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Cranial electrotherapy stimulation alleviates depression-like behavior of post-stroke depression rats by upregulating GPX4-mediated BDNF expression

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ABSTRACT

To elucidate whether cranial electrotherapy stimulation (CES) improves depression-like behavior of post-stroke depression (PSD) via regulation of glutathione peroxidase 4 (GPX4)-mediated brain-derived neurotrophic factor (BDNF) expression. Middle cerebral artery occlusion (MCAO) and chronic unpredictable mild stress (CUMS) were used to develop a rat PSD model. CES was applied, and RAS-selective lethal 3 (RSL3) was injected into the hippocampus to inhibit GPX4 in PSD rats. The depression behavior was detected by sucrose preference and forced swimming tests. The structure and morphology of the hippocampus were observed and analyzed by histopathological hematoxylin-eosin (HE) staining. The mRNA and protein expressions of GPX4 and BDNF in the hippocampus were detected by qRT-PCR, western blot and immunohistochemical analysis.The degeneration and necrosis of hippocampal neurons, the depression-like behavior were severer and the expression of BDNF in the hippocampus were decreased in PSD rats than those in MCAO and control groups. CES promoted the hippocampal neuron repair, alleviated the depression-like behavior and increased the expression of BDNF in PSD rats. The inhibition of GPX4 by RSL3 exacerbated the depression-like behavior and decreased the expression of BDNF in PSD rats. In addition, we found that RSL3 disrupted the positive effects of CES on the PSD rats. Conclusion: CES improves depression-like behavior of PSD rats through upregulation of GPX4-mediated BDNF expression in the hippocampus.

1. Introduction

Stroke has become one of the leading causes of death and disability worldwide[1]. Post-stroke depression (PSD) is an affective disorder, which is a common complication of stroke, characterized by depressed mood, reduced mobility, and delayed thinking[2,3]. PSD retards the recovery of the patient's deficient function, decreases the quality of survival, and even increases mortality[4]. In a previous study, the incidence of depression was 39–52 % within 5 years and 29 % within 10 years after stroke[5]. In addition, another investigation confirmed that the incidence of depression within five years after stroke was 31 %, and approximately 1/3 of the stroke patients developed PSD[6].

The pathogenesis of PSD is complex, and the specific mechanism is still unclear. It is generally considered that its pathogenesis involves the action of many factors, including biological, behavioral, psychological, and social factors[7]. A number of hypotheses regarding the pathogenesis of depression have been proposed, such as the monoamine neurotransmitter, neurotransmitter receptor, neuroendocrine, and neurotrophic hypotheses[8]. The neurotrophic hypothesis suggests that low levels of brain-derived neurotrophic factor (BDNF) can cause neuronal damage in the hippocampal region of the brain, leading to depression[9].

BDNF is synthesized in the neurons and is distributed mainly in the central nervous system. Its concentration is the highest in the hippocampus, which is the part of the brain that predominantly controls emotions, and the cerebral cortex[10]. Moreover, BDNF is involved in the differentiation and growth of nerve cells, and affects the lifespan and physiological activities of mature nerve cells in the brain[11]. Previous studies have found that the BDNF level in the hippocampus of depressed rats was decreased, which caused a decrease in the generation and

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branching of the hippocampal neurons, ultimately leading to adverse changes in their morphology and structure[10,12–14].

Glutathione peroxidase 4 (GPX4), a gene involved in oxidative stress, has been found to play an important regulatory role in the onset of depression[15] , and the whole blood GPX4 activity of depressed patients was significantly lower than that of normal controls[16]. Jiao et al. found that drugs can play an antidepressant effect by enhancing the expression of GPX4 in the hippocampus of depressed mice[17]. In addition, in the process of nerve cell repair, both the expressions of GPX4 and BDNF were found to be increased[18]. However, it is not clear whether GPX4 is involved in the regulation of BDNF in PSD.

Currently, the treatment of PSD includes mainly drug therapy and psychological intervention. However, antidepressants have side effects that hamper the effective recovery of a patient's cognitive and motor functions, whereas psychological interventions have limited therapeutic effects in patients with speech and cognitive impairment. Cranial electrotherapy stimulation (CES) is a physical factor therapy. Previous studies found that CES significantly reduced the symptoms of depression, anxiety, and insomnia[19–21]. Additionally, a randomized controlled clinical trial revealed that CES was effective and safe for the treatment of bipolar depression[22]. However, the specific mechanism of CES treatment of depression is still unclear, and its therapeutic effect in PSD has not been elucidated. Therefore, the aim of this study was to determine the effects of CES on PSD , and whether CES improves the depressive behavior of PSD rats by regulating GPX4-mediated BDNF expression in the hippocampus.

2. Materials and methods

2.1. Experimental animals

A total number of 54 male Sprague-Dawley rats (eight-week-old, weighting 230–260 g) were selected and purchased from Qinglongshan Experimental Animal Center, Nanjing, Jiangsu Province, China. All experimental protocols were approved by the Ethics Committee of Nanjing Hospital, Nanjing Medical University (Nanjing, Jiangsu, China). All rats were isolated in single cages, and food and running water were provided ad libitum.

2.2. Animal grouping

To prove the success of MCAO modeling, twelve rats were randomly divided into 2 groups, including control group and middle cerebral artery occlusion (MCAO) group. Control group was sham operation group, that was, only incision and sutured but no thread plug. MCAO group were treated with MCAO operation.

In order to investigate the changes of BDNF, hippocampal neurons and behavior of rats after PSD modeling and CES intervention, ninety rats were randomly divided into 5 groups, including control group, MCAO group, PSD group, PSD+CES (200 μA) group and PSD+CES (300μA) group, with 18 rats in each group. Control group was sham operation group, that was, only incision and sutured but no thread plug; MCAO group were treated with MCAO operation. PSD group were treated with both MCAO operation and chronic unpredictable mild stress (CUMS). PSD+CES (200 μA) group and PSD+CES (300μA) group were treated with CES at 200 μA and 300μA after PSD model established, respectively.

After completing the above experiments, we want to demonstrate the role of GPX4 in the development of PSD and CES intervention. Seventy-two rats were randomly divided into 4 groups, including PSD group, PSD+CES group, PSD+RSL3 group and PSD+RSL3 +CES group, with 18 rats in each group. PSD group was treated with both MCAO and CUMS. The rats in PSD+RSL3 group and PSD+RSL3 +CES group were injected with RSL3 using brain stereotactic localization after the successful establishment of PSD rat model. The PSD+CES group and the PSD+RSL3 +CES group received CES (300 μA) treatment.

