Journal of Acupuncture and Meridian Studies 13 (2020) 94–103

Contents lists available at ScienceDirect

Journal of Acupuncture and Meridian Studies

journal homepage: www.jams-kpi.com

Research Article

Electroacupuncture Promotes Neural Proliferation in Hippocampus of Perimenopausal Depression Rats via Wnt/β-Catenin Signaling Pathway

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ARTICLE INFO

Article history:
Received 7 November 2019
Received in revised form
13 March 2020
Accepted 27 March 2020
Available online 8 April 2020

Keywords:
Electroacupuncture
Neural Proliferation
Perimenopausal Depression
Wnt/β-catenin Signaling Pathway

ABSTRACT

Background and Objective: Perimenopausal depression is caused by the impaired function of the ovarium before menopause and with a series of symptoms. Electroacupuncture (EA) therapy has been demonstrated to improve clinically depression. However, the mechanism underlying its therapeutic activity remains unknown. This study aimed to investigat the effects of EA treatment on the hippocampal neural proliferation through Wnt signaling pathway.

Methods: Chronic unpredictable mild stress (CUMS) combined with bilateral ovariectomy (OVX) were used to establish a rat model of perimenopausal depression. The open field test (OFT) and sucrose preference test (SPT) were used to assess depression-like behaviors in rats. ELISAs were used to measure estrogen (E2), luteinizing hormone (LH) and gonadotropin-releasing hormone (GnRH) levels in the serum. RT-PCR and Western blot assay were utilized for measuring the mRNA expressions and protein expressions of GSK-3β/β-catenin.

Results: Four-week EA treatment at three points including "Shenshu" (BL23), "Baihui" (GV20) and "Sanyinjiao" (SP6) simultaneously ameliorated depression-like behaviors in rats with CUMS and OVX, whereas rescued the decreased serum level of E2 and prevented the increased serum levels of GnRH and LH. EA treatment ameliorated CUMS and OVX-induced alterations of glycogen synthase kinase-3β (GSK-3β) and β-catenin mRNA levels, β-catenin and phosphorylated β-catenin (p-β-catenin) protein levels.

Conclusions: The results showed that EA treatment promoted hippocampal neural proliferation in perimenopausal depression rats via activating the Wnt/β-catenin signaling pathway, indicating that EA may represent an efficacious therapy for perimenopausal depression.

1. Introduction

Depression is characterized by a persistent feeling of sadness, loss of interest, sleep disorder, unexplained weight gain or loss, hypo- or hyperactivity, inability to concentrate, feeling of guilt and worthlessness, and suicidal thoughts [1]. Perimenopausal depression is caused by the impaired function of the ovarium piror to menopause. Approximately 30% of women experience perimenopausal depression and unpleasant hot flashes in the perimenopausal period[2]. An efficacious treatment for depression is urgently sought. Clinical studies have implicated the impaired function and atrophy of hippocampus in depression, and currently antidepressant drugs have also been demonstrated to improve hippocampal atrophy in depression [3], indicating an important role of hippocampal neurons in depression [4].

Many studies have demonstrated that rodents and humans undergo similar ovarian senescence, beginning with irregular cycling and declining fertility, and ending with total depletion of ovarian follicles. Meanwhile, the acupuncture points in rodents are similar to those in humans. Non-specific acupuncture stimulation could reduce nerve stress and restore abnormal physiological and biochemical processes by activating regulatory functions [5,6]. For example, acupuncture could induce analgesia to improve depression-related behaviors in rodents [7]. The distribution of acupuncture points used to treat perimenopausal depression is

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https://doi.org/10.1016/j.jams.2020.03.065

relatively wide. Liu et al [8] performed “Baihui” and “Anmian” with electroacupuncture (EA) once daily for three consecutive weeks after chronic unpredictable stress. EA treatment significantly reversed the behavioral deficit of depression in a rat model and the decreased glial fibrillar acidic protein (GFAP) level, affecting glial atrophy in the hippocampus. Cheng et al [9] found that the reduced estrogen (E2) increased levels of gonadotropin releasing hormone (GnRH), luteinizing hormone (LH) and follicule stimulating hormone (FSH), which were all decreased after EA treatment. EA at “Sanyinjiao” and “Guanyuan” could increase E2 level by activating the hypothalamus-pituitary-ovary (HPO) axis in castrated rats. In addition, Sun et al [10] found that “Shenshu” and “Sanyinjiao” could alleviate symptoms in patients with perimenopausal depression. Acupuncture treatment relieved depressive symptoms by regulating symptoms such as anxiety, retardation, sleep disorder and cognitive disorder, and had better long-term efficacy. Therefore, this experiment chose “Shenshu” (BL23), “Baihui” (GV20), and “Sanyinjiao” (SP6) as the target of EA points in the present study.

Variations in hormone level during the perimenopausal period can reduce women's capacity to respond to changes in the external environment. Mood changes are a major symptom of menopausal syndrome [11]. Hormone replacement therapy has been widely used and is clinically efficacious, but its use is limited by side effects. An efficacious therapy without side effects is still sought. Acupuncture therapy was proposed for the treatment of 64 different diseases, including depression by world health organization (WHO) at a conference in Milan, Italy in November 1996. EA therapy has been demonstrated to efficiently alleviate menopausal syndrome and depression [12,13]. Xu et al [14] explored the possible mechanism of EA on hippocampal synaptic plasticity. EA treatment was administered once daily for 3 weeks at “Baihui” and “Yintang”, which ameliorated depression-like behaviors by restoring hippocampal synaptic plasticity compared with the sham group. However, the mechanism by which EA therapy improves depression remains undetermined. Since hippocampal neurogenesis has been implicated in depression, this provides a new insight into the antidepressant effects of EA therapy. In fact, the Wnt signaling pathway has previously been implicated to play an important role in neural development, which participated in the process of neural cell differentiation, proliferation, migration, apoptosis, and regulated neural connectivity by controlling axon pathfinding, axon remodeling, dendrite morphogenesis and synapse formation [15,16]. However, the role of Wnt signaling in mechanism by which EA may influence hippocampal neural proliferation remains largely unknown. Therefore, in this study glycogen synthase kinase-3β (GSK-3β) and β-catenin in the hippocampus were measured to investigate the relationship between hippocampal neurogenesis and Wnt signaling in the perimenopausal depression rat model. The study showed that EA treatment promoted hippocampal neural proliferation via the Wnt signaling pathway, which might further illuminate the therapeutic effect of EA therapy in perimenopausal depression.

2. Methods and Materials

2.1. Animals

Thirty Female Sprague Dawley (SD) rats (aged 56-62 d, weighing 200 ± 10 g) were purchased from Beijing (Weitonglihua C.O., SCXK 2012-0001). Rats were housed under SPF conditions with free access to water and food at the Animal Care Laboratory (22-26°C, 40%-50% humidity, and 12 h/12 h light/dark environment, Liaoning University of Traditional Chinese Medicine). All the rats were habituated to adaptive feeding for 3 weeks before the experiment begun (Fig. 1).

Rats were randomly divided into 5 groups including naïve, control + chronic unpredictable mild stress (CUMS), ovariectomy (OVX) + CUMS, OVX + clomipramine hydrochloride (CH) + CUMS, OVX + EA + CUMS. The rats in control + CUMS group received intragastric injection and were subjected to CUMS only. The rats in OVX + CUMS group were subjected to OVX and CUMS. The OVX + CH + CUMS group received intragastric administration of 20 mg/kg CH daily for 28 days after OVX and CUMS. The CH was dissolved in saline. The OVX + EA + CUMS group received EA treatment after OVX and CUMS. The rats in all groups were housed under standard laboratory conditions. At the beginning of the experiment, CUMS animals were transported to another soundproof room individually, which avoided the effect of the actual manipulation of rats, then returned to the original room after CUMS. All experiment procedures were approved by the Liaoning University of Traditional Chinese Medicine (TCM) according to Animal Ethics rules.

