An enriched environment reduces hippocampal inflammatory response and improves cognitive function in a mouse model of stroke

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Graphical Abstract  Enriched and isolated environments can regulate the TRAF6/NF-κBp65 signaling pathway in the hippocampus through miR-146a-5p and affect cognitive function in a stroke mouse model

Abstract

An enriched environment is used as a behavioral intervention therapy that applies sensory, motor, and social stimulation, and has been used in basic and clinical research of various neurological diseases. In this study, we established mouse models of photothrombotic stroke and, 24 hours later, raised them in a standard, enriched, or isolated environment for 4 weeks. Compared with the mice raised in a standard environment, the cognitive function of mice raised in an enriched environment was better and the pathological damage in the hippocampal CA1 region was remarkably alleviated. Furthermore, protein expression levels of tumor necrosis factor receptor-associated factor 6, nuclear factor κB p65, interleukin-6, and tumor necrosis factor-α, and the mRNA expression level of tumor necrosis factor receptor-associated factor 6 were greatly lower, while the expression level of miR-146a-5p was higher. Compared with the mice raised in a standard environment, changes in these indices in mice raised in an isolated environment were opposite to mice raised in an enriched environment. These findings suggest that different living environments affect the hippocampal inflammatory response and cognitive function in a mouse model of stroke. An enriched environment can improve cognitive function following stroke through up-regulation of miR-146a-5p expression and a reduction in the inflammatory response.

Key Words: cognitive function; enriched environment; isolated environment; miR-146a-5p; neuroinflammation; nuclear factor κB p65; photothrombotic model; stroke; tumor necrosis factor receptor-associated factor 6

Introduction

Stroke, the second leading cause of death in the world, is a common acute cerebrovascular disease (Xie et al., 2019), and approximately 80% of cases of stroke are ischemic (Faralli et al., 2013). Studies have found that approximately 50% of patients with stroke have varying degrees of cognitive dysfunction (Sachdev et al., 2006; Mijajlović et al., 2017; Zhang and Bi, 2020), which severely affects patients’ quality of life and survival time. In clinical practice, most patients with stroke do not like to talk to others, lack subjective initiative, and spend most of their time alone, all of which seriously affect the recovery of neurocognitive function. One study has shown that isolated housing can lead to poor recovery and microRNA (miRNA) imbalance, and decrease hippocampal cell proliferation in older female mice models of stroke (Holmes et al., 2020). Therefore, interventions related to the cognitive function of patients with stroke have become a research hot spot.

An enriched environment (EE) is a special environment that can enhance sports ability, cognition, perception, and social interaction (Will et al., 2004). Animal studies have shown that an EE not only improves learning, cognitive, and sports abilities in normal animals, but also improves the cognitive and sports abilities of rats with various central nervous system diseases (Hockly et al., 2002; Bezd et al., 2003; Restivo et al., 2005; Kovesdi et al., 2011). EE is currently used in the clinical rehabilitation of patients with stroke (White et al., 2015), but the specific mechanism underlying its positive effect remains unclear. Our previous study found that an EE can improve post-stroke cognitive impairment (PSCI) by regulating acetylation homeostasis in the cholinergic circuit in mouse models of stroke (Wang et al., 2016). The occurrence of PSCI is closely related to factors such as the inflammatory response (Narasimhalu et al., 2015). As an important immune-related molecule, miR-146a plays a key role in both innate immunity and neuroimmunity, and can inhibit the levels of inflammatory factors such as interleukin 6 (IL-6), IL-8, IL-1β, and tumor necrosis factor-α (TNF-α) by down-regulating tumor necrosis factor receptor-associated factor 6 (TRAF6) expression (Lukew et al., 2008; Aronica et al., 2010).

Therefore, in this study, photothrombotic stroke mouse models were rehabilitated in either EE or isolated environments. Due to the mutual

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interaction between miR-146a-5p and neuroinflammation, as well as their association with cognitive function, we examined the efficacy of an EE in improving cognitive function after stroke, as well as the mechanism underlying this effect, at the levels of miRNA and neuroinflammation.

Materials and Methods

Photothrombotic stroke models and interventions

The animal experiment was approved by the Animal Ethics Committee of Yangzhou University, China (approval No. 20170312001) in March 2017. We used 92 healthy, male, clean-grade C57BL/6J mice aged 2 months (provided by the Animal Experiment Center of Medical College of Yangzhou University, China; license No. SYXK (Su) 2017-0044). Among them, 70 mice were subjected to photothrombotic stroke. Briefly, after anesthesia (intraperitoneal injection of 1% sodium pentobarbital solution, 0.1 mL/10 g, Cat#: P-010, Sigma-Aldrich, St. Louis, MO, USA), Rose Bengal (1%, 1 mg/ml, Cat#: B7767, APEXBio, Houston, TX, USA) was injected intraperitoneally. The mouse was placed in the prone position, the tops of their heads were shaved, and the scalp was cut along the midline to fully expose the bregma (Watson et al., 1985; Labat-gest and Tomasi, 2013). The cold light source probe (Cat#: ULP-L20-5, Bete Jia, Suzhou, China) was placed approximately 2 mm below the left of the bregma. After 5 minutes of Rose Bengal injection, the cold light source was turned on and irradiation was continued for 15 minutes (Udendyks, 2018). Four mice died during the modeling process; the 66 surviving mice were divided into the stroke + standard environment (SE) group, stroke + EE group, and stroke + isolated environment (IE) group using a random number table method. Another 22 mice were selected as the sham operation group. In this sham group, 0.9% normal saline (0.1 mL/10 g) was injected intraperitoneally with the same amount of Rose Bengal without light, and the remaining steps were the same as for stroke model mice. Sham group mice (n = 22) were placed in an SE cage for 4 weeks. SE group mice (n = 22) were also placed in another SE cage for 4 weeks. EE group mice (n = 22) were placed in an EE cage for 4 weeks. IE group mice were individually caged for 4 weeks. The flow chart of this study design is shown in Figure 1.

