

Enriched environment alleviates post-stroke cognitive impairment through enhancing $\alpha 7$ -nAChR expression in rats

Ambiente enriquecido alivia o comprometimento cognitivo pós-AVC em ratos por meio do aumento da expressão de $\alpha 7$ -nAChR

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ABSTRACT

Background: Enriched environment (EE) is a simple and effective intervention to improve cognitive function in post-stroke cognitive impairment (PSCI), partly due to the rebalancing of the cholinergic signaling pathway in the hippocampus. $\alpha 7$ -nicotinic acetylcholine receptor ($\alpha 7$ -nAChR) is a cholinergic receptor whose activation inhibits inflammation and promotes the recovery of neurological function in PSCI patients. However, it is still unclear whether EE can regulate $\alpha 7$ -nAChR and activate the cholinergic anti-inflammatory pathway (CAP) in PSCI. **Objective:** To investigate the effects of EE on cognitive impairment, and the role of $\alpha 7$ -nAChR in PSCI. **Methods:** A PSCI rat model was induced by middle cerebral artery occlusion and reperfusion (MCAO/R) and were reared in standard environment (SE) or EE for 28d, control group with sham surgery. Cognitive function was determined by Morris water maze test. The long-term potentiation (LTP) was assessed by Electrophysiology. Histopathological methods were used to determine infarct volume, $\alpha 7$ -nAChR expression and the cytokines and cholinergic proteins expression. **Results:** Compared with SE group, rats in EE group had better cognitive function, higher expression of $\alpha 7$ -nAChR positive neurons in hippocampal CA1 region. In addition, EE attenuated unfavorable changes induced by MCAO/R in cytokines and cholinergic proteins, and also enhanced LTP promoted by nicotine and attenuated by α -BGT; but showed no significantly difference in infarct volume. **Conclusions:** EE markedly improves cognitive impairment and enhances neuroplasticity in PSCI rats, which may be closely related to enhancement of $\alpha 7$ -nAChR expression.

Keywords: Cognitive Dysfunction; Stroke; Hippocampus.

RESUMO

Introdução: O ambiente enriquecido (AE) é uma intervenção simples e eficaz para melhorar a função cognitiva no comprometimento cognitivo pós-AVC, em parte devido ao reequilíbrio da via de sinalização colinérgica no hipocampo. O receptor nicotínico $\alpha 7$ de acetilcolina ($\alpha 7$ -nAChR) é um receptor colinérgico cuja ativação inibe inflamação e promove a recuperação da função neurológica em pacientes com comprometimento cognitivo pós-AVC. No entanto, ainda não está claro se o AE pode regular $\alpha 7$ -nAChR e ativar a via anti-inflamatória colinérgica (VAC) em comprometimento cognitivo pós-AVC. **Objetivo:** Investigar os efeitos do AE no comprometimento cognitivo e o papel do $\alpha 7$ -nAChR no comprometimento cognitivo pós-AVC. **Métodos:** Modelo de comprometimento cognitivo pós-AVC foi induzido em ratos por oclusão e reperfusão da artéria cerebral média (MCAO/R), que foram criados em ambiente padrão (AP) ou em AE por 28d; grupo controle com cirurgia simulada. A função cognitiva foi determinada pelo teste do labirinto aquático de Morris. A potenciação de longo prazo (PLP) foi avaliada por eletrofisiologia. Métodos histopatológicos foram usados para determinar o volume do infarto, a expressão de $\alpha 7$ -nAChR e a expressão de citocinas e proteínas colinérgicas. **Resultados:** Em comparação com o grupo AP, os ratos do grupo AE tiveram melhor função cognitiva, com maior expressão de neurônios positivos para $\alpha 7$ -nAChR na região CA1 do hipocampo. Além disso, o AE atenuou alterações desfavoráveis induzidas por MCAO/R em citocinas e proteínas colinérgicas, e também aumentou a PLP promovida pela nicotina e atenuada por α -BGT, mas não mostrou nenhuma diferença significativa no volume do infarto. **Conclusão:** O AE melhora acentuadamente o comprometimento cognitivo e aumenta a neuroplasticidade em ratos com comprometimento cognitivo pós-AVC, o que pode estar intimamente relacionado ao aumento da expressão de $\alpha 7$ -nAChR.



Palavras-chave: Disfunção Cognitiva; Acidente Vascular Cerebral; Hipocampo.

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INTRODUCTION

Post-stroke cognitive impairment (PSCI) is a common complication of ischemic stroke. About 25% of patients has severe PSCI three months after stroke, including memory, orientation, language and attention¹. Cognitive impairment is a major contributor to longer duration of hospital stay, lower quality of life and difficulty returning to social life². Prevention for PSCI can be implemented by lowering blood pressure, administration of statin, neuroprotective and anti-inflammatory drugs. However, there is no evidence of convincing efficacy. Recent studies also suggested that lifestyle interventions, physical activity and cognitive training may improve cognition of PSCI, but without sufficient controlled clinical trials³.