2.2. Bilateral OVX

Surgery was performed as described previously [17]. The rats were fasted for 24 h, but provided free access to water before surgery. They were weighed by anesthetized with intraperitoneal injection of 10% chloral hydrate (0.3 ml/100 g), then the skin was sterilized with commercially available alcohol and iodine. The fur over the dorsal lumbar area was shaved. A longitudinal incision (1–2 cm) was made at the midline of the lower abdomen, and the ovaries were removed. The wound was closed with 2-3 drops of penicillin (8 × 106 U penicillin in 5 ml saline). The skin incision was closed by stainless steel wound clips (2 or 3 each side). The same procedure was performed on each side. No treatment was performed in the naïve group, while the control + CUMS group received the same surgery procedure and some adipose tissues around ovaries were removed, but the ovaries were not removed.

2.3. CUMS

The rats in the naïve group were isolated individually. Other rats except the naïve group were subjected to CUMS paradigm modified from a method described previously by Willner *et al.* [18]. The paradigm consisted of seven different procedures including application of a clamp 1 cm from the tail end for 1 min; swimming in 4°C cold water for 5 min; swimming in 45°C hot water for 5 min; 24 h of food deprivation and 24 h of water deprivation; restricted activity for 1 h; cage tilting and damp sawdust for 24 h (200 ml of water per individual cage, sufficient to make the sawdust bedding wet); circadian disruption by inversed light and dark cycle. Rats were individually housed and subjected to a randomly chosen stressor daily for 21 days was shown in Table 1, and the same stressor was not given for two consecutive days. Every stressor was used 3 times at the average.

2.4. EA treatment

According to the publication “Experimental Acupuncture” [19], the locations of three acupuncture points were determined including “Shenshu” (BL23, 5 mm next to the second lumbar vertebra, 6 mm depth), “Sanyinjiao” (SP6, 10 mm upper in the inner malleolus tip of hind limb, 5 mm depth) and “Baihui” (GV20, the center of parietal bone, 2 mm depth). The rats were wrapped with a cloth bag, and left the nose, mouth, hind limbs, and tail exposing. In

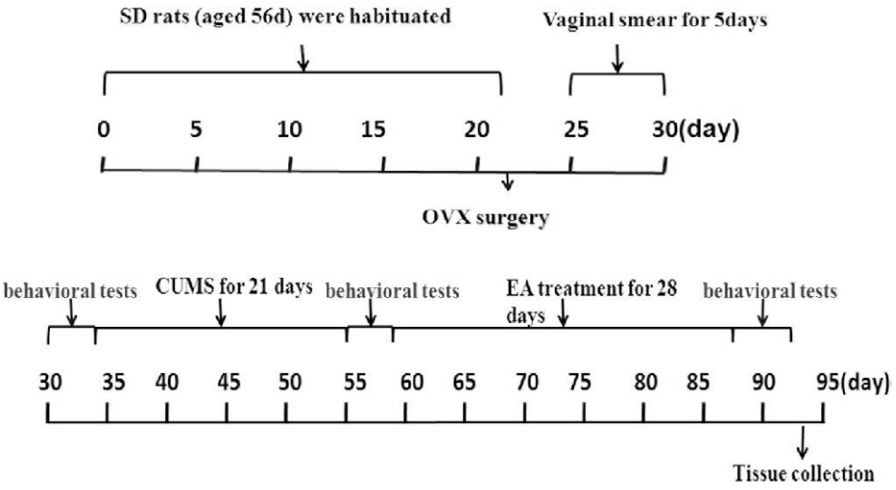

Figure 1. Experimental timing. OVX = Ovariectomy; CUMS = Chronic unpredictable mild stress; EA = Electroacupuncture; SD = Sprague-Dawley.

Table 1
Specific schedules of CUMS.

Stressor day	Tail clamp	Food and water deprivation	Cage tilting and damp sawdust	Swimming in hot water	Swimming in cold water	Restricted activity	Reversal of light and dark
1	*						
2		*					
3				*			
4			*				
5					*		
6							*
7						*	
8					*		
9				*			
10							*
11	*						
12			*				
13		*					
14							*
15	*						
16					*		
17						*	
18				*			
19		*					
20			*				
21						*	

*Respents that the rats were subjected the stressor daily. CUMS = Chronic unpredictable mild stress.

order to perform acupuncture on the hind limbs precisely, the cloth was suspended. After insertion of the acupuncture needle, rats were connected to the electroacupuncture apparatus. Two pairs of electrodes were connected to Sanyinjiao and Shenshu in the same side to prevent electrical current from passing through the heart. The negative pole of another pair of electrodes was connected to Baihui, and the positive pole was connected to the rat tail. An electrical current of 18 V with high/low frequency in 4 Hz and 20 Hz was added. EA was administered daily (20 min per time) for 28 days.

2.5. Estrous cycle assessment

To assess OVX, the estrous cycle was observed using vaginal smears with Giemsa staining [17]. Three days after surgery, the vaginal smears were collected daily for 5 days by inserting saline rinsed cotton to a depth of 0.5-1.0 cm within the vagina while the rat was held and fixed by hand. The cotton was smeared on a glass slide to dry, and fixed in 100% ethanol for 3-5 min after natural evaporation drying, then followed by incubation with Giemsa staining (Beijing Donglinchangsheng Biotechnology Co., Ltd., Catalog No. AR-0751/AR-0752) for 10-30 min and saline rinse. The cell shape was observed under microscopy with 200× magnification after the slides were dried which washed by saline.

2.6. Open field test (OFT)

The time spent in the center and distance traveled during the OFT were used to assess depression-like behaviors in rats. The Plexiglas chamber was equipped with a camera on the top. The open field apparatus (100 cm × 100 cm area and 40 cm high wall) was divided into 25 squares by black lines. An area consisting of the inner five squares (20 cm × 20 cm) which attached to the wall of apparatus was designated as the central part, whereas the area surrounding the center was designated as the peripheral zones. Rats were allowed to move freely in the open field. Rats were habituated to the room containing the open field equipment for 2 h, and then the tests were performed between 9 a.m. and 11 a.m. They were placed in the chamber to explore 3 min freely, and the locomotor activity was monitored by the camera to avoid disturbing

their performance. The 75% ethanol was used to clean the chamber after each test to prevent the scent of previous rat from affecting later rat. The test was conducted after EA or CH treatment respectively.

2.7. Sucrose preference test (SPT)

Rats were individually housed and fasted before the test. To habituate the bottles and sucrose, each rat was housed separately and presented with two bottles filled with 1% (w/v) sucrose (Sigma-Aldrich, MO, USA) dissolved in tap water for 24 h. Subsequently, one bottle was exchanged to tap water, and the other bottle was filled with 1% sucrose for another 24 h. During the test, rats were presented with one bottle containing tap water, and another containing 1% sucrose for 24 h. Within each group, the side of sucrose presentation was randomly assigned for half number of the group. Bottles were weighed before and after these substitutions. The sucrose preference was calculated according to the percentage of the sucrose intake in total liquid intake: Sucrose preference (%) = [Sucrose consumption (g)/Water + Sucrose consumption (g)] × 100 [20].