Morris water maze

After 4 weeks of intervention, eight mice in each group were selected for inclusion in the Morris water maze experiment (Shanghai Keygen Biotechnology Co., Ltd., Shanghai, China). The mice were trained from day 1 to day 4 and tested on days 5 and 6. During training and testing, the water temperature was maintained at 20–22°C, and a platform was placed 2 cm below the water level in one of the four quadrants. During training and testing, each group of mice was put into the water from the four quadrant water inlet points. If the mice did not reach the platform within 60 seconds, they were guided to the platform and stayed there for 30 seconds. Additionally, the escape latency of each group was recorded. On day 6, the platform was removed from the pool, and mice were allowed to swim freely for 60 seconds. The video analysis system automatically recorded the number of times mice crossed the platform zone. After the daily tests, mice were returned to their respective environments.

Histological observation

Two days after completion of the Morris water maze test, six mice in each group were deeply anesthetized by intraperitoneal injection of 10% sodium pentobarbital, and brains were removed after cardiac perfusion. The fresh brain tissue was immediately placed on ice, and then dehydrated in 15%, 20%, and 30% sucrose solution until the specimen sunk to the bottom. The specimen was then removed from the solution, embedded with optimal cutting temperature embedding agent (Servicebio, Cat#: G0605), and placed in a freezing microtome (Dakeke Biotech, Shenzhen, China, Cat#: 3000A). When the brain block had frozen solid, it was fixed on a freezing microtome and sliced. Starting from the optic chiasm, a serial section of the coronal position was obtained, with a section thickness of 4 μm. When the hippocampus appeared, the coronal slice of the hippocampi at the same level were selected for hematoxylin-eosin and Nissl staining in each group.

Hematoxylin-eosin staining

The hippocampal sections were laid flat on a glass slide, fixed for 0.5–1 minute, stained with hematoxylin (Servicebio, Cat#: G1005) for 3–4 minutes, washed with water to remove the dye on the surface, and tap water was applied for 1 to 2 minutes, until the slides turned blue. The slices were dehydrated in 10% (lethal dose) sodium pentobarbital, and then placed in an ethanol series: 75%, 85%, 90%, 95%, and 100%. After dehydration, the slides were placed in xylene for 3 minutes, two grades of xylene for 3 minutes, and finally placed in an absolute ethanol series. The slices were then placed under an optical microscope (Olympus, Tokyo, Japan, Cat#: BX53) to observe and collect images.

Nissl staining

The hippocampal sections in the same location were also laid flat on another glass slide for Nissl staining. Nissl staining solution (Servicebio, Cat#: G1036, Servicebio) was dropped dropwise onto the glass slide, rinsed with distilled water, air-dried, dehydrated in an ethanol series, sealed with neutral gum (Servicebio, Cat#: WG10004160). The sealed slide was then placed under an optical microscope (Olympus, Tokyo, Japan, Cat#: BX53) to observe and collect images.

Construction of mouse cages in different environments

According to our previous research (Wang et al., 2016), the EE cage was improved to comprise a 1.2-m³ three-layer plexiglass cage. Each floor was connected by a plastic corridor. Inside the EE cage, there were three spiral stairs situations (each staircase entrance was equipped with rough carpet), three tunnels of different styles, two walking ways with pipes, two turntables, two swings, toy building blocks, a cabin, a bell sound, and color light stimulation. The EE group mice were held in researchers’ palms and vigorously brushed in order to add stress to the mice. The mice were given toys building blocks, a cabin, a bell sound, and color light stimulation. During training and testing, testing, each group of mice was put into the water from the four quadrant water inlet points. If the mice did not reach the platform within 60 seconds, they were guided to the platform and stayed there for 30 seconds. Additionally, the escape latency of each group was recorded. On day 6, the platform was removed from the pool, and mice were allowed to swim freely for 60 seconds. The video analysis system automatically recorded the number of times mice crossed the platform zone. After the daily tests, mice were returned to their respective environments.