Enriched environment (EE) is an effective rodent rehabilitation treatment method, in which several animals are accommodated in a large space equipped with various toys and receive more sensorial movements, perceptions and social stimulation than in standard conditions. EE has a neuroprotective effect on animal models of cerebral ischemia⁴. There is significant evidence that EE also benefits the clinical rehabilitation of post-stroke patients, including promoting greater exercise, social interaction and personal control⁵. Exploring the underlying mechanisms that EE improves cognitive function in PSCI is of great significance for providing more individualized cognitive rehabilitation programs in stroke patients.

$\alpha 7$ -nicotinic acetylcholine receptor ($\alpha 7$ -nAChR) is a cholinergic receptor that is abundantly-expressed in the hippocampus and the frontal cortex, and has been confirmed to play a critical role in improving cognitive function of learning and memory^{6,7}. As the biological foundation of cognitive processes, synaptic transmission can be modulated by cholinergic pathway and evaluated by long-term potentiation (LTP). Activation of $\alpha 7$ -nAChR contributes to a better induction of LTP⁸, which is also essential for inhibiting cytokine synthesis, such as IL-1 β , IL-6, by the cholinergic anti-inflammatory pathway (CAP)⁹. Activation of $\alpha 7$ -nAChR inhibits inflammation in patients with stroke¹⁰. In the aging process of mice, EE not only increases the expression of choline acetyltransferase (ChAT) and $\alpha 7$ -nAChR in the hippocampus but also improves spatial memory¹¹. EE also significantly improves cognitive impairment in PSCI mice, induces hippocampal LTP, and enhances ChAT promoter acetylation¹².

These studies suggest that activation of cholinergic signals after stroke enhances neuroplasticity and cognitive function. However, it is still unclear whether EE can regulate $\alpha 7$ -nAChR and the role of $\alpha 7$ -nAChR in CAP and LTP of PSCI.

To explore the effects of EE on cognitive function, neuroplasticity and underlying mechanism in PSCI rats, a rat model of ischemic stroke was induced by middle cerebral artery occlusion and reperfusion (MCAO/R). We have characterized the effects of EE on cognitive function, expression of $\alpha 7$ -nAChR protein, induction of LTP in hippocampus, and serum cytokine levels.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (200 \pm 20 g, 10 weeks) were purchased from Shanghai SLRC Laboratory Animal Co., Ltd. (Shanghai, China). Rats were housed under pathogen-free conditions and maintained at controlled temperature (20–24°C) and humidity (40–70%) on a 12h light/dark schedule, with food and water freely available. The experimental procedures were approved by the Animal Ethics Committee of Zhoupu Hospital.

Post-stroke cognitive impairment model

Ischemic stroke model was established through MCAO/R in rats with minor modifications¹³. Briefly, animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (45 mg/kg) and their body were maintained at 37°C of temperature via a thermal pad throughout the process. The midline neck incision was made for each rat to expose the right common carotid artery, internal carotid artery, and external carotid artery. The common carotid artery was ligated near the bifurcation of the carotid artery. A lysine-coated nylon monofilament (0.32 \pm 0.02 mm) (Beijing Sunbio Biotech Co., Ltd., Beijing, China) was inserted into right internal carotid artery via the common carotid artery, and was gently advanced to the origin of the middle cerebral artery. The filaments were pull out 1h later to restore blood flow (reperfusion). Twenty-four hours after MCAO/R, all surviving rats underwent neurobehavioral examination using a longa scoring system: score 0: no neurological deficit; score 1: completely unable to extend right forepaw; score 2: rotated right; score 3: toward right; score 4: can not walk spontaneously and consciousness level declines. Rats with scores of 1–3 were considered successful MCAO/R model and then divided into standard environment (SE) or EE housing condition according to the scores. Sham surgery received the same procedures except for the filament insertion.

Experimental design

Rats were divided into 3 groups according to surgery and housing condition: (1) Control group: sham surgery rats were housed in SE condition, n=16; (2) SE group: MCAO/R rats were housed in SE condition, n=16; (3) EE group: MCAO/R rats were housed in EE condition, n=16. In EE housing condition, 6–8 rats were housing together in a large cage (90 cm long \times 75 cm wide \times 50 cm high) with various objects, including climbing ladders, wood platform, toys or tunnels of different shape and color. These subjects changed 3 times a week to keep novelty. Four rats were housed together in each SE housing condition, which consisted of a standard cage (44 cm long \times 32 cm wide \times 20 cm high) with no objects. To further investigate the role of $\alpha 7$ -nAChR in LTP induction by EE, nicotine ($\alpha 7$ -nAChR activator, 0.5 mg/kg, i.p.) or α -BGT ($\alpha 7$ -nAChR inhibitor, 1.0 μ g/kg, i.v.) were administered daily for 28 days

according to a previously method⁷, 4 rats for each administration. Rats were housed in EE or SE condition for 4 weeks.