2.3. MCAO

The rats were fasted and deprived of water for 12 h before surgery. After anesthesia by intraperitoneal injection of 10 % chloral hydrate (0.4 mL/100 g), the rats were placed on the insulation pad to keep the body temperature at 37 ℃, and fixed on their back, and the middle of the neck was shaved. After routine disinfection, the right common carotid artery, external carotid artery, and internal carotid artery were bluntly separated, and the right vagus nerve was carefully separated. The proximal end of the right common carotid artery, external carotid artery and its branches were ligated. A small incision was cut at the common carotid artery near the bifurcation of the internal and external carotid arteries, and the thread (L3400 or L3600, Jialing Biotechnology Co., Ltd, Guangzhou, Guangdong, China) was inserted into the small incision. The length of the thread plug was inserted along the internal carotid artery approximately 18–20 mm from the bifurcation. In case of a slight sense of resistance, the beginning of the middle cerebral artery was blocked. The thread plug next was ligated and fixed, and the incision was sutured (Fig. 1A). In the sham operation group, the common, internal, and external carotid arteries were separated without a thread plug. After the operation, the incision was covered with sterile gauze and 80,000 units of penicillin were injected intraperitoneally every day for three days. The neurobehavioral performance of the rats were observed and the Zea-Longa scores were performed at 2 h postoperatively.

2.4. Tissue prepration for analysis

The rats were euthanized with excessive anesthesia by intraperitoneal injection of 10 % chloral hydrate. After euthanasia, the rat's head was decapitated between the posterior and cervical spine. The scalp was cut open along the middle of the top of skull and remove the soft tissue. The cranial parietal bone was gradually removed with hemostatic forceps, and the brain was fully exposed. The hippocampus is located at the bottom of the cerebral cortex and is carefully pushed apart with direct forceps to expose the cerebral cortex. Hippocampal tissue was removed and fixed in 4 % paraformaldehyde.

2.5. Triphenyltetrazolium chloride staining (TTC staining)

TTC staining was performed at 24 h after the MCAO operation. After euthanasia, the brain of the rats was directly sliced into seven slices and placed in the previously prepared TTC staining solution (Sigma-Aldrich, St. Louis, MO, USA). The plate containing TTC dye and brain slices was placed in a water bath at 37 °C, and the brain slices were turned once every 5 min to ensure uniform staining. Most of the brain slices were dyed dark red. The white areas represented the infarct areas, whereas the red areas were the non-infarct areas.

2.6. Zea-Longa scoring criteria

The neurobehavioral scores of MCAO rats were evaluated and recorded by the same observer who did not know the grouping situation, and the Zea-Longa scores were measured 2 h after the MCAO operation [23]. The following scoring/grading criteria were employed: 0 points: no symptoms of nerve injury; 1 point: the front paw of the hemiplegia side could not be completely straightened, with mild neurological impairment; 2 points: the resistance in the forelimb of the hemiplegia side decreased, and the rats rotated to the hemiplegia side, with moderate neurological impairment; 3 points: the body of the rats was dumped towards the hemiplegia side, with severe neurological function loss; 4 points: inability to walk spontaneously, loss of consciousness.

2.7. CUMS

On the 7th day after awakening, MCAO rats were subjected to

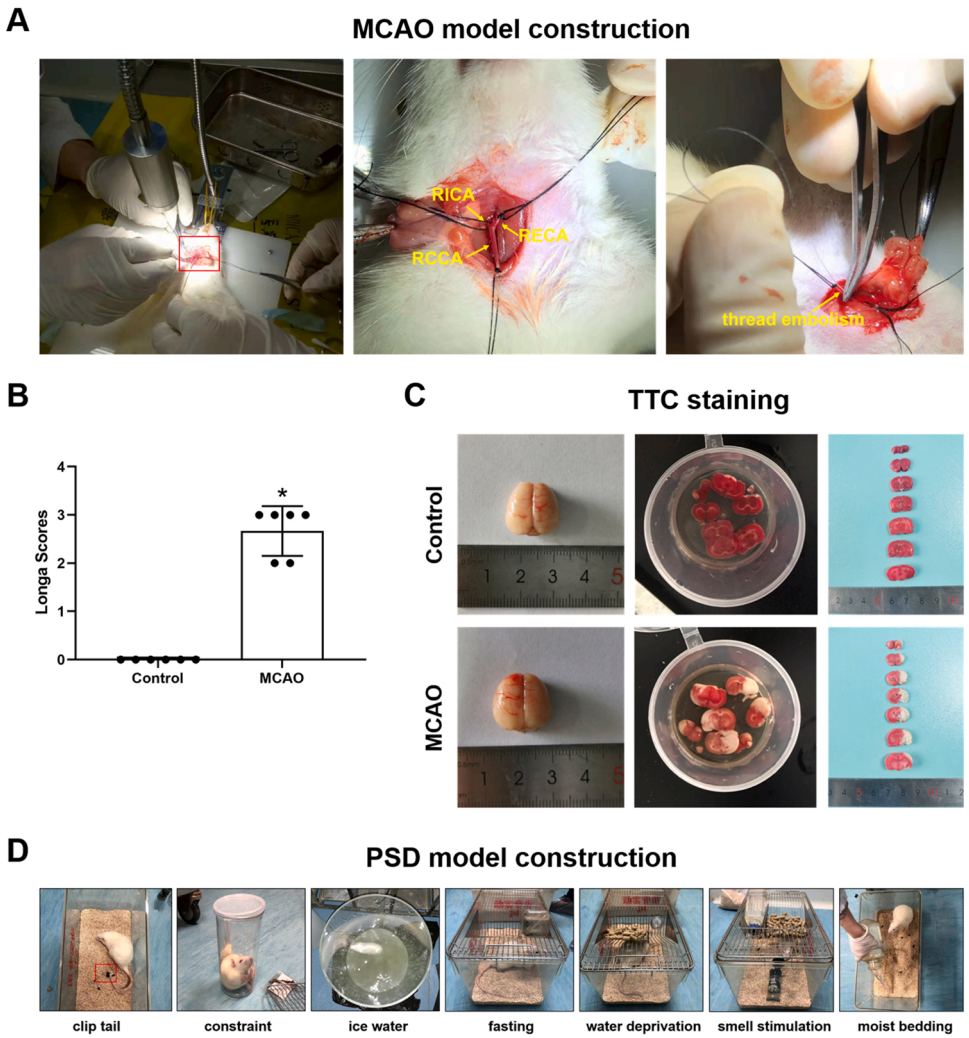

Fig. 1. (A) Middle cerebral artery occlusion (MCAO) was used to develop a rat stroke model. (B) Zea-Longa score was used to evaluate the neurological impairment. (C) Triphenyltetrazolium chloride staining (TTC) of the control and the MCAO groups. (D) Chronic unpredictable mild stress (CUMS) was used to establish a PSD model after the MCAO model was successfully created; n = 6, * $p < 0.05$.

chronic unpredictable mild stress (CUMS)[24,25] to establish a PSD model. Seven stimulation methods (Fig. 1D), including clipping tail, constraint, swimming in ice water at 4 °C, fasting, water deprivation, smell stimulation, and moist bedding, were used to stimulate the rats alternately at a frequency of one stimulus per day for three weeks. All rats were isolated in single cages.