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Nissl staining

The hippocampal sections in the same location were also laid flat on another glass slide for Nissl staining. Nissl staining solution (Servicebio, Cat#: G1038, Servicebio) was dropped dropwise onto the glass slide, rinsed with distilled water, air-dried, dehydrated in an ethanol series, sealed with neutral gum (Servicebio, Cat#: WG10004160). The sealed slide was then placed under an optical microscope (Olympus, Tokyo, Japan, Cat#: BX53) to observe and collect images.
the supernatant was collected. A bicinchoninic acid kit (Cat# K502CA, KeyGen Biotech) was used to detect protein concentration. The samples were heated at 100°C for 10 minutes. The protein samples (20 μL) were subjected to SDS-PAGE and then transferred to a polyvinylidene fluoride membrane (Cat#: 1620112, Bio-Rad, Shanghai, China). The polyvinylidene fluoride membrane was then transferred to nonfat milk-blocking solution for 1 hour at room temperature. The primary antibodies used were NF-κBp65 (1:1000, rabbit, Abcam, Cat#: ab32074, RRID: AB_2630538) and glyceraldehyde-3-phosphate dehydrogenase (1:10,000, rabbit, Abcam, Cat#: ab1801602, RRID: AB_2630358) and anti-histone H3 (1:1000, rabbit, Abcam, Cat#: ab215728, RRID: AB: 2118291) were used as a loading control. The membranes were incubated overnight with primary antibodies at 4°C. Membranes were then washed three times with Tris-buffered saline with Tween-20. The membrane was then incubated with the IgG secondary antibody (1:5000, rabbit, Abcam, Cat#: ab205718, RRID: AB_2191610) for 60 minutes at room temperature. After development, ImageJ software was used to measure the relative optical density of each band.

**Quantitative reverse transcription-polymerase chain reaction**

The left hippocampus tissues of six mice in each group were collected. An RNA extraction kit (Tiangen, Beijing, China, Cat#: DP503) was used to extract total RNA from the hippocampus, and the RNA concentration was determined using a microplate reader (Tecan, Shanghai, China). The concentration of each sample to be tested was diluted to 200 ng/μL.

**miRNA detection**

A 20 μL reaction solution was prepared on ice, comprising total RNA (8 μL), 2× miRNA RT reaction buffer (10 μL), and miRNA RT enzyme mix (2 μL). The reaction mixture was mixed thoroughly, and reverse transcription of miRNA was performed to obtain complementary deoxyribonucleic acid (cDNA). A real-time fluorescent quantitative reverse transcription-PCR (qPCR) plate was used to prepare 20 μL total reaction solution, as follows: 2× miRcute Plus miRNA Premix (with SYBR&ROX; 10 μL), forward primer (10 μM; 0.4 μL), reverse primer (10 μM; 0.4 μL), 50× ROX Reference Dye (2 μL), ddH₂O (5.2 μL), and cDNA (2 μL). The sample was then transferred to a fluorescence qPCR machine (Cat#: StepOnePlus 4376357, Thermo Fisher Scientific, Waltham, MA, USA), and the qPCR reaction was performed with the following cycling conditions: 95°C, 15 minutes for 1 cycle; 94°C, 20 seconds; 64°C, 15 seconds; 94°C, 30 seconds; 72°C, 34 seconds for 5 cycles; 94°C, 20 seconds; 64°C, 30 seconds; 72°C, 34 seconds for 45 cycles. All the reagents were from Tiangen.

**mRNA detection**

A 20 μL reaction solution was prepared on ice, comprising 5× FastKing-RT SuperMix (4 μL), RNase-Free ddH₂O (14 μL), and total RNA (2 μL). The sample was mixed well, and reverse transcription of mRNA was performed to obtain cDNA. Subsequently, a 20 μL system total reaction solution was prepared with a qPCR plate, as follows: 2× SuperReal Premix Mix (10 μL), forward primer (10 μM; 0.6 μL), reverse primer (10 μM; 0.6 μL), CDNA (2 μL), 50× ROX Reference Dye (2 μL), ddH₂O (4.2 μL). The amplification conditions were as follows: 95°C, 15 minutes for 1 cycle; 95°C, 10 seconds; and 63°C, 32 seconds for 40 cycles. All the reagents were from Tiangen.

**Primer sequence**

miR-146a-5p: forward: 5′-TGC GCT GAG AAC TGA ATT CCA T-3′; reverse: 5′-TGG TGT CGT GGA GTG G-3′; internal control U6: forward: 5′-CTC TGC TGT CGG GCA GCA G-3′; reverse: 5′-AAC GCT TCA CGT AGG TCT G-3′; TRAF6: forward: 5′-CGT CTT CTC GAA AGA AAG AAG A-3′; reverse: 5′-GAC AGA GAC GAC CCA GAG A-3′; glyceraldehyde-3-phosphate dehydrogenase: forward: 5′-TGA AGG GTG GAC CAA AAA G-3′; reverse: 5′-AGT CTT CTG GGT GGC AGT GAT-3′. The 2−ΔΔCt method (Schmittgen and Livak, 2008) was used to calculate the relative expression levels of miR-146a-5p and TRAF6 mRNA, where ΔΔCt = Ct_mir-146a−5p − Ct_TRAF6.
Effects of the enriched environment on the protein expression levels of TRAF6, NF-κBp65 (nucleus), TNF-α, and IL-6 in the hippocampus of a mouse model of stroke using Western blot assay.

(A) Bands of TRAF6, TNF-α, and IL-6 protein. (B) Bands of NF-κBp65 (nucleus) protein. (C–F) Quantitative results of TRAF6, TNF-α, IL-6, and NF-κBp65 (nucleus) protein expression. The expression of TRAF6, TNF-α, and IL-6 protein was normalized against GAPDH, and the expression of NF-κBp65 (nucleus) protein was normalized against Histone H3. Data are expressed as the mean ± SD (n = 6). *P < 0.05, vs. sham group; #P < 0.05, vs. SE group (one-way analysis of variance followed by least significant difference test). EE group: stroke + enriched environment group; IE group: stroke + isolated environment group; SE group: stroke + standard environment group.

Figure 2: The different cage environments. The figure shows enriched environment (A and B), standard environment (C and D), and isolated environment (E and F) cages.