Behavioral tests

Assessment of neurological function

Modified neurological severity scores (mNSS)¹⁴ were evaluated on 7, 14, 28 days after MCAO/R to assess the degree of neurological deficits. mNSS is a comprehensive test including motor, sensory, reflex and balance tests and scaled from 0 to 18 score (score 0: normal; score 18: maximal deficit). The higher scores reflect more severe deficits. The scores were evaluated by two researchers who were blinded to the experiment grouping.

Morris water-maze test

Morris water-maze test was performed to measure the spatial learning and memory including two phases. The apparatus consisted of a circular tank (160 cm in diameter and 60 cm in height) that was filled with water at $25\pm 1^\circ\text{C}$ to a depth of 30 cm, and a circular hidden platform (12 cm diameter) was located in the middle of northeast quadrant of the pool and 2 cm below the water level. The first phase involved space learning trainings prior to experimental trail from 3 days after MCAO/R. Rats were randomly placed into pool facing the wall of tank from southeast and southwest directions, and allowed 90 s to reach the hidden platform. Each rat received 4 trainings prior to trail per day for 3 consecutive days. The second phase involved place navigation trial and probe trial at 7, 14 and 28 days after MCAO/R. The place navigation trial was carried out like space learning training and the duration reaching the platform was recorded as the escape latency (maximum 90 s). In the probe trail, platform was removed from the pool and the number of crossing over original platform position were recorded as platform crossing times.

Quantification of infarct volume

At 28 days after MCAO/R, rats were decapitated, and the brains were quickly frozen and sliced into coronal sections (2 mm thickness), and sections were stained with 1.2% 2,3,5-triphenyl tetrazolium chloride (TTC) (Sigma, St. Louis, Mucun, USA) at 37°C for 30 min, and then fixed in 4% paraformaldehyde overnight. Then, sections were observed and photographed. The infarcted tissue showed unstained (white) and normal tissue showed stained (red), and were analyzed using digital image analysis software (SigmaScan Pro, Jandel, San Rafael, CA, USA). The percentage of infarct volume was calculated using the following equation: percentage of infarct volume = (total infarct volume/whole brain section volume) \times 100%. The volume was quantified by summing areas of sections multiplied by the section thickness (2 mm).

Expression of $\alpha 7$ -nAChR in the hippocampus

Rats were decapitated at 28 days after MCAO/R, and the hippocampus was fixed overnight with 4% paraformaldehyde

overnight and embedded with paraffin wax to acquire serial sections (5 μm thickness). After dewaxing, dehydration, incubation with 3% H_2O_2 , and microwave antigen retrieval, the hippocampus serial sections were then incubated with rabbit anti- $\alpha 7$ -nAChR primary antibody (1:100, ab10096, Abcam, UK) at 4°C overnight. After washing with phosphate buffered saline (PBS), sections were incubated with goat-anti-rabbit IgG labelled with HRP as secondary antibody (1:100, ab6721, Abcam, UK) at room temperature for 2h, and then were stained with diaminobenzidine (DAB) (Leica, Germany) and hematoxylin. The DAB stained brown cells are $\alpha 7$ -nAChR positive neurons, which were counted from five random high magnification vision in CA1 region under a microscope (BX51, Olympus, Japan). The cell density was average number of $\alpha 7$ -nAChR positive neurons per high magnification vision (cells/HP).

ELISA for cytokines and cholinergic proteins

Blood samples were obtained from caudal vein of rats at 7, 14 and 28 days after MCAO/R, and the ipsilateral hippocampal tissue at 28 days after MCAO/R. Both serum and hippocampal homogenates were separated by centrifugation at 5000 g for 10 min at 4°C to acquire serum and hippocampal homogenates. ELISA assay kits were used to measure serum levels of interleukin-1 β (IL-1 β), interleukin-6 (IL-6), neuron-specific enolase (NSE) and brain-derived neurotrophic factor (BDNF) (R&D Systems, Minneapolis, MN, USA). Moreover, the ACh content (A105-1), ChAT (A079) and acetylcholinesterase (AChE) (A024) activities in the hippocampus were measured by commercial assay kits (Nanjing Jiancheng Biological Engineering Institute, Nanjing, China). The values of above proteins were determined by measuring the wavelength at 450 nm using a microplate reader (Ricsco RK201, Shenzhen Ricsco Technology Co., Ltd, China).