2.8. Tissue collection

Rats were fasted for 24 h. After rats were anesthetized with 10% chloral hydrate, the abdominal aortat was isolated and blood was collected using negative pressure. Hippocampal tissue was isolated from one half of the brain. The tissue was weighed and homogenized in a certain ratio (Tissue weight: PBS volume = 1:9, for example, 1 g of tissue sample corresponding to 9 ml of PBS) to the precooled PBS homogenate, which followed with centrifuged at 3000 r/min for 10 min, and the supernatant was stored at −80°C. The other half of the hippocampal tissue retained for immunohistochemistry.

2.9. Enzyme-linked immunosorbent assay (ELISA)

ELISAs were used to measure E2, LH and GnRH levels in the serum according to the manufacturer's instructions (Shanghai Lanpai Biotechnology Co., Ltd.Shanghai, China).

2.10. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from hippocampal tissues using Trizol (Invitrogen). The RNA concentration was calculated using the value of OD260 (OD260 = 1 represents 0.04 μg RNA/μl). RNA concentration (μg/μl) = [OD260 × dilution factor × 0.04 μg/μl]. OD260/OD280 ratio indicated the purity of RNA. Take 1 μg of RNA as a template, according to the calculated RNA concentration. The cDNA was synthesized using a ProtoScript® II First Strand cDNA Synthesis Kit (NEB, 600838), according to the manufacturer's instructions.

The synthesized cDNA was appropriately diluted with nuclease-free water, and used in the following qRT-PCR reaction according to the Brilliant II SYBR Green QPCR Master Mix Kit (Agilent, NM_032080.1 and NM_053357) as follows: 95°C, 10 min; 40 cycles for 95°C, 20 s; 59°C, 20 s; 72°C, 30 s. The images were visualized on a 1% agarose gel with Ethidium Bromide (EB) and analyzed by the 4100 Gel imaging system (Tanon Science and Technology Co, Shanghai, China). The primers used in the experiment were described in Table 2. Fluorescent readings from qRT-PCR reactions were quantitatively analyzed by determining the difference between the GSK-3β/β-catenin CTs and the internal GAPDH control (delta Ct).

2.11. Western blot

Tissues were homogenized in lysis buffer and phosphatase inhibitor cocktail (P1260, Beijing Solarbio Science & Technology). Homogenates were centrifuged at 13000×*g* at 4°C for 15 min and supernatants were collected. Proteins were analyzed using BCA work solution assay (BioRad, Hercules, CA). Proteins were separated by 6% SDS-PAGE TM gels, transferred to PVDF membranes at 100 V for 1.5 h. After blocking with 5% BSA, the membranes were incubated with the primary antibody goat anti-rabbit IgG (1:500, Santa Cruz), rabbit β-catenin (1:1000, Cell Signaling Technology), rabbit phosphorylated β-catenin (p-β-catenin) antibody (1:1000, Cell Signaling Technology), β-actin (1:500, Santa Cruz, CA) overnight at 4°C. After washing with TBST for 3 times, membranes were incubated with the secondary antibodies including anti-rabbit and anti-goat for 2 h at room temperature. Blots were developed using chemiluminescent detection reagents, and chemiluminescent bands were detected on an Image Station FluorChem Q (Nature Gene Corp) and quantified using Image J software.

2.12. Statistical analysis

All statistical analyses were performed using SPSS 19.0 software. The data was tested for normality and expressed as mean ± SD. More than two groups were compared using One-Way analysis of variance (ANOVA) followed by LSD post-hoc test at $\alpha = 0.05$. When the variance is equal ($p > 0.05$), LSD test was used; while the variance is not uniform ($p < 0.05$), Tamhane's T 2 test was used. The differences were considered statistically significant if $p < 0.05$.

3. Results

3.1. Vaginal smear results

Estrous cycles of each rat were monitored daily by microscopic examination of vaginal cytology beginning at the vaginal opening. Vaginal smears were categorized into 4 stages: proestrus, estrus, metestrus and diestrus. As shown in Fig. 2, in the naïve group, vaginal smears mainly contained nucleated epithelial cells in the stage of proestrus; irregular keratinized epithelial cells in the stage of estrus; irregular keratinized epithelial cells, nucleated epithelial cells and white blood cells in the stage of metestrus; white blood cells in the stage of diestrus. After OVX surgery, vaginal smears did not have mature exfoliated cells, showing no change in estrous cycle for five consecutive days in Fig. 2.

Table 2
PCR primer.

Symbol	Gene Bank	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product size
GSK-3β	NM_032080.1	GGGCACCAGAGCTGATCTTT	GCCGAAAGACCTTCGTCCA	252 bp
β-catenin	NM_053357	CACCATCGAGAGGGCTTGTT	CGCACTGCCATTTTAGCTCC	155 bp
GAPDH	NM_008084.2	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA	150 bp

GSK-3β = Glycogen synthase kinase-3β; PCR = Polymerase chain reaction; GAPDH = Glyceraldehyde-3-phosphate dehydrogenase.

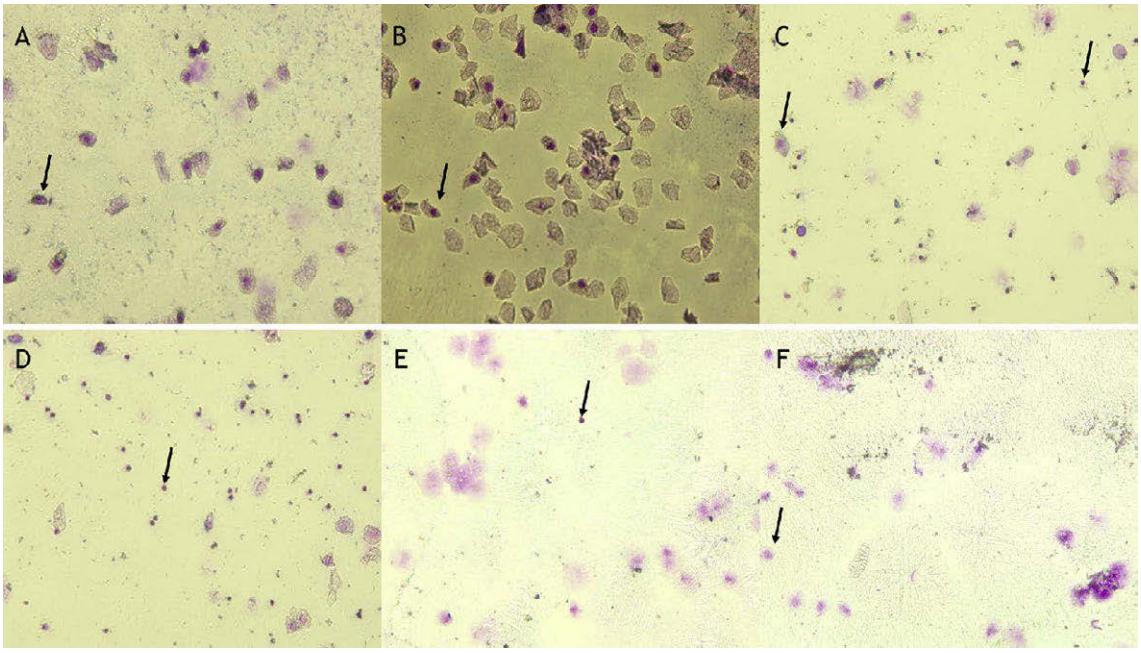

Figure 2. Vaginal smears. (A) Proestrus in the naïve group. There are nucleated epithelial cells (arrow) in the stage of proestrus. (B) Estrus in the naïve group. There are irregular keratinized epithelial cells (arrow) in the stage of estrus. (C) Metestrus in the naïve group. There are irregular keratinized epithelial cells, nucleated epithelial cells, and white blood cells (arrows) in the stage of metestrus. (D) Diestrus in the naïve group. There are white blood cells (arrow) in the stage of diestrus. (E) and (F) Vaginal smears in the OVX + CUMS group. After OVX surgery, the representing images showed white blood cells (arrows), but not epithelial cells. OVX = Ovariectomy; CUMS = Chronic unpredictable mild stress.