2.8. Sucrose preference test

Rats were given sucrose adaptation training two days before the experiment. Each rat was fed separately and simultaneously provided with two bottles of water, one bottle of sucrose water, and one bottle of tap water. The two bottles of water were alternately placed every 30 min. After the adaptation training, access to water and food was ceased for 24 h, followed by a sucrose preference test. The specific process was as follows: rats were separately placed in cages and given two bottles of quantitative liquid, one bottle of 2 % sucrose solution and one bottle of tap water (Fig. 2B). After 1 h, the consumption of sugar water and tap water was measured. The sucrose preference rate was determined using the following formula: Sucrose preference rate = sugar water consumption (mL)/ total liquid consumption (mL)* 100 %. Notably, a lower sucrose preference rate was associated with more severe depression.

2.9. Forced swimming test

The rats were placed in a cylindrical glass bucket filled with clean water, one rat per bucket. On the first day of the experiment, each rat should swim for 15 min. Then, it was taken out, dried, and placed in a cage. The rats were placed in the water again, and the time was recorded again. The rats were continuously observed for 6 min, and the duration of each rat in an immobile state was recorded (Fig. 2C). The immobile state meant that the rat gave up actively struggling, and its body was in a state of floating without twisting. Intriguingly, we found that longer immobility time was associated with more severe depression.

2.10. Stereotactic injection of GPX4 inhibitor into the brain

RAS-selective lethal 3 (RSL3) is an inhibitor of GPX4[18]. The PSD rats were anesthetized by intraperitoneal injection of 10 % chloral hydrate (0.4 mL/100 g). Their heads were shaved and disinfected, and fixed on a stereotaxic device. The skin and fascia were then cut, and the anterior fontanelle was used as the origin to locate the right hippocampus of the rat. According to the Rat Brain in Stereotaxic Coordinates [26], the prepared in advance 10 μL of RSL3(50 mM, S8155, Selleck, Houston, TX, USA) dissolved in solvent dimethylsulfoxide(DMSO) was slowly injected into the hippocampus of the rats (injection coordinates (AP: −3.00 mm, R: 3.00 mm, d: 3.60 mm)) with a microsyringe for

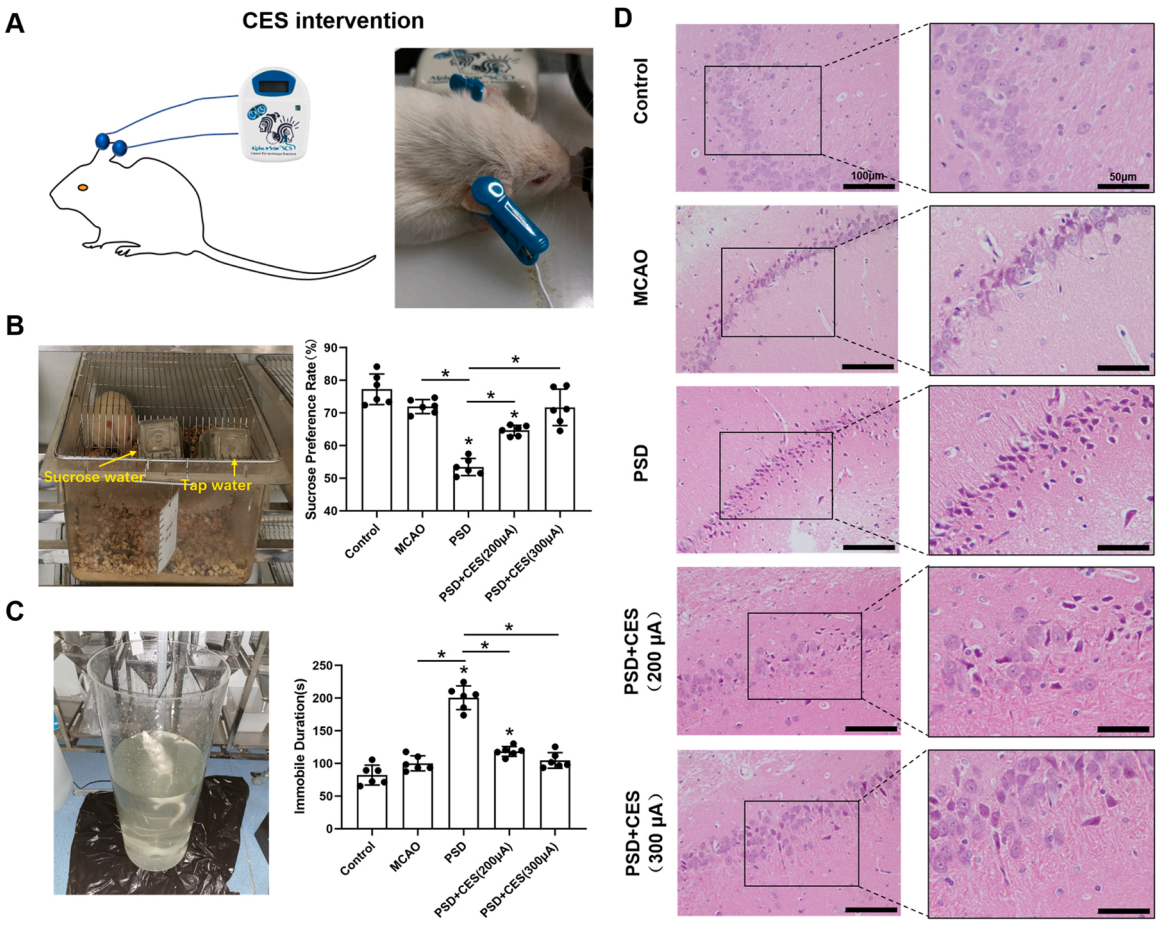

Fig. 2. Effects of CES on the depressive-like behavior of PSD rats, in which CES promoted hippocampal neuron repair. (A) We used the fifth-generation Alpha-Stim®SCS, and two ear-clip electrodes were utilized to clamp both ears of the rat. (B) A bottle of sucrose water and a bottle of tap water were provided simultaneously to detect the sucrose preference rate. The bar chart represents the relative level of the sucrose preference rate in each group of rats. (C) The rats were subjected to forced swimming test in a cylindrical glass bucket filled with clean water. The bar chart indicates the relative level of immobility time in each group during swimming. (D) HE staining showed the changes of the number, arrangement, and morphology of the hippocampal neurons in each group; scale bars = 100 and 50 μm; n = 6, * $p < 0.05$.

10 min and then stopped for 10 min (Fig. 4A). Each rat was injected only once. After the injection, the wound was closed with bone wax, and the skin was sutured.