Electrophysiology

Rats were decapitated at 28 days after MCAO/R, brains were rapidly removed and the ipsilateral hippocampus region was cut into transverse slices (400 μm thickness). Slices were continuously perfused with ice-cold ACSF composed of the following: NaCl 124 mM, CaCl_2 2.0 mM, KCl 4.5 mM, MgCl_2 1.0 mM, NaHCO_3 26 mM, NaH_2PO_4 1.2 mM, D-glucose 10 mM, and pH 7.4. A bipolar electrode was inserted into the ipsilateral Schaffer collaterals using microscope (Olympus BX50-wI, Olympus, Japan) to deliver the orthorhombic stimulus by a stimulator (SEN-3301, Nihon Kohden, Japan), and a recording electrode was inserted into the ipsilateral CA1 region to record the field excitatory postsynaptic potential (fEPSP). Baseline responses were recorded for 20 minutes prior to beginning the experiment with a constant current pulse (frequency 0.1 Hz, pulse duration 0.25 ms). Then, LTP was induced by high frequency stimulation (HFS, 100 Hz). LTP was recorded every 5 min for 120 min with the same stimulation intensity as pre-HFS. To further investigated the role of $\alpha 7$ -nAChR in LTP induction by EE, α -BGT (1.0 $\mu\text{g}/\text{kg}$, i.v.) and nicotine (0.5 mg/kg, i.p.) were administered daily for 28 days.

Statistical analysis

All quantitative data were presented as mean±standard deviation (SD) and analyzed by SPSS 19.0 statistical software (SPSS, USA). The differences among groups in Morris water-maze test and serum cytokines were analyzed using two-way analysis of variance (ANOVA) with repeated measures, with following Bonferroni test for multiple comparisons. One-way ANOVA was applied to compare the differences in other data, followed by Bonferroni's post hoc. For statistically significance, $p < 0.05$ was considered as criteria.

RESULTS

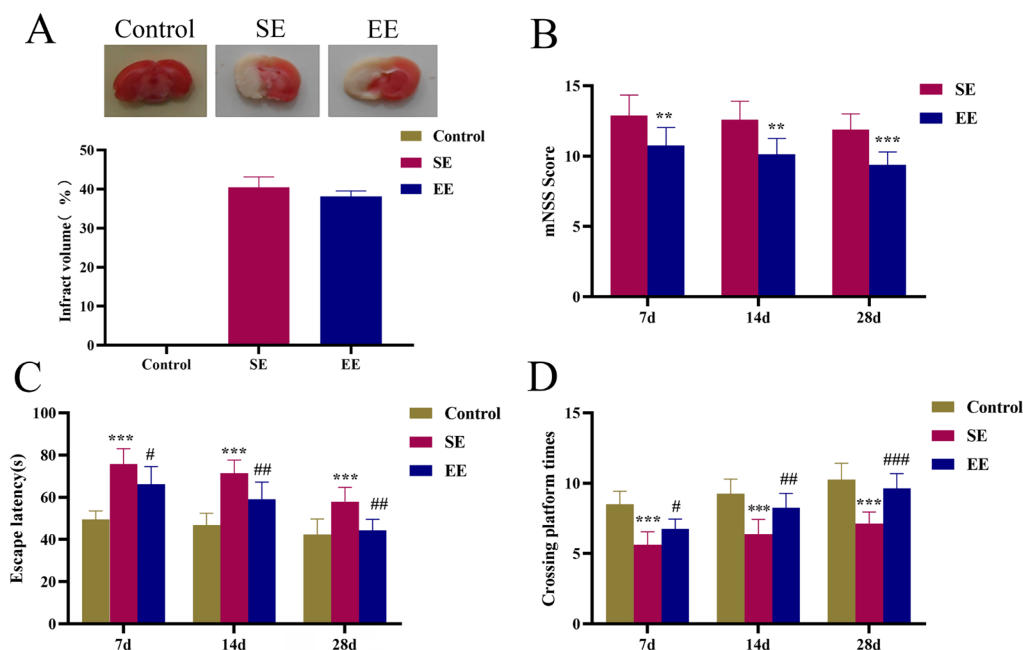
Enriched environment ameliorated cognitive deficits in ischemic stroke rats

At 28 days after MCAO/R, the infarct volume of EE group ($38.13 \pm 1.44\%$; $n=4$) was slightly smaller than SE group ($40.48 \pm 2.69\%$; $n=4$). However, with no significant difference ($p=0.189$) (Figure 1A). In terms of the functional recovery, mNSS scores were significantly decreased by 16.5, 19.6 and 21.1% at 7, 14 and 28 days after MCAO/R, respectively, compared with SE group ($p < 0.001$; $n=8$ rats per group) (Figure 1B). In terms of learning and memory, data of Morris water-maze test suggested that, compared with control group, escape latency time was significantly increased and crossing platform

times were significantly decreased in rats of SE group at different time points. Moreover, results were reversed significantly by EE treatment ($p < 0.05$; $n=8$ per group) (Figures 1C and 1D). These results indicate that EE is capable of alleviating cognitive impairment induced by ischemic lesion.