Figure 3: Triphenyl tetrazolium chloride staining of the brain in control mice (A) and photothrombotic stroke model mice (B). The non-ischemic necrosis area was red, and the avascular necrosis area was white. The brain tissue of mice in the sham mice was normal with red color (A), while the left cortex and subcortex in mice with photothrombotic stroke were white (B) under triphenyl tetrazolium chloride staining, which suggests ischemic infarction.

Figure 4: The enriched environment improved learning and memory of a mouse model of stroke in the Morris water maze. (A) Average escape latency on days 1–5. (B) The number of platform zone crossings on day 6. Data are expressed as the mean ± SD (n = 8). *(P < 0.05, vs. sham group; #P < 0.05, vs. SE group; †P < 0.05, vs. EE group) (one-way analysis of variance followed by least significant difference test). EE group: stroke + enriched environment group; IE group: stroke + isolated environment group; SE group: stroke + standard environment group.

Figure 5: Effects of the enriched environment on the swimming trajectory of a mouse model of stroke on day 5 of the Morris water maze test. (A) Sham group. (B) SE group. (C) EE group. (D) IE group. After stroke, the swimming trajectory of the mice looking for the platform was extended. The enriched environment shortened the swimming trajectory of mice, and the isolated environment further extended the swimming trajectory of mice. The blue dot indicates the point at which mice were placed into the water; the red dot indicates the end of the swimming track; the big red circle indicates the edge of the pool; the small red circle indicates the platform. EE group: stroke + enriched environment group; IE group: stroke + isolated environment group; SE group: stroke + standard environment group.

Figure 6: Effects of the enriched environment on the histopathology in the hippocampal CA1 region of a mouse model of stroke. (A–D) Hematoxylin-eosin (A1, B1, C1, D1) and Nissl (A2, B2, C2, D2) staining in the hippocampal CA1 region in the sham (A), SE (B), EE (C), and IE (D) groups (original magnification 400×). Red arrows indicate nerve cells that were obviously irregular, with nuclear pyknosis and vacuoles; and green arrows indicate normal nerve cells. The morphology of hippocampal CA1 cells in the sham group was normal, hippocampal CA1 cells in the SE group were damaged, the morphology of CA1 cells in the EE group was improved compared to SE, and hippocampal CA1 cells in the IE group were more damaged compared to SE. (E) Number of Nissl bodies in the hippocampal CA1 region (per 400-fold field). Data are expressed as the mean ± SD (n = 6). *P < 0.05, vs. sham group; #P < 0.05, vs. SE group (one-way analysis of variance followed by least significant difference test). EE group: stroke + enriched environment group; IE group: stroke + isolated environment group; SE group: stroke + standard environment group.

Figure 7: Effects of the enriched environment on the protein expression levels of TRAF6, NF-κBp65 (nucleus), TNF-α, and IL-6 in the hippocampus of a mouse model of stroke using Western blot assay.

(A) Bands of TRAF6, TNF-α, and IL-6 protein. (B) Bands of NF-κBp65 (nucleus) protein. (C–F) Quantitative results of TRAF6, TNF-α, IL-6, and NF-κBp65 (nucleus) protein expression. The expression of TRAF6, TNF-α, and IL-6 protein was normalized against GAPDH, and the expression of NF-κBp65 (nucleus) protein was normalized against Histone H3. Data are expressed as the mean ± SD (n = 6). *P < 0.05, vs. sham group; #P < 0.05, vs. SE group (one-way analysis of variance followed by least significant difference test). EE group: stroke + enriched environment group; IE group: stroke + isolated environment group; SE group: stroke + standard environment group; TNF-α: tumor necrosis factor-α; TRAF6: tumor necrosis factor-associated factor 6.
The hippocampus, located in the temporal lobe of the brain, plays an important role in the formation of normal learning and memory. Previous studies have shown that the hippocampal microstructure of rats will be damaged to varying degrees after stroke (Morrison et al., 1990; Hinojosa et al., 2011; Stepete et al., 2013). Compared with age-matched healthy individuals, stroke survivors experience increased social isolation (Ebrahim et al., 1986). Studies have shown that EE treatment has a neuroprotective effect on animal models of cerebral ischemia (Li et al., 2016) and can improve the learning and memory dysfunction of mice after stroke by promoting plasticity of neuronal circuits (Nithianantharajah and Hannan, 2006) and hippocampal dentate gyrus neurogenesis (Brown et al., 2003). Alternatively, long-term social isolation can increase the risk of vascular and nervous system diseases (Friedler et al., 2015), accelerate the rate of memory decline in older adult people (Bassuk et al., 1999; Ertel et al., 2008), and reduce synaptic plasticity, hippocampal neurogenesis, and spatial cognition (Quan et al., 2010; Kamal et al., 2014; Almeida et al., 2020). In this study, the Morris water maze test was used to detect the effects of different living environments on learning and memory abilities of a mouse model of stroke. We found that in the SE, the post-stroke cognitive function of mice decreased. The EE significantly improved the learning and memory ability of a mouse model of stroke, while the IE aggravates the PSCI of mice.

