Early exposure to enriched environment protects apoptosis and improves behavioral improvement by downregulating Fas/FasL signaling pathway after hypoxic-ischemic brain injury

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Abstract

Early rehabilitation presents favorable outcomes for stroke patients. However, the optimal strategy for early rehabilitation is unclear currently due to the current limitation in the data on the effects of early initiation of rehabilitation based on voluntary exercise. During the hyperacute phase of ischemic stroke, upregulation of Fas/FasL-mediated apoptosis is observed. Environmental enrichment (EE) is a therapeutic paradigm for laboratory animals that consists of complex combinations of physical, cognitive, social stimuli and voluntary exercise. Few studies delineated the effect of EE on apoptosis in an experimental model of hyperacute stroke. The aim of the study is to determine whether hyperacute exposure to EE can effectively regulate Fas/FasL-mediated apoptosis following hypoxic-ischemic brain injury and improve neurobehavioral function. C57Bl/6 mice were randomly assigned to either EE or standard cage (SC) within 3 hours or on day 3 after hypoxic-ischemic brain injury for 2 weeks. Neurobehavioral tests, transcriptome analysis, Western Blot, and immunohistochemistry were performed in the brain samples of cerebral cortex and hippocampus, and total infarct volume was calculated. Compared with SC, hyperacute exposure to EE was associated with greater improvement in anxiety, motor function, cognitive ability, reduced total infarct volume, and decreased neuronal death. It significantly downregulated Fas/FasL-mediated apoptosis, decreased expression of Fas, FADD, cleaved caspase-8/caspase-8, cleaved caspase-3/caspase-3, as well as Bax/Bcl-2 in both regions. Overall, the results of this study demonstrates that the early exposure to EE is a neuroprotective therapeutic translation for stroke rehabilitation through effective inhibition of extrinsic as well as intrinsic apoptotic pathways.

Introduction

Stroke is a leading cause of serious long-term disabilities, especially in more than 80% of all cases of ischemic attacks [1–3]. Ischemic stroke results in a sudden neurologic deficit, including impaired motor response, cognitive ability, communication and mood, directly reducing patients' quality of life with a heavy burden on family and community[4, 5]. Therefore, effective strategies for functional recovery of patients with stroke are imperative.

Acute brain ischemia triggers an "ischemic cascade" of pathophysiological events such as energy failure, excitotoxicity, oxidative stress, inflammation, and apoptosis, resulting in neuronal cell death. Ample evidence suggests the critical role of apoptosis in the pathophysiology of acute brain ischemia, resulting in a significant loss of brain cells [4, 6]. The ischemic core of the brain experiences a sudden reduction of blood flow, just minutes after ischemic attack with irreversible injury and subsequent cell death. However, apoptosis within the ischemic penumbra may occur after several hours or days, which may be reversible [6, 7]. Early animal and human studies revealed different apoptotic mechanisms during acute brain ischemia, as well as the efficacy of anti-apoptotic agents and inhibitors of apoptosis ameliorating brain tissue injury and reducing morbidity [8–13]. Therefore, the inhibition of apoptosis may be a promising neuroprotective rehabilitation strategy, suggesting the need to elucidate the anti-apoptotic mechanisms involved.

Ischemic stroke triggers two main pathways of apoptosis. The intrinsic pathway is initiated by the disruption of mitochondria and the release of cytochrome C, which is mediated by members of the Bcl-2 family such as antiapoptotic protein B cell lymphoma/leukemia-2 (Bcl-2) and proapoptotic protein Bcl-2-associated X protein (Bax) [7]. The extrinsic pathway is triggered by signaling cell death receptors in the plasma membrane including tumor necrosis factor (TNF)-receptor 1, apoptosis antigen-1 (APO1/Fas/CD95), and TNF-related apoptosis-inducing ligand receptor (TRAIL-R) [7, 14]

Fas is a cell surface death receptor belonging to the TNF receptor superfamily. Fas ligand (FasL) binds to the Fas receptor, which triggers recruitment of the cytoplasmic adaptor protein Fas-associated death domain protein (FADD), and initiates apoptosis [15]. The “death effector domain” at the N terminus of FADD binds to procaspase-8 [16]. This complex (FasL–Fas–FADD–procaspase-8), also referred to as death-inducing signaling complex (DISC), is assembled within seconds of Fas receptor engagement. The DISC catalyzes the proteolytic cleavage and transactivation of procaspase-8 to generate caspase-8 [16]. The activated caspase-8 is released from the DISC complex into the cytoplasm to initiate the downstream cleavage of caspase-3, which leads to the execution phase of apoptosis, resulting in nDNA damage and apoptosis [17].