Enriched environment enhanced $\alpha 7$ -nAChR expression and cholinergic pathway of hippocampus

The density of $\alpha 7$ -nAChR in the hippocampal CA1 region was detected by immunohistochemistry. Representative pictures were shown for the $\alpha 7$ -nAChR positive neurons in control group (Figure 2A, photo 1), SE group (Figure 2A, photo 2) and EE group (Figure 2A, photo 3). Compared with the control group ($46.75 \pm 4.88\%$), the cell density of $\alpha 7$ -nAChR positive neurons was decreased in SE group ($18.08 \pm 3.16\%$; $p < 0.001$), and then restored by EE ($36.43 \pm 2.73\%$; $p < 0.001$ vs. SE group). It was then measured the ACh content, ChAT and AChE activities of hippocampal tissues with ELISA. MCAO/R significantly decreased the ACh content (43.93%; $p < 0.001$) (Figure 2B) and ChAT activity (49.10%; $p < 0.001$) (Figure 2C), but increased AChE activity (153.83%; $p < 0.001$) (Figure 2D) in the hippocampal tissues. Moreover, these changes were significantly reversed by EE (ACh content, $p < 0.001$; ChAT activity, $p=0.003$; AChE activity, $p < 0.001$), $n=4$ per group. All these findings suggest that EE enhances $\alpha 7$ -nAChR expression and cholinergic pathway of hippocampus.



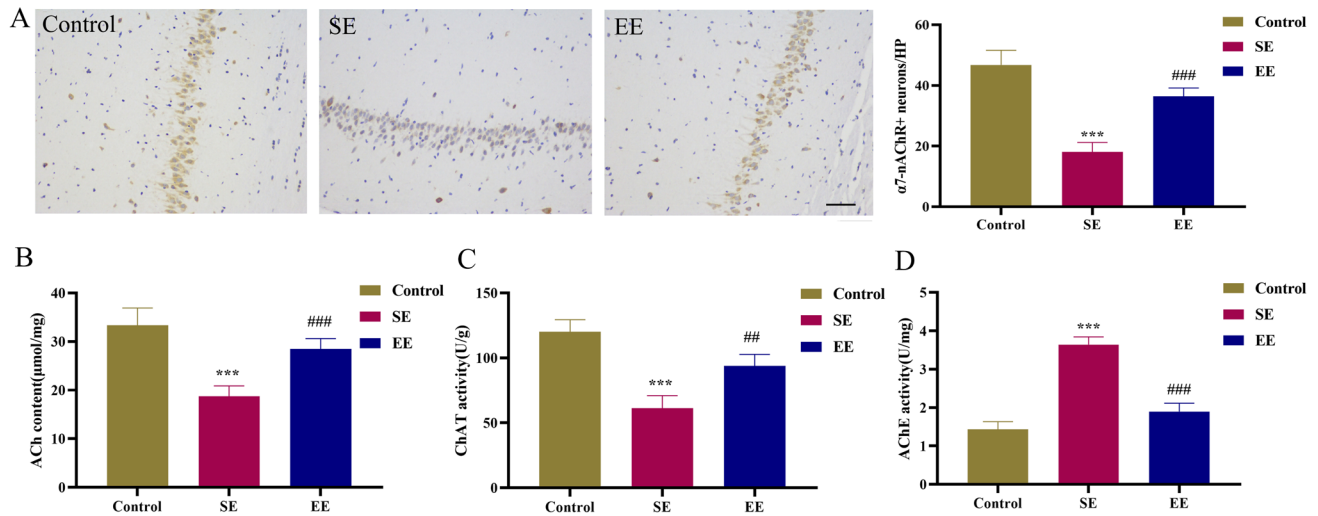
(A) Representative photographs of brain slices that are stained with TTC at 28 days after MCAO/R. The white areas are infarcted tissue. EE slightly reduces infarct size, with no significant difference ($n=4$ rats per group). (B) EE significantly decreases mNSS score at 7, 14 and 28 days after MCAO/R. Data are expressed as mean±SD. $**p < 0.01$, $***p < 0.001$ vs. SE group of the same time points ($n=8$ rats per group). (C-D) EE significantly decreases escape latency time and increases crossing platform times. Data are expressed as mean±SD. $***p < 0.001$ vs. control group of the same time points; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ vs. SE group of the same time points ($n=8$ rats per group). SE: standard environment; EE: enriched environment, mNSS: Modified neurological severity scores.

Figure 1. Effects of enriched environment on infarct volume, neurological deficit scores and cognitive impairment.