2.11. CES treatment

We used a fifth-generation Alpha-Stim®SCS device (Electromedical Products International, Inc., Mineral Wells, TX, USA) and two ear-clip electrodes were used to clamp the ears on both sides of the rat (Fig. 2A). The stimulation frequency of CES was 0.5 Hz, and the output intensity was from 0 to 500 μA. Previously reported and recommended current intensities 200 and 300 μA were employed in our study[27–29]. On the third day after the PSD model was established(the end of CUMS), the rats were treated for 20 min, once a day for four weeks.

2.12. Hematoxylin-eosin (HE) staining

The hippocampal tissue of rats was fixed in 4 % paraformaldehyde for more than 24 h, and then the trimmed tissue was dehydrated and embedded to prepare wax blocks and be cut into slices with a thickness of approximately 4 μm. The slices were transferred into an oven and baked at 60 °C. After the paraffin sections were dewaxed, hematoxylin (Baso Biotechnology Co., Ltd, Zhuhai, Guangdong, China) was added for 3–8 min to stain the nucleus, and eosin solution (Baso Biotechnology Co., Ltd) was then added for 1–3 min to stain the cytoplasm. Further, the sections were dehydrated and sealed. Under a microscope(H550S and DS-Ri2, Nikon, Japan), images were collected and analyzed, in which the nucleus was stained in blue, whereas the cytoplasm was red.

2.13. Immunohistochemical analysis

The prepared paraffin sections of rat hippocampal tissue were baked in an oven at 65 °C and dewaxed to water. The sections were repaired by high pressure in ethylene diamine tetraacetic acid (EDTA) (Maxim Biotechnology Development Co., Ltd, Fuzhou, China) antigen repair solution. After natural cooling, the slices were incubated with 3 % hydrogen peroxide for 10 min at room temperature, and then sealed with 5 % bovine serum albumin (BSA) (Sigma-Aldrich) for 20 min. After the removal of the BSA solution, rabbit monoclonal anti-BDNF antibody (1:500 , ab108319, Abcam, Cambridge, UK) or rabbit monoclonal anti-GPX4 antibody (1:250 , ab125066, Abcam) was added to cover the tissue, and the specimen was kept at 4 °C overnight. After three-fold washing, secondary goat anti-rabbit antibody (1:5000, A0208, Beyotime Bio, Inc.) was added, followed by incubation at 37 °C for 30 min. After another three times of washing, diaminobenzidine (DAB) solution (Dako A/S, Glostrup, Denmark) was added. After the color was completely developed, the slices were rinsed with distilled water and redyed with hematoxylin dye (Baso Biotechnology Co., Ltd). After dehydration, drying, and sealing, the slices were observed and photographed under the microscope(H550S and DS-Ri2, Nikon).

2.14. Western blot analysis

Protein was extracted from the rat hippocampus by the addition of RIPA lysate (Enhancer Bio-Technology Co., Ltd, Nanjing, Jiangsu, China). The extracted protein was transferred to the protein assay kit (Beyotime Bio, Inc., Songjiang, Shanghai, China) for protein content determination, and then SDS-PAGE(Mini Protean 3, Bio-Rad, USA) was performed. After electrophoresis, the proteins were transferred to PVDF membranes (Millipore Corp, Billerica, MA, USA) under wet conditions (170–3930, Bio-Rad, USA). The membrane was transferred to TBST solution containing 5 % skimmed milk powder (Enhancer Bio-Technology Co., Ltd) and sealed at room temperature for 1 h. A anti-glyceraldehyde 3-phosphate dehydrogenase (GAPD H) mouse monoclonal antibody(1:1000 , A01020, Abbkine,Inc. China), anti-BDNF rabbit monoclonal antibody (1:1000 , ab108319, Abcam) or anti-GPX4 rabbit monoclonal antibody (1:1000 , ab125066, Abcam) antibody was added and incubated overnight at 4 °C, decolorized at room temperature and washed in a shaker three times. Then, goat anti-rabbit secondary antibody (1:5000, A0208, Beyotime Bio, Inc.) was added, followed by incubation at 37 °C for 2 h. Then, decolorization was conducted at room temperature, and the membrane was washed in a shaker three times. Finally, the membrane was exposed to a chemiluminescence reagent (ECL kit, Tanon Science & Technology Co., Ltd., Minhang, Shanghai, China) and photographed under a chemiluminescence detection system (Tanon 5200, Tanon Science & Technology Co., Ltd.).

2.15. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the hippocampus using TRIzol reagent (Takara Bio, Inc., Otsu, Shiga, Japan), and cDNA was synthesized by reverse transcription using the PrimeScript™ RT reagent Kit with the gDNA Eraser Transcription Kit (Takara Bio, Inc.). Based on the cDNA sequences obtained from the NCBI sequence database, PCR primers (Table 1) for GAPDH, BDNF and GPX4 were designed using Primer Express® software. According to Ensembl database, there are 9 exons of the BDNF gene in rats. The PCR amplified gene position in our experiment is bound to the No. 6 exon, and the downstream primer is bound to the No. 9 exon. qRT-PCR was then performed using SYBR® Green PCR Mix (Takara Bio, Inc.) on the ABI Prism 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). To quantify the relative expression of each gene, Ct values were normalized against the endogenous reference ($\Delta Ct = Ct_{target} - Ct_{GAPDH}$) and were compared with a calibrator using the $2^{-\Delta\Delta Ct}$ method ($\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{calibrator}$).

2.16. Statistical analysis

The data obtained in this study were analyzed with SPSS 26.0 statistical software and the figures were created with Graphpad Prism 9.0 software. All data are expressed as mean ± standard deviation and tested for normal distribution and Levene's analysis. Mann-whitney U test was applied to compare data between the two groups, and One-way analysis of variance (ANOVA) followed by Tukey test(equal variance) or Tamhane's T2 test(heterogeneity of variance) was applied to compare data between more than two groups. $p < 0.05$ was considered to indicate statistically significant differences.

Table 1
Primer sequences for qRT-PCR.

Gene	Primer sequences
GAPDH	Forward:5′-TGATGGGTGTGAACCACGAG-3′
GAPDH	Reverse:5′-AGTGATGGCATGGACTGTGG-3′
BDNF	Forward:5′-TCCGAGAGCTTTGTGTGGAC-3′
BDNF	Reverse:5′-TCGTCAGACCTCTCGAACCT-3′
GPX4	Forward:5′-ACGCCAAAGTCCTAGGAAGC-3′
GPX4	Reverse:5′-CTGCGAATTCGTGCATGGAG-3′

GAPDH: glyceraldehyde 3-phosphate dehydrogenase
BDNF: brain-derived neurotrophic factor
GPX4: glutathione peroxidase 4

3. Results

3.1. Longa score and TTC staining after MCAO modeling

We used the Zea-Longa score for neurological impairment assessment. MCAO group and control group were 6 rats in each group. Our results showed that the MCAO group had significantly higher score than the control group ($p < 0.05$) (Fig. 1B). TTC staining revealed a distinct white infarct area in the left brain of the MCAO group, whereas no infarct area was observed in the brain slices of the control group (Fig. 1C).

