

## Ethics approval and consent to participate

All experimental protocols involving animal tissue samples were approved by the Ethics Committee of Nanjing University of Chinese Medicine.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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