Enriched environment suppressed proinflammatory cytokines, brain damage and improved nerve regeneration

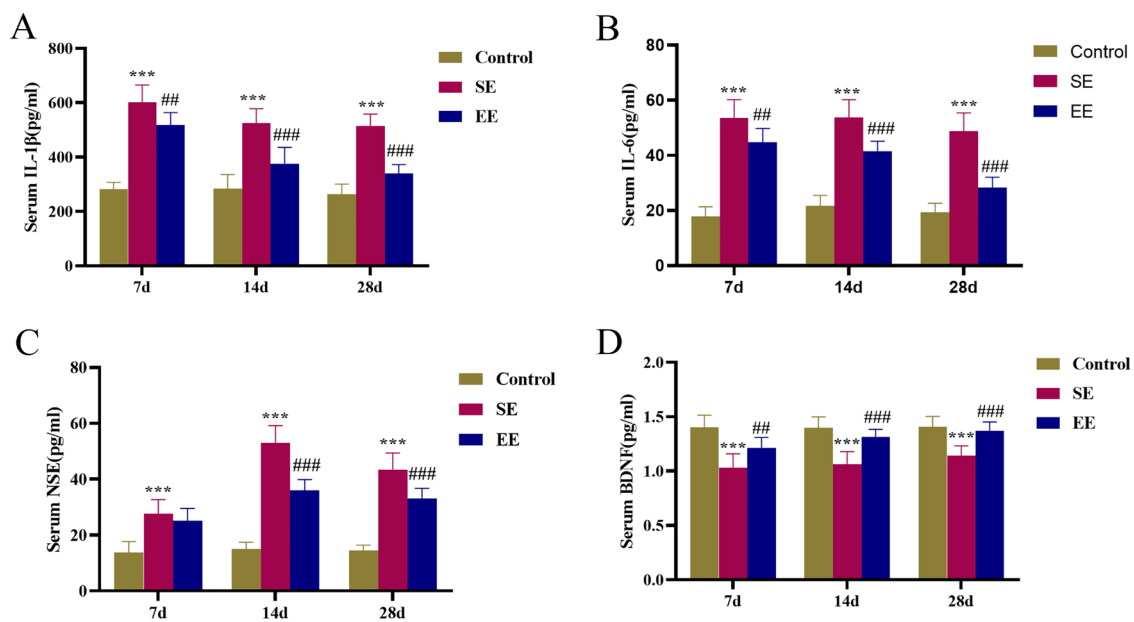
ELISA immunoassay were performed to detect the expression levels of serum proinflammatory cytokines (IL-1 β and IL-6) (Figures 3A and 3B), NSE (Figure 3C) and

BDNF (Figure 3D) at 7, 14 and 28 days after MCAO/R. Compared with control group, the level of proinflammatory cytokines and NSE were much higher ($p < 0.001$) and BDNF was decreased significantly ($p < 0.001$) at all time points after MCAO/R. EE significantly attenuated MCAO/R-induced change of proinflammatory cytokines, NSE and BDNF in



(A) Representative images of hippocampal $\alpha 7$ -nAChR positive neurons in the hippocampal CA1 region are shown in rats of control group, SE group and EE group at 28 days after MCAO/R ($\times 200$). Scale bar = 10μ m. EE increases the cell density of $\alpha 7$ -nAChR positive neurons in the hippocampus ($p < 0.05$). EE increases ACh content (B) and ChAT activity (C), and decreases AChE activity (D) in hippocampal tissue of MCAO/R rats. Data are expressed as mean \pm SD, *** $p < 0.001$ vs. control group; ## $p < 0.01$, ### $p < 0.001$ vs. SE group. $n = 4$ samples from 4 rats per group. ACh, acetylcholine; ChAT, choline acetyltransferase; AChE, acetylcholinesterase.

Figure 2. The effects of enriched environment on cholinergic pathway in the hippocampal tissue of middle cerebral artery occlusion and reperfusion rats.



EE significantly decreases serum IL-1 β (A), IL-6 (B) and NSE (C) levels, and increases BDNF (D) level in rats at 7, 14 and 28 days after MCAO/R. Data are expressed as mean \pm SD, *** $p < 0.001$ vs. control group of the same time points; ## $p < 0.01$, ### $p < 0.001$ vs. SE group of the same time points. $n = 8$ samples from 8 rats per group. NSE, c.

Figure 3. Effects of enriched environment on serum cytokine levels in middle cerebral artery occlusion and reperfusion rats.

serum, n=8 per group. These results suggest that EE is capable to suppress proinflammatory cytokines, brain damage and improve nerve regeneration.