The hippocampus, located in the temporal lobe of the brain, plays an important role in the formation of normal learning and memory. Previous studies have shown that the hippocampal microstructure of rats will be damaged to varying degrees after stroke (Morrison et al., 1990; Hinojosa et al., 2011; Stepete et al., 2013). To study the mechanism of the effect of an EE and IE on the behavior of a mouse model of stroke, and have a significant protective effect on stroke. We found that after EE intervention, the expression of TRAF6 and NF-κBp65 signaling pathway proteins in the hippocampus of the SE group was significantly higher than that in the sham group. The present study found that the expression of TRAF6 in the hippocampus of mice in the SE group was upregulated, nuclear NF-κBp65 was increased, and the expression of miR-146a-5p was significantly decreased compared with the controls. The expression of pro-inflammatory factors (IL-6 and TNF-α) was upregulated, which indicates that the TRAF6/NF-kBp65 signaling pathway is activated and the neuroinflammatory response is enhanced in the hippocampus of mice after stroke. These results are consistent with the above research conclusions.

Studies have shown that EE treatment can reverse hippocampal neuroinflammation (Jung and Johnson, 2012) and reduce cognitive dysfunction after stroke (Gonçalves et al., 2018). In contrast, previous work has reported glial cell activation and increased IL-1β, IL-6, and TNF-α in the prefrontal cortex and hippocampus of IE mice (Wang et al., 2018; Alshammarri et al., 2020). We found that EE intervention, the expression of pro-inflammatory factors (IL-6 and TNF-α) in the hippocampus of a mouse model of stroke was reduced, NF-κBp65 nuclear transport was reduced, and hippocampal neuroinflammation was reversed. The expression of TRAF6/NF-κBp65 signaling pathway proteins in the hippocampus of mice in the SE group was higher than that in the sham group. Moreover, studies have also reported that isolated feeding can cause cognitive impairment caused by diabetes (Kubota et al., 2018). Additionally, researchers such as Avenoso et al. (2021) found that during the hyaluronic acid 6-mer-induced inflammatory response, the upregulation of miR-146a reduced the inflammatory cascade of NF-κB signaling. The evidence revealed that the expression of miR-146a in the hippocampus of the SE group was higher than that in the sham group. We believe that, under normal circumstances, the mouse hippocampal NF-κB signaling pathway and the level of inflammatory factors such as TNF-α and IL-1β are in a relatively stable balance. In the SE group, the increased expression of miR-146a in the hippocampus of mice may be caused by activation of the NF-κB signaling pathway induced by stroke, following which the increase in miR-146a negatively regulates the NF-κB signaling pathway through the targeting effect of TRAF6, which controls neuroinflammation of the hippocampus to a certain extent. Taken together, this process may function as a negative feedback regulation mechanism of the body's anti-inflammatory response. However, the increase in miR-146a caused by stroke is insufficient to prevent the cascade effect of neuroinflammation during stroke. Considering that the role of miRNAs in the regulation of neuroinflammation is complex and dynamic, both pro- and anti-inflammatory miRNAs often activate in parallel (Gaudet et al., 2014), the existence of pro-inflammatory miRNAs that promote neuroinflammation remains a possibility.

Interestingly, studies have found that miRNAs can regulate many aspects of social interaction (Bahi, 2016, 2017; Fregace et al., 2016). A social environment can also directly affect the expression of miRNAs and, in turn, triggers the production of downstream genes. Studies have shown that isolated feeding can cause cognitive impairment caused by diabetes (Kubota et al., 2018). Moreover, miR-146a is associated with the neuroinflammatory response (Aronica et al., 2010) and can prevent the decline in cognitive function caused by surgical trauma by inhibiting hippocampal neuroinflammation in a mouse model of postoperative cognitive dysfunction (Chen et al., 2019). TRAF6 is a key signaling molecule in the NF-κB signaling pathway. Through a database comparison analysis, we found that TRAF6 was a target gene of miR-146a-5p according to the principle of base complementary pairing (A=T, C=G).
that case, the low level of miR-146a would be unable to inhibit the NF-κB signaling pathway and the cascade amplification effect of inflammation in the hippocampus of mice after stroke by targeting TRAF6, thus aggravating the hippocampal neuroinflammation in mice after stroke.

In summary, EE, a behavioral therapy, has been used in the rehabilitation of various nervous system diseases after years of continuous practice and improvement. In this study, we investigated the effects of an EE and an IE on the cognitive function and hippocampal cell morphology of a mouse model of stroke, and explored the underlying molecular mechanisms of these effects. Our data showed that neuroinflammation occurred in the hippocampus of mice after stroke, the TRAF6/NF-κB signaling pathway was activated, miR-146a-5p expression was up-regulated, and cognitive function was decreased. An IE aggravated hippocampal neuroinflammatory response, nerve damage and functional impairment in a mouse model of stroke, which may have been caused by a low level of miR-146a-5p in the hippocampus. However, the EE may increase the expression of miR-146a-5p in the hippocampus of a mouse model of stroke, inhibit TRAF6/NF-κB signaling, reduce the neuroinflammatory response in the hippocampus, alleviate the pathological state of hippocampal damaged nerve cells, and improve cognitive impairment of mice. In conclusion, we found that different living environments can affect hippocampal neuroinflammation and cognitive function in a mouse model of stroke by regulating miR-146a-5p. Thus, miR-146a-5p may represent a potential target for treating PSCI. These findings further explore the mechanism underlying EE-induced improvements in PSCI and could help to develop more effective rehabilitation methods.