3.2. Effect on the serum hormone levels of perimenopausal depression rats

In the control + CUMS group, the rats were subjected to CUMS and the ovaries were not removed. So serum hormone tests showed there were slightly fluctuating, but no significant difference, compared with the naïve group ($p > 0.05$). After OVX surgery, serum level of E2 was decreased significantly in the OVX + CUMS group (Fig. 3A, naïve: 5.89 ± 0.30; OVX + CUMS: 1.83 ± 0.11). CH or EA treatment ameliorated serum level of E2 (Fig. 3A, OVX + CH + CUMS: 3.05 ± 0.13; OVX + EA + CUMS: 3.54 ± 0.33; df = 4, F = 360.38, $p < 0.05$). Compared with the naïve group, serum level of LH was increased after OVX and CUMS (Fig. 3B, naïve: 428.69 ± 6.72; OVX + CUMS: 1112.99 ± 3.31). CH or EA treatment reduced serum level of LH (Fig. 3B, OVX + CH + CUMS: 538.52 ± 15.70; OVX + EA + CUMS: 480.75 ± 10.59; df = 4, F = 4335.27, $p < 0.05$). Compared with the naïve group, serum level of GnRH was increased after OVX and CUMS (Fig. 3C, naïve: 62.08 ± 3.52; OVX + CUMS: 172.26 ± 4.02). CH or EA treatment reduced serum level of GnRH (Fig. 3C, OVX + CH + CUMS: 102.46 ± 1.30; OVX + EA + CUMS: 85.57 ± 2.95; df = 4, F = 1661.65, $p < 0.05$).

3.3. Effect on the depression-like behaviors of perimenopausal depression rats

In the open field test, the exploration activity in the center zone of OFT chamber was assessed as a measure of depression-like behaviors in rats before and after OVX and CUMS and/or EA treatment. We found that the rats in the OVX + CUMS group spent less time in the center zone of OFT chamber than that of rats in the control + CUMS or naïve group, suggesting that OVX and CUMS increased depression level. CH and EA increased the time spent in the center zone of OFT chamber (Fig. 4A, OVX + CUMS: 3.85 ± 1.77; OVX + CH + CUMS: 8.15 ± 2.23; OVX + EA + CUMS: 7.67 ± 3.09; df = 4, F = 3.93, $p < 0.01$). Similarly, rats in the OVX + CUMS group traveled less distance compared with control + CUMS group, while EA rescued the decrease in the distance compared with OVX + CUMS group (Fig. 4B, OVX + CUMS: 1447.04 ± 593.99; OVX + CH + CUMS: 4461.56 ± 548.03; OVX + EA + CUMS: 2641.14 ± 236.57; df = 4, F = 36.22, $p < 0.01$).

Next, the effect of EA on anhedonia behaviors of the perimenopausal depression animal model was assessed using SPT. We found that the rats in OVX + CUMS group showed significantly lower sucrose preference than control + CUMS group. However, four-week CH and EA treatment significantly increased the sucrose preference from the third week compared with OVX + CUMS group (Fig. 4C, OVX + CUMS: 64.77 ± 6.10; OVX + CH + CUMS: 81.18 ± 3.07; OVX + EA + CUMS: 81.03 ± 1.67; df = 4, F = 26.1, $p < 0.01$). Taken together, these results suggested that EA treatment could rescue the depression-like behaviors caused by OVX and CUMS in the perimenopausal depression rat model.

3.4. Effect on the GSK-3β and β-catenin mRNA expression of perimenopausal depression rats

To further explore the reason of EA treatment in promoting neurogenesis in the hippocampus, the experiment detected the crucial genes of Wnt signaling, which were well known as the critical pathway involved in cell proliferation and differentiation. The amplification efficiency was 95%-100%. Result from the melt curve we could see that the curve had only a single characteristic peak. It indicated single peak for each pair of primes used in this study suggesting the good specificity of the primers, and the primer design was reasonable. In the OVX + CUMS group, GSK-3β mRNA expression in the hippocampus was decreased compared with the naïve group (Fig. 5A, naïve: 1.00 ± 0.08; OVX + CUMS: 0.39 ± 0.02; $p < 0.01$). However, EA or CH treatment significantly increased GSK-3β mRNA expression compared with OVX + CUMS group, which was comparable to naïve group (Fig. 5A, OVX + CH + CUMS:

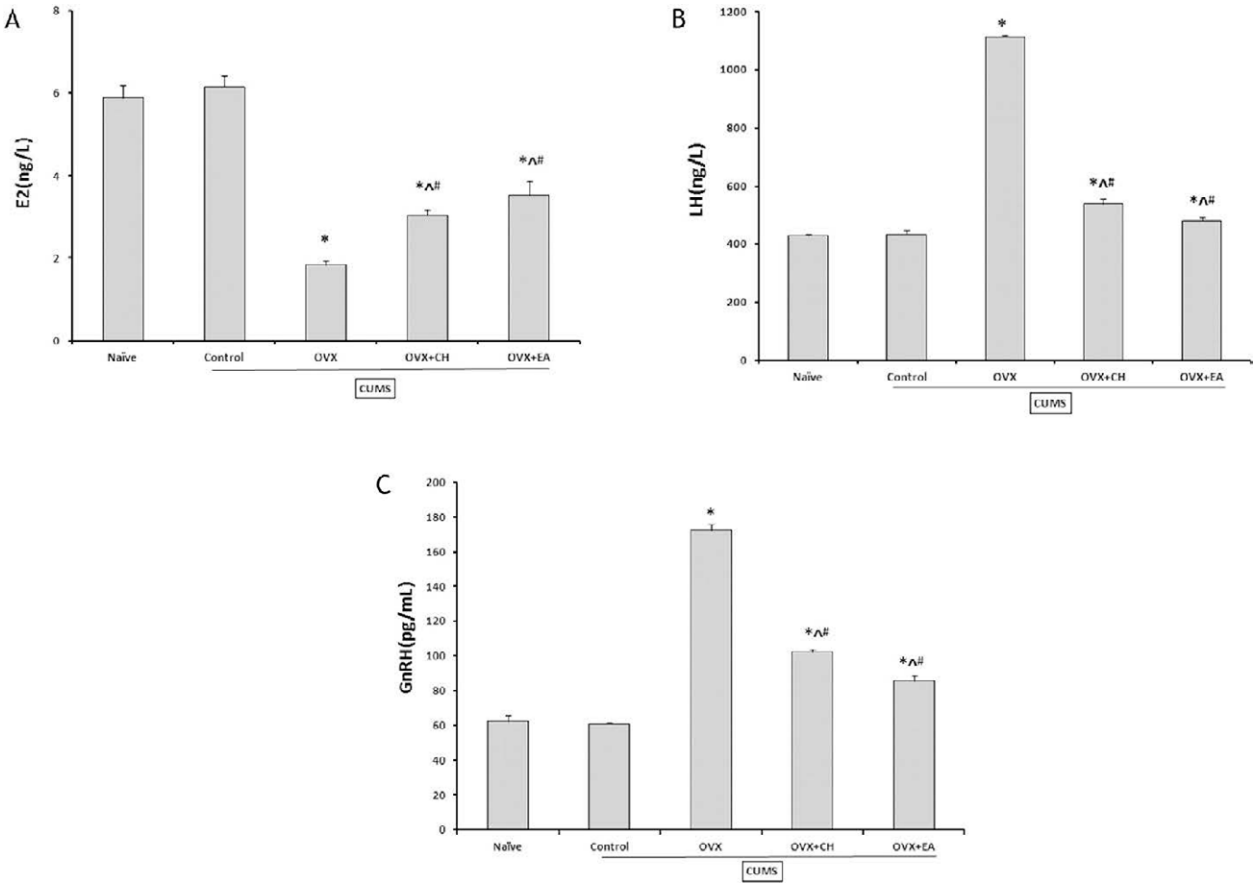

Figure 3. The effect of EA treatment on the serum levels of E2, LH, and GnRH after OVX and CUMS. (A) The representing graph showed the serum levels of E2 in the naïve, control + CUMS, OVX + CUMS, OVX + CH + CUMS, and OVX + EA + CUMS group. (B) The representing graph showed the serum levels of LH in the naïve, control + CUMS, OVX + CUMS, OVX + CH + CUMS, and OVX + EA + CUMS group. (C) The representing graph showed the serum levels of GnRH in the naïve, control + CUMS, OVX + CUMS, OVX + CH + CUMS, and OVX + EA + CUMS group. N = 6 in each group. Data were expressed as mean ± standard deviation (SD). $^{*}p < 0.05$ compared with the naïve group; $^{\wedge}p < 0.05$ compared with the control + CUMS group; $^{\#}p < 0.05$ compared with the OVX + CUMS group. LH = Luteinizing hormone; E2 = Estrogen; GnRH = Gonadotropin releasing hormone; OVX = Ovariectomy; CUMS = Chronic unpredictable mild stress; EA = Electroacupuncture; CH = Clomipramine hydrochloride.

0.70 ± 0.03; OVX + EA + CUMS: 0.76 ± 0.13; df = 4, F = 75.18, $p < 0.01$). On the other hand, β-catenin was measured, which was the downstream gene of GSK-3β. The data revealed the increased β-catenin mRNA expression in the hippocampus in OVX + CUMS group compared with that in naïve group (Fig. 5B, naïve: 1.00 ± 0.03; OVX + CUMS: 1.88 ± 0.08; $p < 0.01$). However, EA or CH treatment rescued the increased β-catenin mRNA expression after OVX and CUMS (Fig. 5B, OVX + CH + CUMS: 1.37 ± 0.09; OVX + EA + CUMS: 1.33 ± 0.15; df = 4, F = 111.07, $p < 0.01$).

3.5. Effect on the β-catenin and p-β-catenin protein expression of perimenopausal depression rats

Next, the protein level of p-β-catenin in the hippocampus was examined since it was reported to correlate with Wnt signaling [21]. Western blotting results showed that total β-catenin protein expression in the hippocampus was increased after OVX and CUMS (Fig. 6A and D, naïve: 42.35 ± 3.70; OVX + CUMS: 55.73 ± 0.62; $p < 0.01$), while EA or CH treatment ameliorated the increased β-catenin protein expression in the hippocampus (Fig. 6A and D, OVX + CH + CUMS: 46.61 ± 2.77; OVX + EA + CUMS: 48.27 ± 2.84; df = 4, F = 31.03, $p < 0.01$). However, although the p-β-catenin protein expression was decreased in the OVX + CUMS group compared with naïve group (Fig. 6B and D, OVX + CUMS: 20.53 ± 3.58; naïve: 30.04 ± 5.62; df = 4, F = 5.6, $p < 0.01$), EA or CH treatment didn't rescue the effect and level in the OVX + CUMS, OVX + CH + CUMS or OVX + EA + CUMS group did not differ significantly ($p > 0.05$). We revealed that OVX + EA + CUMS group significantly increased the ratio of p-β-catenin to total β-catenin compared with the OVX + CUMS group (Fig. 6C).

4. Discussion

This study aimed to explore the effects of EA on hippocampal neural proliferation and Wnt signaling in perimenopausal depression rats, which were established by OVX + CUMS induction, and to examine the contribution of Wnt signaling pathway to this process. The results demonstrated that EA treatment ameliorated the increased depression-like behaviors induced by OVX + CUMS, and activated Wnt signaling of hippocampus inducing GSK-3β and β-catenin expression to regulate neural proliferation. Taken together, the findings indicated that EA treatment might represent an efficacious therapy for perimenopausal depression.