3.2. Effects of CES on the depression-like behavior in the PSD rats

We used the sucrose preference and forced swimming tests to detect the changes of depression-like behavior in each group of rats. A lower sucrose preference rate and a longer immobility time during swimming led to a more serious degree of depression. There were significant differences(sucrose preference rate: F=34.14, df=4, $p < 0.05$; immobility time during swimming: F=71.73, df=4, $p < 0.05$) between the groups.In the PSD and PSD + CES (200 μA) groups, the sucrose preference rates were lower ($p < 0.05$) than that of the control group, whereas the immobility time during swimming in the PSD and PSD + CES (200 μA) groups was significantly longer ($p < 0.05$), with no significant difference from the control in the MCAO and PSD + CES (300 μA) group ($p > 0.05$). In the PSD group, the sucrose preference rate was significantly lower ($p < 0.05$), and the immobility time during swimming was significantly longer ($p < 0.05$) than the respective values in the MCAO group. Additionally, in the PSD + CES (200 μA) and the PSD + CES (300 μA) groups, the sucrose preference rates were significantly higher ($p < 0.05$) and the immobility time during swimming was significantly shorter ($p < 0.05$) than that of the PSD group. The effect in the PSD + CES (300 μA) group was more obvious, but with no significant difference from the PSD + CES (200 μA) group ($p > 0.05$) (Fig 2B and C).

3.3. CES promoted hippocampal neuron repair

HE staining showed that more neurons with a normal structure and orderly arrangement were present in the control group. The arrangement of the hippocampal neurons in the MCAO group was disordered, and some neurons had shrinkage, degeneration, and even necrosis. In the PSD group, the hippocampal neurons were more disorderly. The cell body of these neurons was wrinkled, with cytoplasmic red staining and nuclear pyknosis; more necrotizing neurons appeared. In the PSD + CES (200 μA) and PSD + CES (300 μA) groups, the arrangement of the hippocampal neurons tended to be normal and their appearance was improved (Fig. 2D).

3.4. CES increased the expression of BDNF in the hippocampus of PSD rats

We evaluated the effect of CES on the BDNF expression in the hippocampus of PSD rats. Immunohistochemical analysis showed that the numbers of BDNF+ cells were significant differences (F=39.91, df=4, $p < 0.05$) between the groups. The numbers of BDNF+ cells in the MCAO, PSD, PSD + CES (200 μA), and PSD + CES (300 μA) groups were lower than that in the control group. The number of BDNF+ cells in the PSD group was lower than that in the MCAO group. In addition, the numbers of BDNF+ cells in the PSD + CES (200 μA) and PSD + CES (300 μA) groups were higher than that in the PSD group (Fig. 3A).

Western blot results revealed that the protein expression of BDNF were significant differences (F=42.09, df=4, $p < 0.05$) between the groups. The expression levels of BDNF in the MCAO, PSD, and PSD

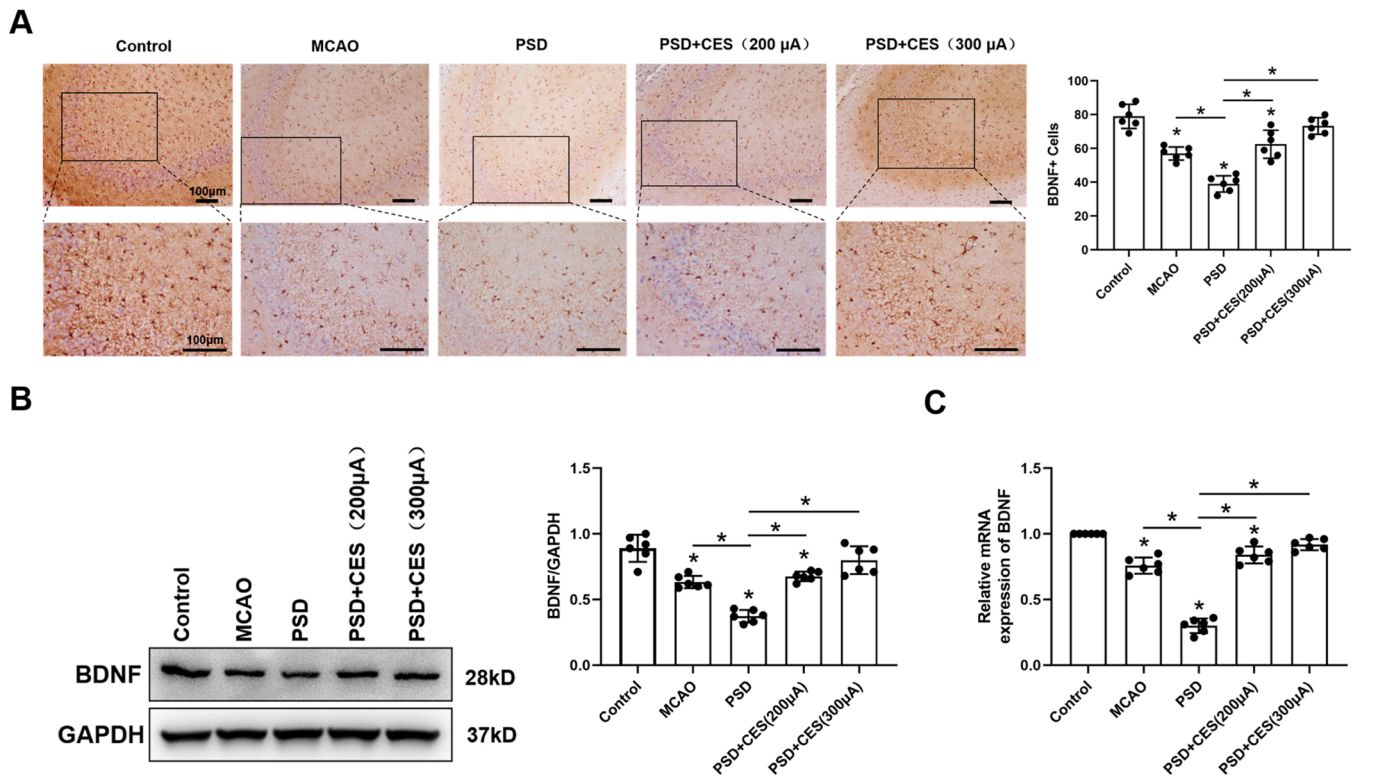

Fig. 3. CES increases the expression of BDNF in the hippocampus of PSD rats. (A) Immunohistochemical results showed the number of BDNF positive cells (BDNF+ cells) in the hippocampus of each group, scale bars = 100 μm. (B) Western blot results showing the expression level of BDNF in the hippocampus of each group; the GAPDH was used as an internal reference. (C) Quantitative PCR results indicating the expression level of BDNF mRNA in the hippocampus of each group; n = 6, * $p < 0.05$.