In future research, we will extend the intervention cycle of a mouse model of stroke, pay more attention to the effects of the EE at different time points, and study the dynamic changes of various observation indicators. We would also like to explore the relationship between peripheral inflammation and neuroinflammation in a mouse model of stroke; lentiviral miR-146a (agonist) induced the up-regulation of miR-146a into the hippocampus of mice to further verify the role of this EE-regulated signaling pathway. In terms of clinical research, we hope to design more complete and enriched rehabilitation programs based on EE elements to better reflect the important role of environmental intervention in clinical practice.

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Availability of data and materials: All data generated or analyzed during this study are included in this published article and its supplementary information files.

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References
Almeida FB, Nin MS, Barros HMT (2020) The role of allopregnanolone in depressive-like behaviors: Focus on neurotrophic proteins. Neurobiol Stress 12:100218.
Alshammari TK, Alghamdi H, Alkhader LF, Alqattan O, Alrashef NM, Yacoub H, Aimea N, AlNakhi M, Alshammar MA (2020) Analysis of the molecular and behavioral effects of acute social isolation on rats. Behav Brain Res 377:112191.
Aronica E, Fluitet K, Iyer A, Zurolo E, Vreijling J, Baayen JC, Gorter JA (2010) Expression pattern of miR-146a, an inflammation-associated microRNA, in experimental and human temporal lobe epilepsy. Eur J Neurosci 31:1100-1107.
Avenoso A, D’Ascola A, Scurchi M, Mandrapprof G, Campo S, Campo GM (2021) miR146a up-regulation is involved in small HA oligosaccharides-induced pro-inflammatory response in human chondrocytes. Biochim Biophys Acta Gen Subj 1865:129731.
Bah A (2016) Sustained lentiviral-mediated overexpression of microRNA124a in the dentate gyrus exacerbates anxiety- and autism-like behaviors associated with neonatal isolation in rats. Behav Brain Res 311:298-308.
Bah A (2017) Hippocampal BDNF overexpression or microR124a silencing reduces anxiety- and autism-like behaviors in rats. Behav Brain Res 326:281-290.
Barrientos RM, Hein AM, Frank MG, Watkins LR, Maier SF (2012) Intracranial interleukin-1 receptor antagonist prevents postoperative cognitive decline and neuroinflammatory response in aged rats. J Neurosci 32:14641-14648.
Bassuk SS, Glass TA, Berkman LF (1999) Social disengagement and incident cognitive decline in community-dwelling elderly persons. Ann Intern Med 131:165-173.
Betto LEB, Rajendran L, Gil-Mohapel J (2017) The effects of aging in the hippocampus and cognitive decline. Neurosci Biobehav Rev 79:66-86.
Bezard E, Doverso S, Belin D, Ducqcon S, Jackson-Lewis V, Przudborski S, Piazza P, Giv CE, Jaber M (2003) Enriched environment confers resistance to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and cocaine: involvement of dopaminergic transporters and trophic factors. J Neurosci 23:10999-11007.
Bhaluk D, Scott GK, Schokrur SP, Patil CK, Campisi J, Benz CC (2008) Expression of microRNA-146a suppresses NF-kappaB activity with reduction of metastatic potential in breast cancer cells. Oncogene 27:5643-5647.
Brenes JC, Lackminger M, Höglinger GU, Sacht G, Schwartz K, Wühr M (2016) Differential effects of social and physical environmental enrichment on brain plasticity, cognition, and ultrasonic communication in rats. J Comp Neurol 524:1586-1607.
Brown J, Cooper-Kuhn CM, Kempermann G, Van Praag H, Winkler J, Gage FH, Kuhn HG (2003) Enriched environment and physical activity stimulate hippocampal but not olfactory bulb neurogenesis. Eur J Neurosci 17:2042-2046.
Chen L, Dong R, Lu Z, Zhou Y, Li K, Zhang Z, Peng M (2019) MicroRNA-miR-146a protects against cognitive decline induced by surgical trauma by suppressing hippocampal neuroinflammation in mice. Brain Behav Immun 78:188-201.
Du J, Li M, Huang Q, Liu W, Li WQ, Li YJ, Gong ZC (2019) The critical role of microRNAs in stress response: Therapeutic prospect and limitation. Pharmacol Res 142:294-302.
Ebrahim S, Barer D, Nouri F (1986) Use of the Nottingham Health Profile with patients after a stroke. J Epidemiol Community Health 40:166-169.
Ertel KA, Glymour MM, Berkman LF (2008) Effects of social integration on preserving memory function in a nationally representative US elderly population. Am J Public Health 98:1215-1220.
Fan W, Liang C, Ou M, Zou T, Sun F, Zhou H, Cui L (2020) MicroRNA-146a is a widely-reaching neuroinflammatory regulator and potential treatment target in neurological diseases. Front Mol Neurosci 13:90.
Faralli A, Bigoni M, Mauro A, Rossi E, Carulli D (2013) Noninvasive strategies to promote functional recovery after stroke. Neural Plast 2013:854597.
Ferrante M, Conti GO (2017) Environment and neurodegenerative diseases: an update on miRNA role. Microrna 6:157-165.
Flannery S, Bowie AG (2010) The interleukin-1 receptor-associated kinases: critical regulators of innate immune signalling. Biochem Pharmacol 80:1981-1991.
Fregace J, Colleaux L, Nguyen LS (2016) The emerging roles of microRNAs in autism spectrum disorders. Neurosci Biobehav Rev 71:729-738.
Friedlér B, Caperer J, McCullough L (2015) One is the deadliest number: the detrimental effects of social isolation on cerebrovascular diseases and cognition. Acta Neuropathol 129:493-509.
Gaudet AD, Fonken LK, Watkins LR, Nelson RJ, Popovich PG (2018) MicroRNAs: roles in regulating neuroinflammation. Neuroscientist 24:221-245.
Geng W, Tang H, Luo S, Li Y, Li J, Kang X, Hong W (2019) Exosomes from miRNA-126-modified ADSCs promotes functional recovery after stroke in rats by improving neurogenesis and suppressing microglia activation. Am J Trans Res 11:780-792.
Gonçalves U, Heringer AL, Ferreira TAA, Cotohino JB, Pires RGW, Martins-Silva C (2018) Environmental enrichment cognitive neuroprotection in an experimental model of cerebral ischemia: biochemical and molecular aspects. Behav Brain Res 348:171-183.
Hinojosa R, Hauj J, Hinojosa MS, Rittman M (2011) Social isolation poststroke: relationship between race/ethnicity, depression, and functional independence. Top Stroke Rehabil 18:79-86.
Hockly E, Cordero PM, Woodman B, Mahal A, van Dellen A, Blakemore C, Lewis CM, Hannan AJ, Bates GP (2002) Environmental enrichment slows disease progression in R6/2 Huntington’s disease mice. Ann Neurol 51:235-242.
Holmes A, Xu Y, Lee J, Maniskas ME, Zhu L, McCullough LD, Venra VR (2020) Post-stroke social isolation reduces cell proliferation in the dentate gyrus and alters miRNA profiles in the aged female mouse brain. Int J Mol Sci 22:99.
Hu J, Li C, Hua Y, Liu P, Gao B, Wang Y, Bai Y (2020) Constraint-induced movement therapy improves functional recovery after ischemic stroke and its impacts on synaptic plasticity in sensorimotor cortex and hippocampus. Brain Res Bull 160:8-23.
Jurgens HA, Johnson RW (2012) Environmental enrichment attenuates hippocampal neuroinflammation and improves cognitive function during influenza infection. Brain Behav Immun 26:1006-1016.
Kamal A, Ramakers GM, Attemsbliek B, Kas MJ (2014) Social isolation stress reduces hippocampal long-term potentiation: effect of animal strain and involvement of glucocorticoid receptors. Neuroscience 256:262-270.