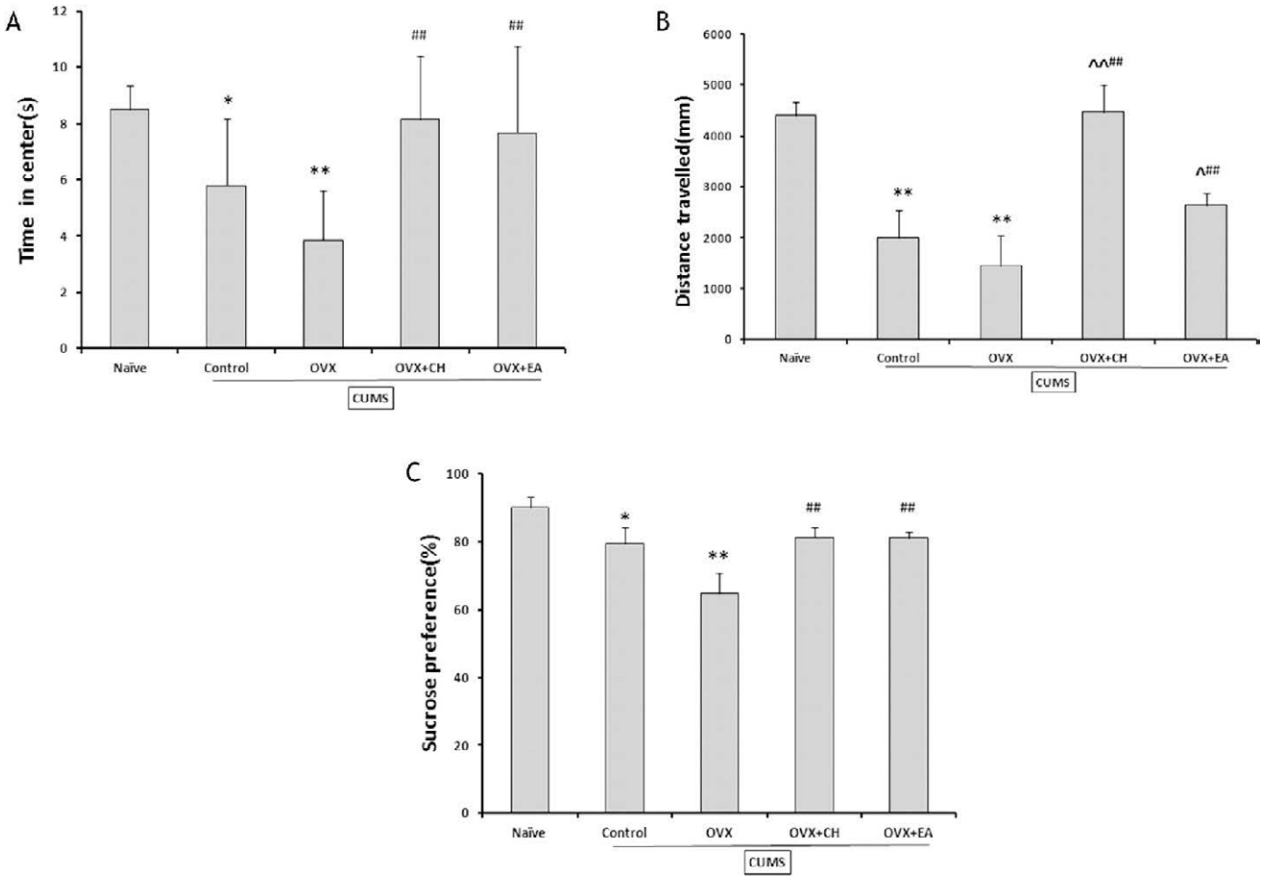

Figure 4. The effect of EA treatment on open-field test (OFT) and sucrose preference test after OVX and CUMS. (A) The fraction of the first 3 min that rats spent in the open-field test central zone. (B) The distance travelled during the OFT. (C) The sucrose preference measured before OVX and CUMS, and after EA or CH treatment. N = 6 in each group. Data were expressed as mean ± standard deviation (SD). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the naïve group; $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$ compared with the control + CUMS group; $^{\#\#}p < 0.01$ compared with the OVX + CUMS group. OVX = Ovariectomy; CUMS = Chronic unpredictable mild stress; EA = Electroacupuncture; CH = Clomipramine hydrochloride.

OVX and CUMS were used to establish a rat model of perimenopausal depression in current study [22]. Many studies confirmed that female SD rats showed four estrus cycles and sexual maturity at around 65 days of age. In this study, rats were precultured to 77 days or older to ensure sexual maturity in rats. The vaginal smear of normal rats had periodic changes in the estrous cycle, which was one of the physiological characteristics of female rats. After OVX surgery, rats had the menstrual cycle disorder. We detected the vaginal exfoliated cells of rats for five consecutive days after OVX, and found that vaginal exfoliated cells disappeared in the

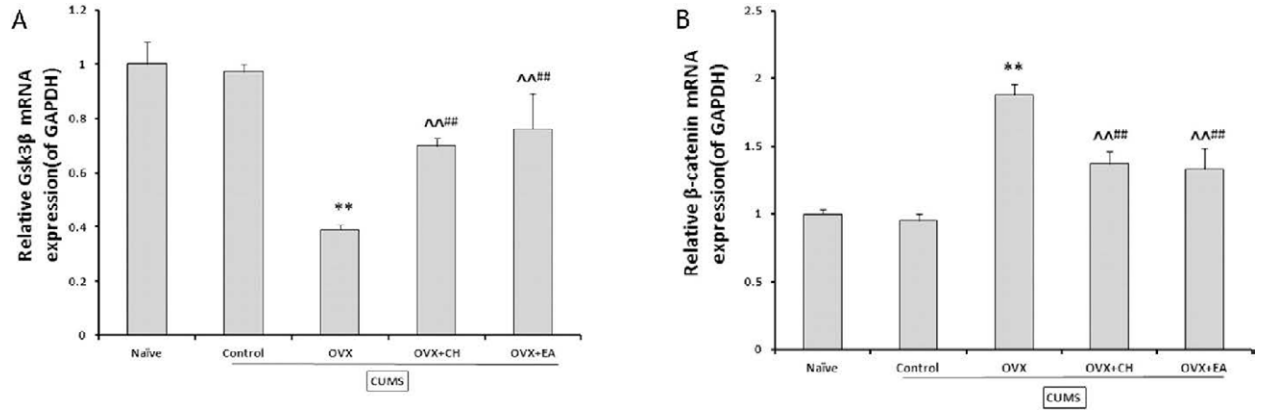

Figure 5. The effects of EA on hippocampal GSK-3β mRNA and β-catenin mRNA levels in perimenopausal depression rats. (A) The relative hippocampal GSK-3β mRNA expression. (B) The relative hippocampal β-catenin mRNA expression. N = 6 in each group. Data were expressed as mean ± standard deviation (SD). $^{**}p < 0.01$ compared with the naïve group; $^{\wedge\wedge}p < 0.01$ compared with the control + CUMS group; $^{\#\#}p < 0.01$ compared with the OVX + CUMS group. OVX = Ovariectomy; CUMS = Chronic unpredictable mild stress; EA = Electroacupuncture; CH = Clomipramine hydrochloride.