The incidence of ischemic stroke is highly associated with the expression of Fas, FADD and caspase-8, independent of several potential confounding factors [18]. Furthermore, the Fas signaling pathway has been suggested as a critical inducer of apoptotic signals in acute ischemia [19–21]. The first day of ischemic stroke onset is marked by significantly higher levels of serum and cerebrospinal fluid sFas/APO1 in patients with stroke than in control subjects, followed by a gradual decline [20]. Analysis of post-mortem tissues derived from ischemic stroke victims revealed the highest levels of active caspase-8 on day 1 of onset, followed by a slow decrease with time [22]. Some early studies showed that therapeutic neutralization of FasL or Fas or under-expression of FADD or caspase-8 reduced the levels of stroke-related damage and neurologic deficit in rodent models of focal cerebral ischemia [23–25]. The extrinsic pathway of apoptosis also represents a potent therapeutic target in acute traumatic brain injury, acute spinal cord injury (SCI), seizure, acute organ injury or parasite infection [26–33]. Thus, it is reasonable to hypothesize that rehabilitation targeting downregulation of Fas/FasL-mediated apoptosis in early phases of ischemic stroke leads to neuroprotection and functional recovery.

The optimal time to initiate rehabilitation after stroke has yet to be established, although evidence increasingly suggests the benefit of early, organized, and interprofessional stroke rehabilitation within the first 2 weeks of stroke [34]. However, increasing evidence suggests that commencing high-dose, very early mobilization within 24 hours of stroke onset may adversely affect patient outcomes [35–37]. Most clinical and preclinical studies investigating exercise-induced effects after stroke utilized the forced exercise (FE) paradigm. The results of A Very Early Rehabilitation Trial after stroke (AVERT) trial demonstrated that high-dose and forced mobilization protocol within 24 hours of stroke onset was less favorable than the usual care [38]. In experimental animal stroke models, FE in hyperacute phase exacerbated brain damage, increased apoptotic cell death, and delayed functional recovery [39–43]. However, the effects of hyperacute exposure to environmental enrichment (EE), including voluntary

exercise (VE), on neuroprotection and functional recovery have yet to be fully investigated. In contrast to previous studies, the current study focuses on the effects of exposure to EE on the inhibition of apoptosis during hyperacute phase of ischemic stroke [44].

EE is an invasive approach based on voluntary physical activity and non-stressful conditioning through provision of a larger space with various objects such as toys, tunnels, and running wheels, allowing greater social interaction and stimulating rodent exploratory behavior [45–47]. While most of the studies investigating the potential mechanisms underlying the effects of EE focused on neurogenesis, synaptogenesis or angiogenesis, the importance of neuronal survival has yet to be fully elucidated [48–50]. Limited studies delineated the mediating effect of EE on extrinsic apoptosis in an experimental stroke model.

The study sought to determine the neuroprotective effects of early exposure to EE following hypoxic-ischemic brain injury and compare the mechanisms associated with delayed exposure to EE in adult mice. We focused on the effects of altered expression of apoptosis-related genes on neuronal survival and functional recovery and explored novel neuroprotective rehabilitation strategies after stroke.

Materials And Methods

Construction of an adult hypoxic–ischemic (HI) brain injury model

Ischemic brain damage was induced in 6-week-old C57BL/6 mice via unilateral right carotid artery ligation under anesthesia with a mixture of Ketamine and Rompun. Hypoxic brain injury (8% O_2 for 30 minutes) was also generated as previously described [51]. Visual inspection of the wide-field microscopic images was used to assess the severity of brain injury at the end of the study.

Experimental procedures and cage condition

To establish a hyperacute EE model, a total of 70 C57BL/6 mice were randomly housed in standard cages (SC) or very early EE (n = 35 per group) within 3 hours of exposure to hypoxic-ischemic brain injury for 2 weeks (Fig. 1a). To develop a delayed EE model, a total of 40 C57Bl/6 mice were randomly assigned to either delayed EE or SC (n = 20 per group), 3 days after exposure to HI brain injury for 2 weeks (Fig. 1b).