Kim H, See JS, Lee SY, Ha KT, Choi BT, Shin YJ, Yu Yun Y, Shin HK (2020) AIM2 inflammasomes contributes to brain injury and chronic post-stroke cognitive impairment in mice. Brain Behav Immun 87:765-776.

Kovesdi E, Goygory AB, Kwon SK, Wingo DL, Kamaknash A, Long JH, Kasper CE, Agoston DV (2011) The effect of enriched environment on the outcome of traumatic brain injury; a behavioral, proteomics, and histological study. Front Neurosci 5:42.

Kubota K, Nakano M, Kobayashi E, Mizuse Y, Chikenui T, Otani M, Nagashii K, Fuyimia M (2018) An enriched environment prevents diabetes-induced cognitive impairment in rats by enhancing exosomal miR-146a secretion from endogenous bone marrow-derived mesenchymal stem cells. PLoS One 13:e0204252.

Labat-gest V, Tombai S (2013) Photothrombotic ischemia: a minimally invasive and reproducible photochemical cortical lesion model for mouse stroke studies. J Vis Exp 50370.

Lee HM, Kim TS, Jo EK (2016) MIR-146 and miR-125 in the regulation of innate immunity and inflammation. BMB Rep 49:311-318.

Leonard M, Avgier M, Katsarou M, Droukalis N, Sloutzani A, Ventenziot A, Alexoudi A, Frytraki A, Petkelis P, Vassilopoulos D, Gatzonis S, Sideris DC (2020) Circulating mir-146A and mir-134 in predicting drug-resistant epilepsy in patients with focal impaired awareness seizures. Epilepsia 61:959-970.

Li YW, Li QY, Wang JH, Xu XL (2016) Contribution of p38 MAPK to the ameliorating effect of enriched environment on the cognitive deficits induced by chronic cerebral hyperfusion. Cell Physiol Biochem 40:549-557.

Lu Y, Cao DL, Jiang Y, Yang T, Gao YJ (2015) MicroRNA-146a-5p attenuates neuropathic pain via suppressing TRAF6 signaling in the spinal cord. Brain Behav Immun 49:119-129.

Lukwi WJ, Zhao Y, Cui JG (2008) An NF-kappaB-sensitive micro RNA-146a-mediated inflammatory circuit in Alzheimer disease and in stressed human brain cells. J Biol Chem 283:31151-31122.

Marevsa P, Tomzone S, Lameski P, Marduriva E, Chorbev I, Trajkovik V, Ellens M, Rodile K (2018) Technological solutions for older people with Alzheimer’s disease: review. Curr Alzheimer Res 15:975-983.

Mens MM, Ghanbari M (2018) Cell cycle regulation of stem cells by microRNAs. Stem Cell Rev Rep 14:309-322.

Majlovic MD, Pavlovic A, Brannin M, Heiss WD, Quinji TJ, Ihle-Hansen HB, Hermann DM, Assayag EB, Richard E, Thiel A, Kliper E, Shin YJ, Kim YH, Choi S, Jung S, Lee YB, Sinanovic O, Levine DA, Schlesinger I, Mead G, et al. (2017) Post-stroke dementia: a comprehensive review. BMC Med 15:11.