+ CES (200 μA) groups were significantly lower ($p < 0.05$) than that in the control group, but no significant difference was found between the PSD + CES (300 μA) group ($p > 0.05$) and the control. Compared with the MCAO group, the expression of BDNF in the PSD group was significantly lower ($p < 0.05$). The expression of BDNF in the PSD + CES (200 μA) and the PSD + CES (300 μA) groups was significantly higher ($p < 0.05$) than that in the PSD group. The effect in the PSD + CES (300 μA) group was more obvious, but no significant difference was observed as compared with the PSD + CES (200 μA) group ($p > 0.05$) (Fig. 3B).

The qRT-PCR results showed that the mRNA expression of BDNF were significant differences (F=175.69, df=4, $p < 0.05$) between the groups. The BDNF mRNA expression levels in the MCAO, PSD, and PSD + CES (200 μA) groups were significantly lower ($p < 0.05$) than that in the control group, but no significant difference was found between the PSD + CES (300 μA) and the control groups ($p > 0.05$). Compared with the MCAO group, the BDNF mRNA expression in the PSD group was significantly lower ($p < 0.05$). The BDNF mRNA expression levels in the PSD + CES (200 μA) and the PSD + CES (300 μA) groups were significantly higher ($p < 0.05$) than that in the PSD group. The effect of the PSD + CES (300 μA) was more considerable, but with no significant difference from the PSD + CES (200 μA) ($p > 0.05$) (Fig. 3C).

3.5. CES promoted hippocampal neuron repair and improved depression-like behavior by upregulation of GPX4 in PSD

There were significant differences (sucrose preference rate: F=44.99, df=3, $p < 0.05$; immobility time during swimming: F=63.97, df=3, $p < 0.05$) between the 4 groups. In the PSD+CES group, the sucrose preference rate was significantly higher ($p < 0.05$) and the immobility time during swimming was significantly shorter ($p < 0.05$) than that in the PSD group. In the PSD + RSL3 group, the sucrose preference rate was significantly lower ($p < 0.05$) and the immobility time during swimming was significantly longer ($p < 0.05$) than that in the PSD group. There was no significant difference in the values of these parameters between PSD+RSL3 +CES groups and PSD group ($p > 0.05$). Compared with the PSD + CES group, the sucrose preference rate in the PSD + RSL3 and PSD + RSL3 + CES groups was significantly lower ($p < 0.05$), whereas the immobility time during swimming was significantly longer ($p < 0.05$). There were no significant differences in the sucrose preference rate and the immobility time during swimming between the PSD + RSL3 + CES and the PSD + RSL3 groups ($p > 0.05$) (Fig. 4B, C).

HE staining showed obvious shrinkage, degeneration, and necrosis of the hippocampal neurons in the PSD group , which was more severe in the PSD+RSL3 group. An improvement was observed in the PSD + CES group as compared with the PSD group: the arrangement, appearance, and morphology of the hippocampal neurons tended to be normal. The PSD + RSL3 + CES group showed no improvement after the CES treatment (Fig. 4D).

3.6. CES increased the BDNF expression by GPX4 upregulation

Immunohistochemistry analysis showed that the numbers of BDNF+ cells and GPX4 + cells were significant differences (BDNF: F=52.13, df=3, $p < 0.05$; GPX4: F=49.51, df=3, $p < 0.05$) between the groups. The number of BDNF+ cells and GPX4 + cells in the PSD + CES group was larger than those in the PSD group, which was lesser in the PSD + RSL3 group than those in the PSD group. No significant changes were observed between the PSD + RSL3 + CES group and the PSD group. Compared with the PSD + CES group, the number of BDNF+ cells and GPX4 + cells in the PSD + RSL3 and the PSD + RSL3 + CES group were less. There was no significant difference between the values of these parameters in the PSD + RSL3 + CES and the PSD + RSL3 groups (Fig. 5A).

The ANOVA results of western blot results showed significant

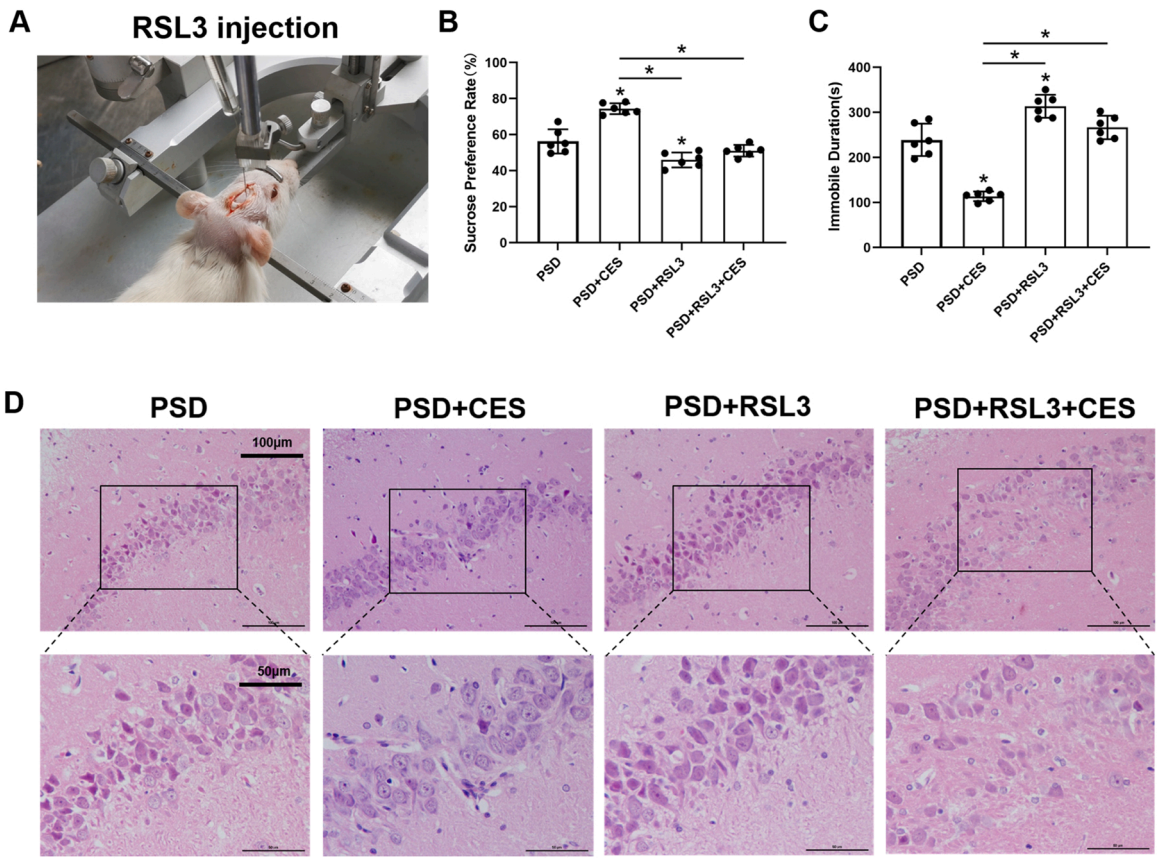

Fig. 4. CES promotes hippocampal neuron repair and improves depressive behavior by upregulation of GPX4 in PSD. (A) RSL3 was injected into the right hippocampus of PSD rats using brain stereotactic localization. (B) The bar chart illustrates the relative level of the sucrose preference rate in each group of rats. (C) The bar chart shows the relative level of immobility time in each group during swimming. (D) HE staining reveals the changes in the number, arrangement, and morphology in the hippocampal neurons in each group; scale bars = 100 and 50 μm; n = 6, * $p < 0.05$.