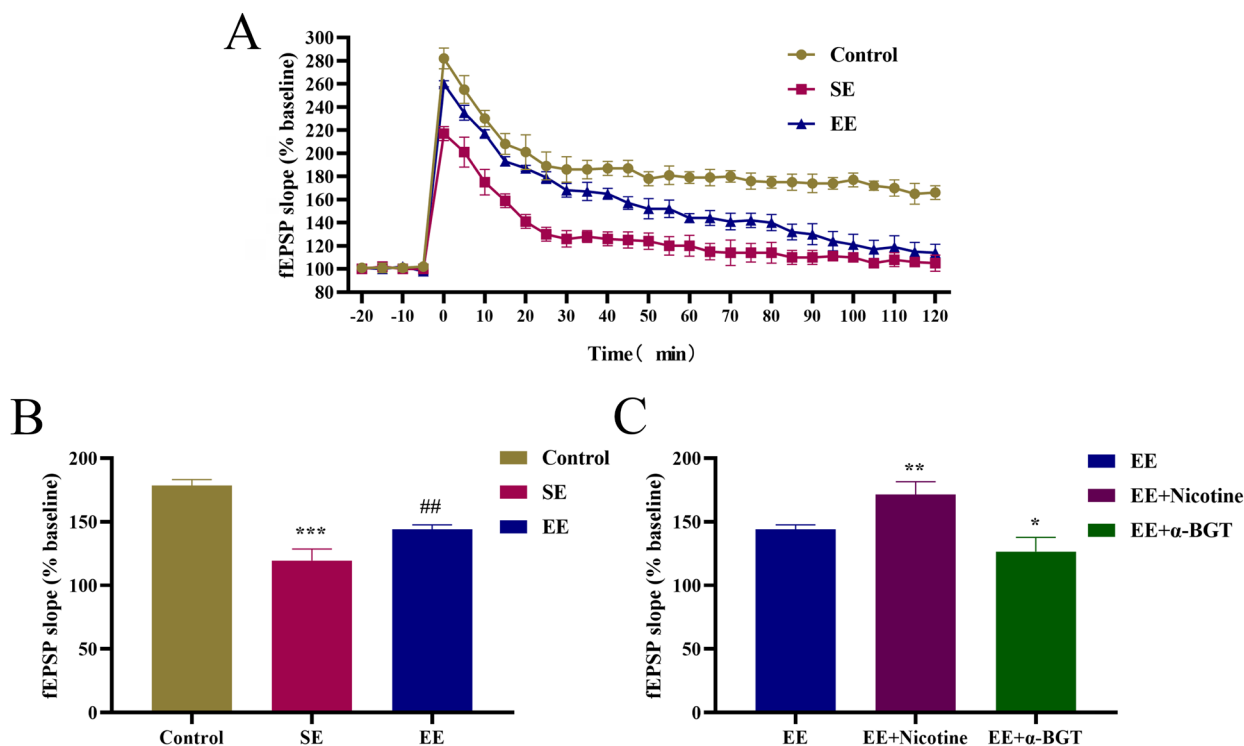
Enriched environment enhanced LTP in $\alpha 7$ -nAChR dependent manner

To investigate the effect of EE on neural plasticity, hippocampal slices were stimulated by HFS to measure LTP. In the 5 min after HFS, the fEPSP slope of control group sharply increased compared baseline and then slowly decreased. EE could increase fEPSP slope in MCAO/R rats, especially before 90 min compared with SE group (Figure 4A). This indicates that ischemic stroke induces obvious damage on LTP, while EE enhances LTP after HFS. Average fEPSP amplitude at 60 min after HFS was significantly increased by 20.50% with EE, compared with SE group ($p=0.002$) (Figure 4B), and this increase was further enhanced by cotreatment with nicotine (19.10%; $p=0.002$ vs. EE group), and attenuated by cotreatment with α -BGT (12.15%; $p=0.026$ vs. EE group) (Figure 4C), n=4 per group. These results suggest that EE enhances synaptic plasticity and the effect is dependent on $\alpha 7$ -nAChR.

DISCUSSION

The present study demonstrated effects and related mechanisms of EE in PSCI rats induced by MCAO/R. EE significantly improves neurological function and attenuates cognitive impairment in MCAO/R rats. In addition, $\alpha 7$ -nAChR expression, hippocampal ACh content and ChAT activity was increased due to EE, and AChE activity was decreased due to EE, compared with SE group. EE also suppresses neuroinflammation, as evidenced by reduced serum levels of IL-1 β and IL-6. NSE levels and BDNF level were reversed by EE after MCAO/R. Moreover, LTP was enhanced by EE in hippocampal slices, which was further enhanced by nicotine and attenuated by α -BGT. Together, these results provide evidence that EE has neuroprotective effects on PSCI rats. What's more, the mechanisms underlying this protection may be associated with enhanced expression of $\alpha 7$ -nAChR, suppression of inflammation, and enhanced induction of LTP.

No significant difference in infarction volume was observed between EE and SE groups in our study, which is similar to recent studies in PSCI. For example, infarct volume on day 31 after MCAO in EE group is also smaller slightly, but no



(A) Time course and extent of LTP induction in rats 28 days after MCAO/R. fEPSP amplitude is normalized to average baseline. (B) EE increases the fEPSP slope. Data are expressed as mean \pm SD, *** $p<0.001$ vs. control group, ## $p<0.01$ vs. SE group. (C) The effects of EE on LTP are dependent on $\alpha 7$ -nAChR. Average fEPSP amplitude at 60 min after HFS by EE are enhanced by cotreatment with nicotine, and attenuated by cotreatment with α -BGT. Data are expressed as mean \pm SD, * $p<0.05$, ** $p<0.01$ vs. EE group. n=4 slices from 4 rats per group. α -BGT, α -bungarotoxin; LTP, long-term potentiation; HFS: high frequency stimulation; fEPSP: field excitatory postsynaptic potential.