Morris PL, Robinson RG, Raphael B (1990) Prevalence and course of depressive disorders. J Clin Epidemiol 43:1097-1108.

Maresova P, Tomsone S, Lameski P, Madureira J, Mendes A, Zdravevski E, Chorbev I, Lukiw WJ, Zhao Y, Cui JG (2008) An NF-kappaB-sensitive micro RNA-146a-mediated inflammatory circuit in Alzheimer disease and in stressed human brain cells. J Biol Chem 283:31151-31122.

Peltonen M, Rauramaa R, Stigsdotter-Neely A, Strandberg T, Tuomilehto J, Soininen H, et al. (2015) A 2 year multidomain intervention of diet, exercise, cognitive training, and vascular risk monitoring versus control to prevent cognitive decline in at-risk elderly people (FINGER): a randomised controlled trial. Lancet 385:2255-2263.

Quan MN, Tian YT, Xu KH, Zhang T, Yang Z (2010) Post weaning social isolation influences spatial cognition, prefrontal cortical synaptic plasticity and hippocampal potassium ion channels in Wistar rats. Neuroscience 169:214-222.

Restivo L, Ferrari F, Passino E, Sbigio C, Bock J, Oostra BA, Bagni C, Ammassari-Teule M (2005) Enriched environment promotes behavioral and morphological recovery in a mouse model for the fragile X syndrome. Proc Natl Acad Sci U S A 102:11557-11562.

Sachdev PS, Brodaty H, Valenzuela MJ, Lorentz E, Looi JC, Bernard K, Ross A, Wen W, Zagami AS (2006) Clinical determinants of dementia and mild cognitive impairment following ischaemic stroke: the Sydney Stroke Study. Dement Geriatr Cogn Disord 21:275-283.

Schmittdiel JD, Livak KJ (2008) Analyzing real-time qPCR data by the comparative C(T) method. Nat Protoc 3:1101-1108.

Steptoe A, Shankar A, Demakakos P, Wardle J (2013) Social isolation, loneliness, and all-cause mortality in older men and women. Proc Natl Acad Sci U S A 110:5797-5801.

Sun JH, Tan L, Yu JT (2014) Post-stroke cognitive impairment: epidemiology, mechanisms and management. Ann Trans Med 2:80.

Sun L, Dong R, Xu X, Yang J, Peng M (2017) Activation of cannabinoid receptor type 2 attenuates surgery-induced cognitive impairment in mice through anti-inflammatory activity. J Neuroinflammation 14:138.

Sventek JL, Tsen ME, Cobb MH, Thomas JA (2000) IL-1 receptor-associated kinase modules host responsiveness to endotoxin. J Immunol 164:4301-4306.

Taganov KD, Boldin MP, Chang KJ, Balthore PM (2006) NF-kappaB-dependent induction of microRNA-miR-146; an inhibitor targeted to signaling proteins of innate immune response. Proc Natl Acad Sci U S A 103:12481-12486.

Teo WP, Muthalib M, Yamin S, Hendy AM, Bramstedt K, Kotsopoulos E, Perrey S, Ayaz H (2016) Does a combination of virtual reality, neuromodulation and neuroimaging provide a comprehensive platform for neurorehabilitation? - A narrative review of the literature. Front Hum Neurosci 10:284.

Udzensky AB (2018) Photorefractive stroke as a model of ischemic stroke. Transl Stroke Res 9:437-451.

Wang L, Cao M, Pu T, Huang H, Marshall C, Xiao M (2018) Enriched physical environment attenuates spatial and social memory impairments of aged socially isolated mice. Int J Neuropsychopharmacol 21:1114-1127.

Wang X, Chen A, Wu H, Ye M, Cheng H, Jiang X, Wang X, Zhang X, Wu D, Gu X, Shen F, Shan C, Yu D (2016) Enriched environment improves post-stroke cognitive impairment in mice by potential regulation of acetylation homeostasis in cholinergic circuits. Brain Res 1650:232-242.

Wang Z, Yuan Y, Zhang Z, Ding K (2019) Inhibition of miRNA-27b enhances neurogenesis via AMPK activation in a mouse ischemic stroke model. FEBs Open Bio 9:859-869.

Watson BD, Dietrich WD, Busto R, Wachtel MS, Ginsberg MD (1985) Induction of TNF-α, IL-6, and IL-1β. Neurobiol Aging 33:1364-1378.

White JH, Bartley E, Janssen H, Jordan LA, Spratt N (2015) Exploring stroke survivor awareness seizures. Epilepsia 61:959-970.

Will B, Galani R, Keilce C, Rosenzweig MR (2004) Recovery from brain injury in animals: mechanisms of actions, and circulation. Front Endocrinol (Lausanne) 9:402.

Wu X, Lu Y, Dong Y, Zhang G, Zhang Y, Xu Z, Culley DJ, Crosby G, Marcantonio ER, Tanzi RE, Shukitt-Hale B, et al. (2015) A 2 year multidomain intervention of diet, exercise, cognitive training, and vascular risk monitoring versus control to prevent cognitive decline in at-risk elderly people (FINGER): a randomised controlled trial. Lancet 385:2255-2263.

Xie Z (2012) The inhalation anesthetic isoflurane increases levels of proinflammatory cytokines and oxidative stress by activating Nrf2-ARE pathway. Int J Neurosci 131:641-649.