differences between groups in the protein expression of BDNF and GPX4 (BDNF: F=197.20, df=3, $p < 0.05$; GPX4: F=481.75, df=3, $p < 0.05$). The expression of BDNF and GPX4 in the PSD + CES group were significantly higher than those in the PSD group ($p < 0.05$), and the expression of BDNF and GPX4 in the PSD + RSL3 group were significantly lower than those in the PSD group ($p < 0.05$). There was no significant difference between the PSD + RSL3 + CES groups and the PSD group ($p > 0.05$). The expression of BDNF and GPX4 in the PSD + RSL3 and the PSD + RSL3 + CES group were significantly lower than those of the PSD + CES group ($p < 0.05$). The expression of BDNF and GPX4 in the PSD + RSL3 + CES group showed no statistically significant difference from that in the PSD + RSL3 group ($p > 0.05$) (Fig. 5B).

The ANOVA results of qRT-PCR showed significant differences between groups in the mRNA expression of BDNF and GPX4(BDNF: F=91.99, df=3, $p < 0.05$; GPX4: F=73.19, df=3, $p < 0.05$). The mRNA expression of BDNF and GPX4 in the hippocampus of the PSD + CES group were significantly higher than those in the PSD group ($p < 0.05$) , and the mRNA expression of BDNF and GPX4 in the PSD + RSL3 group were significantly lower than those in the PSD group ($p < 0.05$). There was no significant difference between the PSD + RSL3 + CES groups and the PSD group ($p > 0.05$). The mRNA expressions of BDNF and GPX4 in the PSD + RSL3 group and the PSD + RSL3 + CES group were significantly lower than those in the PSD + CES group ($p < 0.05$). No significant difference was observed in the mRNA expression of BDNF and GPX4 between the PSD + RSL3 + CES and PSD + RSL3 groups (Fig. 5C).

4. Discussion

The purpose of our study was to investigate the effect of CES on depression-like behavior in PSD rats, and to elucidate whether it promotes BDNF expression and neuronal cell repair in the hippocampus by regulating GPX4. We found that GPX4 is involved in the regulation of BDNF expression and hippocampal neuron repair in PSD rats, and CES increased the expression of BDNF by GPX4 upregulation, promoted hippocampal neuron repair and improved the depression-like behavior of PSD rats.

Ischemic stroke accounts for more than 80 % of all strokes and affects predominantly the middle cerebral artery (MCA) area[30]. The MCAO animal model is one of the best models used to simulate human ischemic stroke[31], which has been widely used in ischemic stroke research[32]. Zea-Longa score is utilized in MCAO rats to judge the degree of the neurological deficit [33]. A higher score indicates a more severe neurological deficit. The results of our study showed that the scores of the MCAO group were significantly higher than those of the control group, with a certain degree of neurological deficit. TTC staining is commonly employed to evaluate cerebral ischemia injury. In this method, brain tissue sections are stained, whereas the ischemic brain tissue remains unstained[34,35]. Here, we observed white ischemic areas without stain in the brain slices of the MCAO group, indicating the success of MCAO modeling.

Currently, the PSD rat model is established based on a MCAO model in which the animals have been subjected to chronic unpredictable mild stress (CUMS)[24,36,37]. Seven different stimulation methods were selected in our investigation, and the rats were alternately stimulated every day for three weeks. We also performed the sucrose preference and