Figure 4. The effects of enriched environment on long-term potentiation of middle cerebral artery occlusion and reperfusion rats.

statistically significant difference compared with SE group¹⁵. This result is also observed at 14 days after MCAO¹⁶, possibly due to that the neuronal death is irreversible. Moreover, studies also found that pretreatment with EE for 2 or 5 weeks significantly decreases infarct volume induced by ischemic stroke in rats, suggesting that pretreatment with EE protect neuronal death from stroke^{17,18}. However, a previous systematic review found that EE increases infarct volume of some degree without significantly statistic difference compared with SE group in two third of stroke-related studies, possibly due to the stress of new environment and cortical hyperexcitability¹⁹. Given that the small sample size in our study and no consensus on the effect of infarct volume by EE in PSCI, the effect of EE on infarct volume still need further research.

The anti-inflammatory effects of EE at different time point after stroke in this study is in accordance with some previous studies. For example, at four weeks after ischemic stroke, EE attenuates histopathological and oxidative damage to the brain, thereby improving cognitive function in chronic cerebral hypoperfusion (CCH) rats²⁰. In an earlier phase of stroke, EE significantly decreases the expression of IL-1 β at 5d after MCAO²¹. These show similar results with our study and indicate that suppressed neuroinflammation is an important mechanism of EE. The hippocampus is likely a good target of anti-inflammatory responses given that hippocampus is the main brain area responsible for cognitive function.

The present study shows that EE activates cholinergic pathway in MCAO/R rats. Agonists of $\alpha 7$ -nAChR has an improvement effect on cognition, while reduced $\alpha 7$ -nAChR expression has been found in various neurological disorders²². $\alpha 7$ -nAChR is a major component of CAP and controls inflammation in central nervous system²³. In ischemic stroke, $\alpha 7$ -nAChR activates CAP and suppresses neuroinflammation, thus improving cognitive impairment²⁴. These indicate that enhanced hippocampal $\alpha 7$ -nAChR expression in our study may contribute to inhibition of neuroinflammation and improvement of cognitive function. $\alpha 7$ -nAChR also might mediate the cognitive effect of EE. To our knowledge, our study is the first report on the up-regulation of $\alpha 7$ -nAChR by EE in ischemic stroke. However, the intermediate links between EE and $\alpha 7$ -nAChR remain largely unknown.

This study shows that EE increased ACh content and ChAT activity, and decreased AChE activity in the hippocampus of MCAO/R rats. ACh is a neurotransmitter and

are associated with cognitive ability²⁵, who is regulated by two enzymes: ACh is synthesized by ChAT and degraded by AChE²⁶. One report showed that acetylation of histones bound to the ChAT gene promoter and cholinergic circuits are enhanced by EE in PSCI mice, which supports our study. Increased AChE activity in the prefrontal cortex is associated with age-related cognitive decline²⁷, while decreased AChE activity and enhanced memory are found in physically enriched rats compared with rats reared in social environment²⁸. These suggest that EE increases hippocampal ACh contents by increasing ChAT and decreasing AChE activities, thereby improving cognitive function.

EE enhances LTP in MCAO/R rats in this study. LTP manifests persistent strengthening of synapses and subsequent enhanced signaling between two neurons. Suppressed hippocampal LTP can result in cognitive impairment²⁹. Hippocampal LTP is reduced in rats of CCH and reversed by EE³⁰. Enhanced LTP indicates higher synaptic plasticity, and might be associated with increased BDNF by EE³¹. Furthermore, there is inter-regulation between BDNF and $\alpha 7$ -nAChR. Choline could induce higher $\alpha 7$ -nAChR activity and then lead to increased BDNF level³². Conversely, BDNF up-regulated $\alpha 7$ -nAChR levels on subpopulations of hippocampal interneurons, which are important target cells that participate LTP^{33,34}. The regulatory role of $\alpha 7$ -nAChR in LTP was supported by our study, which is that LTP enhancement in hippocampal slices by EE was dependent on $\alpha 7$ -nAChR.

In summary, EE demonstrates a marked preventive effect against cognitive impairment of MCAO/R rats. The mechanism underlying EE may be associated with enhanced expression of $\alpha 7$ -nAChR, activation of CAP, and enhanced synaptic plasticity. The study reveals that EE may be a promising therapeutic method for PSCI patients, and provides potential possibility for the combination of EE with cholinergic activating agents.

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