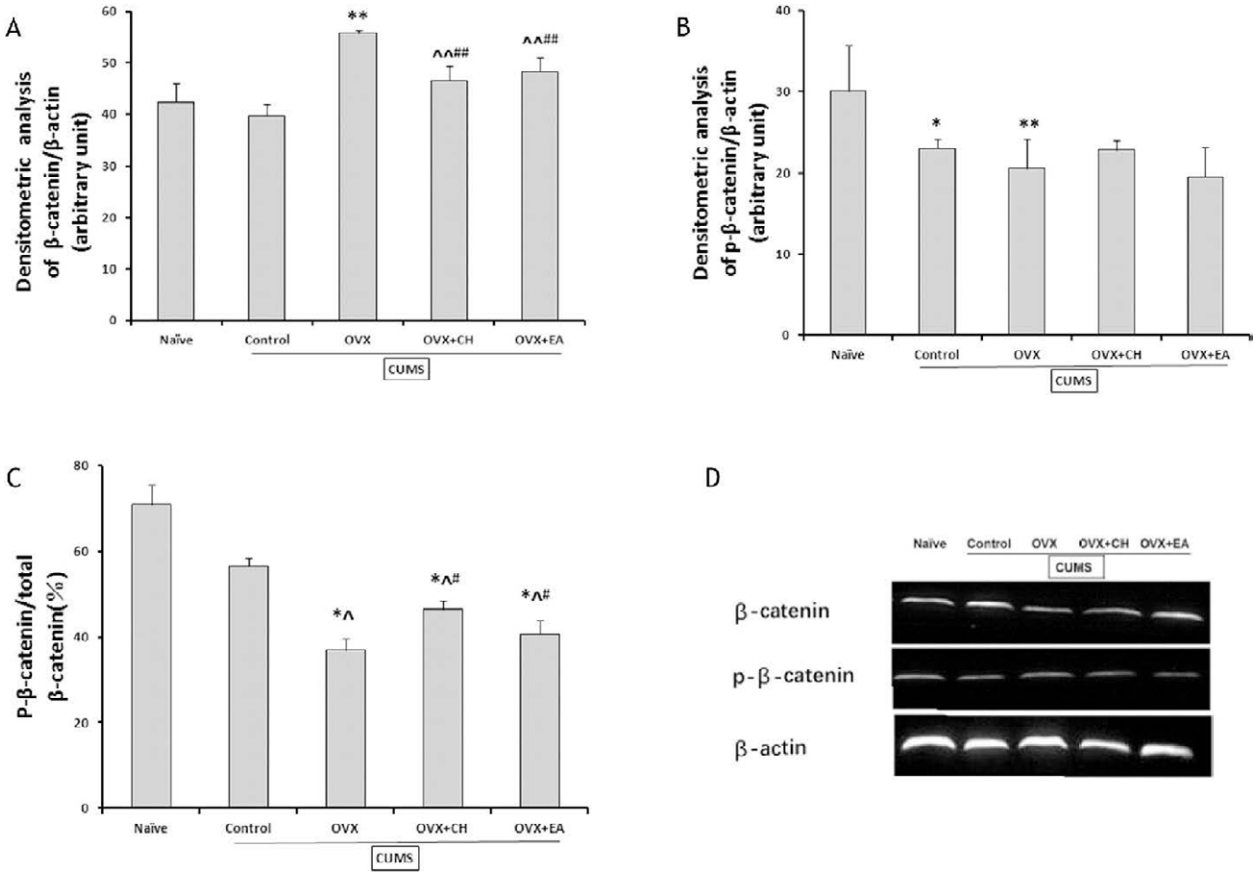

Figure 6. The effects of EA on hippocampal β-catenin and p-β-catenin protein levels in perimenopausal depression rats. (A) The quantitative analysis of β-catenin expression in (D). (B) The quantitative analysis of p-β-catenin expression in (D). (C) The ratio of p-β-catenin to total β-catenin in perimenopausal depression rats. (D) Representative Western blot of β-catenin and p-β-catenin staining in the hippocampus. N = 6 in each group. Data were expressed as mean ± standard deviation (SD). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the naïve group; $^{\wedge\wedge}p < 0.01$ compared with the control + CUMS group; $^{\#\#}p < 0.01$ compared with the OVX + CUMS group. OVX = Ovariectomy; CUMS = Chronic unpredictable mild stress; EA = Electroacupuncture; CH = Clomipramine hydrochloride.

OVX + CUMS, OVX + CH + CUMS and OVX + EA + CUMS groups, which had similar performance during perimenopause patients in clinical too. Meanwhile, the serum hormone levels of rat were also assessed to ascertain the success of OVX model. In the perimenopausal period, follicles continue to decline, which lead to the decrease of E2 level. The feedback inhibition of hypothalamus and pituitary is weakened, and the secretion of LH and GnRH are excessive. The results of this investigation showed that the serum hormone levels significantly changed in the OVX + CUMS rats. The content of E2 decreased, LH and GnRH increased at different degrees, which indicated the hormonal disorder. EA and CH treatment improved E2 level, decreased LH and GnRH levels to different degrees. The difference was statistically significant. EA and CH treatment could improve the hormone level and adjust the HPO axis function to alleviate the symptoms. After modeling successfully, we used OFT and SPT to detect behavioral changes of rats. OFT is a classical method for determining the behavior of rats in depression, which can evaluate the spontaneous activity and active exploration qualitatively and quantitatively. OFT is also used to assess anxiety by observing the time spent in the center zone. Rats preferred to hide in the shadows on the edge of the apparatus, and at the same time had exploration behaviors in the open field. When the rats had less anxiety, they stayed longer in the center to evaluate the degree of anxiety. SPT is the most reliable behavioral index in depression, mainly manifested as lack of pleasure. SPT was carried out for 24 h at night in the absence of external human activities. Rats lost interest in sweets which meant a certain degree of depression. The data from OFT and SPT were consistent. Before modeling, there was no difference between the rats in each group. After modeling, the behavioral tests in other groups were significantly worse than the naïve group, indicating that the rats showed decreased autonomic behavior and lack of pleasure similar to clinical patients. The results of OFT and SPT demonstrated the increased anxiety levels in the perimenopausal depression rats could be prevented by EA treatment, which indicated the success of the combination model of OVX and CUMS, and the efficacious effect of EA treatment.

Wnt signaling pathway is highly conserved and closely correlated with CNS development [23], playing critical role in the proliferation and differentiation of neural stem cells in adult hippocampal neurogenesis [24]. Many studies demonstrated that GSK-3β/β-catenin signaling had been implicated in both the pathophysiology and treatment of depression [25,26], Wnt/β-catenin signaling could regulate proliferation and differentiation of adult hippocampal neural stem cells in vivo and vitro [27]. During the early development of embryo, the Wnt signaling pathway could inhibit the differentiation from embryonic stem cells to neural stem cells. During the subsequent differentiation of nervous system, the continuous activation of Wnt could promote the differentiation

from neural stem cells to sensory nerve cell. GSK-3β and β-catenin are key proteins in Wnt signaling, which comprise a degradation complex to p-β-catenin to be ubiquitined. In the cytoplasm, GSK-3β, Axin, casein kinase 1ε (CK1ε) and protin phosphatase-A (APC) form a polyprotein complex that promotes β-catenin degradation. CK1ε phosphorylates 45-bit serine of β-catenin through Axin. GSK-3β phosphorylates other sites at the amino terminal of β-catenin. β-transducing repeat-containing protein (β-TrCP) is an ubiquitin protein that recognizes the phosphorylation site of β-catenin and connects it with the ubiquitin proteasome to degrade β-catenin, thus limiting cellular level of β-catenin to ensure the physiological and biochemical functions of normal cells [28]. The level of free β-catenin in normal cytoplasm is extremely low, which is not sufficient to produce Wnt signaling. When the component of signaling pathway changes in quality and quantity, β-catenin rapidly increases and moves into the nucleus, which affects the proliferation and differentiation of stem cells. When the activity of GSK-3β is inhibited, thereby blocking the phosphorylation and degradation of β-catenin in the cytoplasm by degradation complex to promote the accumulation of β-catenin furtherly, and then transfer into the nucleus. The entry of β-catenin into the nucleus indicates activation of Wnt signaling pathway. The antidepressants have been demonstrated to suppress GSK-3β and promote β-catenin expression [24,29]. In clinical, EA treatment for depression is effective, which can significantly reduce the symptoms of perimenopausal depression [10]. TCM theory considers the pathogenesis of perimenopausal depression is the deficiency of kidney essence. Under the guidance of the " Kidney-Brain theory", this study chose Shenshu, Baihui and Sanyinjiao to treat perimenopausal depression. Three groups of acupoints respectively belong to the upper, middle and lower part of the body. EA therapy could activate the kidney essence, and push kidney essence toward brain to play the role of tonifying the kidney and brain, calming the mind and relieving depression. The results showed that EA promoted cell proliferation by regulating Wnt signaling pathway [30]. However, there are limited studies on the mechanism of EA treatment for depression focusing on Wnt signaling pathway. Considering the prevalence of perimenopausal depression, the goal of the present study was to determine whether Wnt signaling was required for the antidepressant-like effects of EA in perimenopausal depressive-like rats. GSK-3β mRNA expression was decreased but β-catenin was enhanced after 28 d EA treatment, which showed the activation of Wnt signaling pathway. Meanwhile, the protein level of β-catenin was increased but p-β-catenin was decreased, also demonstrated the activation of Wnt signaling pathway. β-catenin is a downstream protein of GSK-3β. In this study, the mRNA and protein expression levels of β-catenin were significantly increased in the OVX + CUMS group, which showed consistency. EA treatment reduced the mRNA and protein expression levels of β-catenin. As the expression level of β-catenin gradually decreased, showing the Wnt signaling pathway was gradually moving from the activation state to the inhibition process. The expression of β-catenin showed antidepressant effect, suggesting that EA can stimulate neurogenesis by Wnt/β-catenin signaling pathway to protect hippocampal neurons from depressive rats, and produce antidepressant effect at a certain degree. In summary, this investigation reported that EA treatment at three points including "Shenshu", "Baihui" and "Sanyinjiao" improved depression-like behaviors, and protected against the impairments associated with perimenopausal depression induced by OVX and CUMS, the underlying mechanism of which might be the promoting adult hippocampal neural proliferation by EA through Wnt signaling pathway. The treatment of depression remains challenging due to the low diagnosis rate and limited knowledge of depression patients. EA, as an external use way, is a potential therapy which is safe and effective without side effects. It can not only treat perimenopausal depression, but also play a preventive role. This study provided a new insight to treat perimenopausal depression patients in clinical.