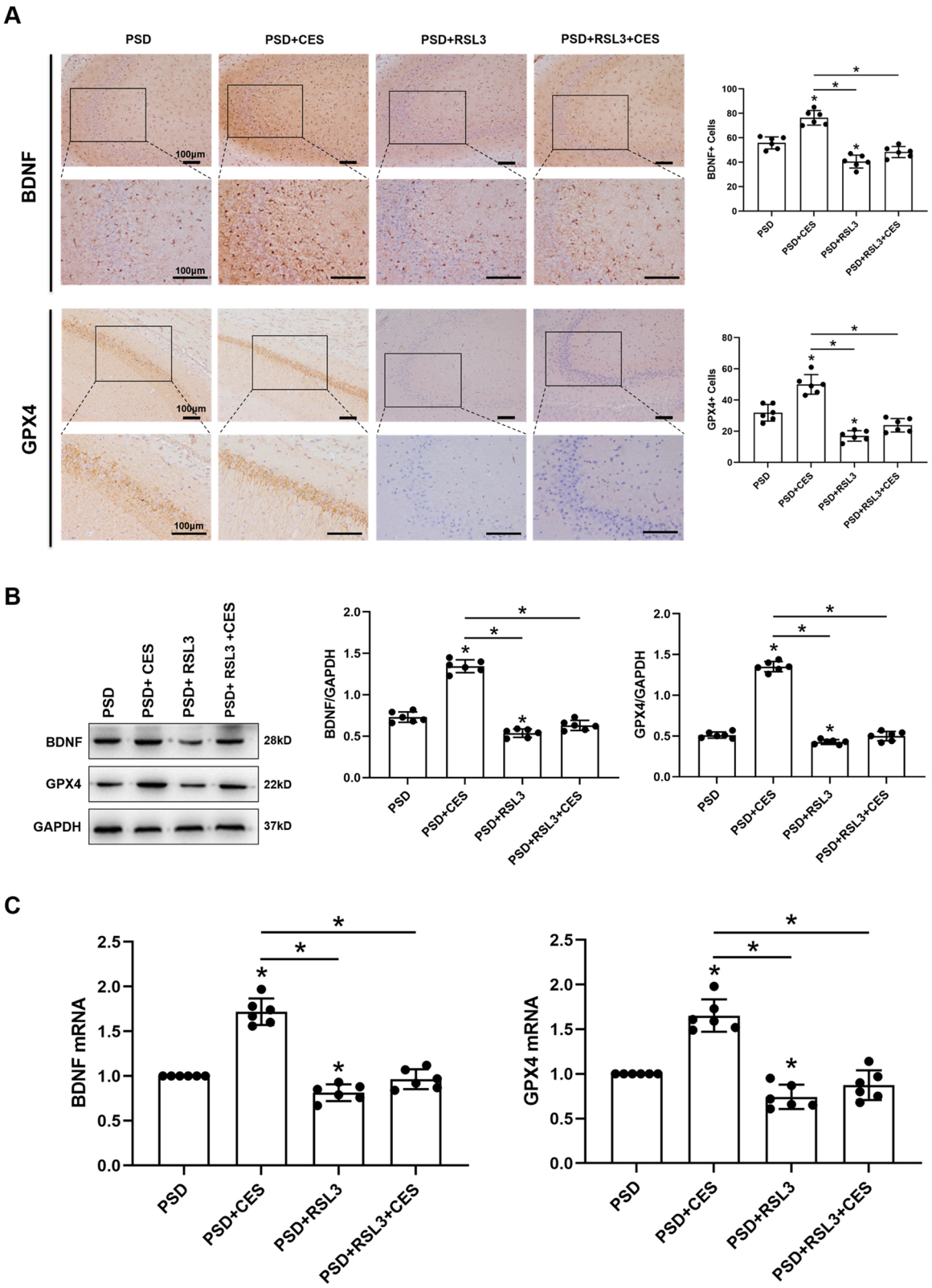

Fig. 5. CES increases BDNF expression by upregulation of GPX4. (A) Our immunohistochemical results showing the number of BDNF+ cells and GPX4 + cells in the hippocampus of each group, scale bars = 100 μm. (B) Western blot results showing the expression of BDNF and GPX4 in the hippocampus of each group; GAPDH was used as an internal reference. (C) qRT-PCR results indicating the mRNA expression levels of BDNF and GPX4 in the hippocampus of each group.; n = 6, * $p < 0.05$.

forced swimming tests to detect depressive behavior in rats[38]. We found that the sucrose preference rate of PSD rats was significantly lower, whereas the immobility time during swimming was significantly longer than those of the control group, indicating that the rats had depressive behavior.

PSD is a common post-stroke complication with a complex etiology [3], which may be the outcome of biological, social psychological, and environmental factors[7]. BDNF is a coding gene which is located on human chromosome 11 and is mainly distributed in the central nervous system tissues, with the highest content in the hippocampus and cerebral cortex. This gene is involved in nerve cell differentiation and growth. Studies have shown that low levels of BDNF can cause damage to the neurons in the hippocampus, which can lead to depression[9]. In recent years, increasingly more studies have also evidenced that BDNF is closely related to the development and prognosis of PSD. The level of BDNF was significantly decreased in PSD patients or mice, whereas the overexpression of BDNF was associated with the significantly improved depressive behavior in PSD rats[39,40]. Our study found that the expression of BDNF in the hippocampus of PSD rats was decreased, and the arrangement of hippocampal neurons was scattered. Their number was significantly reduced; the cell body was wrinkled, with a red-stained cytoplasm and nuclear pyknosis, and a large number of necrotizing neurons. These results also demonstrated that PSD has BDNF down-regulation and hippocampal neuron damage.

Currently, the main therapy of PSD includes drug and psychological therapy[41]. CES is a non-invasive and low-side-effect treatment for depression. By brain stimulation with low intensity micro-electric current, CES directly regulates the limbic and reticular systems, which are responsible for mental and emotional activities. This approach can quickly increase endogenous morphine peptide secretion, improve abnormal brain waves, reversing them to normal or close to normal brain waves, achieving a relaxed, happy, and clear thinking alpha-state [42–44]. Recently, Price et al. conducted a meta-analysis including 1415 subjects in a total number of 17 studies showing that CES had a significant effect on the depressive symptoms in moderate to severe patients in the population[45]. Furthermore, Yennurajalingam et al. found that four weeks of CES treatment in patients with advanced cancer (ACPs) significantly improved depressive mood[20]. However, the therapeutic effects of CES on PSD has not been reported in previous literatures. Our study revealed that a four-week treatment with 200 and 300 μA CES significantly increased the expression of BDNF in the hippocampus, promoted the repair of hippocampal neurons, and improved the depression-like behavior of PSD rats. The above results confirmed the therapeutic effects of CES on PSD and suggested that these effects may be related to BDNF upregulation and hippocampal neuron repair.

The GPX4 gene is located on human chromosome 19 and encodes selenoprotein. GPX4 catalyzes the reduction of hydrogen peroxide, organic hydrogen peroxide, and lipid peroxides through glutathione oxidation[15]. GPX4 expression was negatively correlated with depressive symptoms[16]. Wang et al. detected an increase in GPX4 protein expression during the use of drugs to treat depressive behavior in type 1 diabetes[46]. Qiu et al. found that the levels of GPX4 and BDNF increased in the process of promoting nerve cell repair[18]. Our study found that the inhibition of GPX4 by RSL3 downregulated BDNF expression, inhibited hippocampal neuronal repair and exacerbated depression-like behavior in PSD rats. In addition, the expression of GPX4 and BDNF in the hippocampus was significantly increased, and the degeneration and necrosis of the hippocampal neurons and depression-like behavior were improved after the CES treatment. However, these effects of CES on PSD rats were disrupted by the RSL3 injection. These results suggested that the BDNF expression and hippocampal neuron repair were regulated by GPX4, the down-regulation of GPX4 reduced the BDNF expression and prevented the hippocampal neuron repair. CES can regulate the expression of BDNF in the hippocampus through GPX4 and then promote the repair of hippocampal neurons, ultimately improve the depression-like behavior in PSD rats.

5. Conclusion

In conclusion, our findings suggest that GPX4 is critically involved in the regulation of BDNF expression and hippocampal neuron repair, and CES increased the expression of BDNF by upregulation of GPX4, promoted the repair of hippocampal neurons, and thus improved the depression-like behavior in PSD rats. Our results clarified the efficacy of CES on PSD and elucidated the mechanism of the effect of CES on PSD through upregulating GPX4 and BDNF expression. However, this study is an animal experiment, the therapeutic effects of CES on PSD and the repair effect and mechanism of CES on hippocampal neurons still need to be further verified in human clinical trials.

Ethics approval and consent to participate

The experimental protocol associated with research work on rats was approved by the Nanjing First Hospital Ethics Committee of Nanjing Medical University (Nanjing, Jiangsu, China).

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CRediT authorship contribution statement

Xiaoju Wang (First Author): Conceptualization, Methodology, Software, Investigation, Formal analysis, Writing – original draft. **Peng Xia:** Data curation, Writing – original draft. **Jiulong Song:** Visualization, Investigation. **Ting Yang:** Resources, Supervision. **Changjun Yu**: Software, Validation. **Kai Cheng:** Writing – review & editing, **Anling Chen:** Visualization, Writing – review & editing. **Xueping Li** (Corresponding Author): Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing. All authors read and approved the final version of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Data Availability

No data was used for the research described in the article.

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Author Contributions Statement

XW and XL conceived and designed the study. XW and PX performed the experiments. XW wrote the manuscript. KC, AC and XL reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

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