Author Contributions

QJ, LR, XD and NZ designed the study. XD, MF, GW, XRJ and SRL collected and analyzed the data. CRM advised on histological staining and analysis. SRL contributed samples collection. QJ drafted and wrote the manuscript. LR and NZ revised the manuscript critically for intellectual content. All authors gave intellectual input to the study and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (81373718), the National Natural Science Foundation of China (81503273), The research of Traditional Chinese Medicine "Kidney Essence life fire" Viscera Theory related diseases and syndromes (2010CB530401973), and the Program for Excellent Talents of Province Educational Commission (LR2014027). Medjaden provided writing assistance and language editing.

References

[1] World Cancer Report 2014 Chapter 1.1. World Health Organization; 2014. ISBN 9283204298.
[2] Bromberger JT, Matthews KA, Schott LL, Brockwell S, Avis NE, et al. Depressive symptoms during the menopausal transition:TheStudy of Women'sHealth Across the Nation. J.Affective Disord. 2007;103:267–72.
[3] Morais M, Santos PA, Mateus-Pinheiro A, Patricio P, Pinto L, Sousa N, et al. The effects of chronic stress on hippocampal adult neurogenesis and dendritic plasticity are reversed by selective MAO-A inhibition. J Psychopharmacol 2014;28:1178–83.
[4] Kim YK, Na KS, Myint AM, Leonard BE. The role of pro-inflammatory cytokines in neuroinflammation, neurogenesis and the neuroendocrine system in major depression. Prog Neuropsychopharmacol Biol Psychiatry 2016;64:277–84.
[5] Zhang ZJ, Ng R, Man SC, Li TY, Wong W, Tan QR, et al. Dense cranial electroacupuncture stimulation for major depressive disorder–a single-blind, randomized, controlled study. PLoS One 2012;7:e29651.
[6] Kaptchuk TJ. Acupuncture: theory, efficacy, and practice. Ann Intern Med 2002;136:374–83.
[7] Han JS. Acupuncture analgesia: areas of consensus and controversy. Pain 2011;152:S41–8.
[8] Liu Q, Li B, Zhu HY, Wang YQ, Yu J, Wu GC. Glia atrophy in the hippocampus of chronic unpredictable stress-induced depression model rats is reversed by electroacupuncture treatment. J Affect Disord 2011;128:309–13.
[9] Cheng K, Tian SL. Effects of preventive-electroacupuncture of "Guanyuan" (CV 4) and "Sanyinjiao" (SP 6) on hypothalamus-pituitary-ovary axis in ovariectomized rats. Zhen Ci Yan Jiu 2012;37:15–9. 45.
[10] Sun Z, Jin Y, Xiang H, Liu F. Clinical observation on mild perimenopausal depression of kidney deficiency and liver stagnation syndrome treated with acupoint catgut implantation. Zhongguo Zhen Jiu 2015;35:443–6.
[11] Joffe H, Petrillo LF, Koukopoulos A, Viguera AC, Hirschberg A, Nonacs R, et al. Increased estradiol and improved sleep, but not hot flashes, predict enhanced mood during the menopausal transition. J Clin Endocrinol Metab 2011;96:E1044–54.
[12] Chen XL, Luo RH. Clinical Observation of Electroacupuncture Treatment for Menopausal Syndrome. Journal of Clinical Acupuncture & Moxibustion 2013;8:35–7.
[13] Liu R. The 36 samples from differential treatment of electroacupuncture in menopausal syndrome. Chinese acupuncture 2000;8:40.
[14] Han X, Wu H, Yin P, Chen Z, Cao X, Duan Y, et al. Electroacupuncture restores hippocampal synaptic plasticity via modulation of 5-HT receptors in a rat model of depression. Brain Res Bull 2018;139:256–62.
[15] Davis EK, Zou Y, Ghosh A. Wnts acting through canonical and noncanonical signaling pathways exert opposite effects on hippocampal synapse formation. Neural Dev 2008;3:32.
[16] Ciani L, Salinas PC. WNTs in the vertebrate nervous system: from patterning to neuronal connectivity. Nat Rev Neurosci 2005;6:351–62.

[17] Li LH, Wang ZC, Yu J, Zhang YQ. Ovariectomy results in variable changes in nociception, mood and depression in adult female rats. PLoS One 2014;9:e94312.
[18] Willner P, Towell A, Sampson D, Sophokleous S, Muscat R. Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant. Psychopharmacology (Berl) 1987;93:358–64.
[19] Li ZR. The experimental acupuncture. Beijing: China press of traditional Chinese medicine; 2003. p. 147–8.
[20] Kurhe Y, Mahesh R, Gupta D, Devadoss T. QCM-4, a serotonergic type 3 receptor modulator attenuates depression co-morbid with obesity in mice: an approach based on behavioral and biochemical investigations. Eur J Pharmacol 2014;740:611–8.
[21] Willert K, Nusse R. Beta-catenin: a key mediator of Wnt signaling. Curr Opin Genet Dev 1998;8:95–102.
[22] Recamier-Carballo S, Estrada-Camarena E, Reyes R, Fernandez-Guasti A. Synergistic effect of estradiol and fluoxetine in young adult and middle-aged female rats in two models of experimental depression. Behav Brain Res 2012;233:351–8.
[23] Costa V, Lugert S, Jagasia R. Role of adult hippocampal neurogenesis in cognition in physiology and disease: pharmacological targets and biomarkers. Handb Exp Pharmacol 2015;228:99–155.
[24] Wexler EM, Geschwind DH, Palmer TD. Lithium regulates adult hippocampal progenitor development through canonical Wnt pathway activation. Mol Psychiatry 2008;13:285–92.
[25] Crofton EJ, Nenov MN, Zhang Y, Scala F, Page SA, McCue DL, et al. Glycogen synthase kinase 3 beta alters anxiety-, depression-, and addiction-related behaviors and neuronal activity in the nucleus accumbens shell. Neuropharmacology 2017;117:49–60.
[26] Xu LZ, Xu DF, Han Y, Liu LJ, Sun CY, Deng JH, et al. BDNF-GSK-3beta-beta-Catenin Pathway in the mPFC Is Involved in Antidepressant-Like Effects of Morinda officinalis Oligosaccharides in Rats. Int J Neuropsychopharmacol 2017;20:83–93.
[27] Zhang X, Chen L, Wang Y, Ding Y, Peng Z, Duan L, et al. Macrophage migration inhibitory factor promotes proliferation and neuronal differentiation of neural stem/precursor cells through Wnt/beta-catenin signal pathway. Int J Biol Sci 2013;9:1108–20.
[28] Neth P, Ciccarella M, Egea V, Hoelters J, Jochum M, Ries C. Wnt signaling regulates the invasion capacity of human mesenchymal stem cells. Stem Cells 2006;24:1892–903.
[29] Zhu Z, Kremer P, Tadmori I, Ren Y, Sun D, He X, et al. Lithium suppresses astrogliogenesis by neural stem and progenitor cells by inhibiting STAT3 pathway independently of glycogen synthase kinase 3 beta. PLoS One 2011;6:e23341.
[30] Chen B. Electroacupuncture regulates WNT signaling pathways to promote the proliferation of neural precursor cells after cerebral ischemia and reperfusion. Fujian University of Traditional Chinese Medicine; 2014.