The EE mice were housed in a large cage (86 × 76 × 31 cm^3) containing novel objects, such as tunnels, shelters, toys, and running wheels for VE and social interaction (12–15 mice/cage), whereas the control mice were housed in SCs (27 × 22.5 × 14 cm^3) without social interaction (4–5 mice/cage). The brain regions were dissected based on the gross anatomy of the mouse brain atlas, and the stereotaxic coordinates of the cerebral cortex and hippocampus were (ML = − 1.0, AP = 0.1, DV = 1.0) and (ML = − 1.0, AP = − 2.0, DV = 2.0), respectively. All experiments were performed with C57BL/6 mice housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and provided with food and water ad libitum under alternating 12-h light/dark cycles, according to animal

protection regulations. The experimental procedure was approved by the Institutional Animal Care and Use Committee (IACUC) of Yonsei University Health System (permit number:2021 – 0182).

Behavioral assessments

The cylinder test, open field test, ladder walking, and Y-maze tests were performed before surgery and 14 days after exposure to EE or SC conditions (Fig. 1c-f). The cylinder test is designed to assess anxiety in the animal disease model (Fig. 1c) [47]. Mice placed in a transplant plexiglass cylinder measuring 8 cm in diameter and 18 cm in height stood spontaneously and used their forepaws for support. In this test, the number of mouse forelimbs touching the wall of the cylinder (Jeung Do B&P, Seoul, Korea) was counted in the standing position over a period of 5 min. Open field test is generally used to evaluate locomotor activity and anxiety in a novel environment (Fig. 1d) [52]. Activity was monitored in an area measuring 30×30.5×31 cm^3. Mice were placed individually in the periphery of the area and explored freely for 25 minutes, while being monitored with a video camera. The total distance traveled by each mouse was recorded for 25 min as an index of hyperactivity [53]. The resulting data were analyzed using the Smart Vision 2.5.21 (Panlab, Barcelona, Spain) video tracking system. The ladder rung walking task can be used to distinguish subtle disturbances in motor function based on qualitative and quantitative analyses of skilled walking (Fig. 1e) [54]. During the test, the mice were required to walk three times for one meter on a horizontal ladder equipped with metal rungs (Jeung Do B&P) located at various distances. The number of slips from the transverse rungs with each forelimb were measured with a videotape. The control and EE groups were compared by measuring the difference in the percentage of slips on the transverse rungs of the ladder relative to the total number of steps taken by each forelimb. Y-maze test is used to evaluate cognition and short-term spatial memory (Fig. 1f) [55]. This test was carried out in an enclosed Y-shaped maze (Jeung Do B&P). Normal mice tend to visit the arms of the maze one after the other. This behavior is called spontaneous alternation and used to assess short-term spatial memory in a new environment. The number of each arm entries, spontaneous alterations, and percent alterations were recorded for 8 min. The percent alteration was calculated as follows: [Number of spontaneous alterations / (Number of total arm entries − 2)] × 100. At the end of each trial, the urine and feces on the maze were cleaned with 70% ethanol.

Transcriptome analysis

Total RNA was prepared from cerebral cortex and hippocampus using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturers' protocols. For quality control, RNA quality and quantity were evaluated via 1% agarose gel electrophoresis and the ratio of absorbance at 260 and 280 nm was determined with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

RNA sequencing was performed by Macrogen Inc (Seoul, Korea). The mRNA was transcribed into a library of templates. The successive cluster generation using the reagents was achieved using the Illumina® TruSeq™ RNA Sample Preparation Kit [56]. We performed the transcriptome analysis via RNA-seq and data handling procedures. The detailed RNA-seq protocol was performed according to the manufacturer's

instructions. First, the TruSeq mRNA library construction was accomplished in 8 steps: mRNA purification and fragmentation, synthesis of first-strand cDNA, synthesis of second-strand cDNA, end repair, single 3' adenylation, ligation of adapters, enrichment of DNA fragments, followed by validation of enriched library.

Gene ontology (GO) is widely used to describe protein function in a standardized format. GO analysis of the identified proteins was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) software (http://david.abcc.ncifcrf.gov/). Functional annotation clustering was used to identify enriched GO terms for biological process, molecular function and cellular components to obtain an overview of the main biological processes involving these proteins. In addition, we also performed pathway enrichment analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway.

Western blotting

For each sample, 50 μg of total protein and a pre-stained protein-weight marker (Bio-Rad) were separated on a 10% SDS-PAGE gel and transferred onto a PVDF membrane (0.45 μm, Millipore) in Tris-glycine buffer with 20% (vol/vol) methanol. The membrane was blocked in 5% nonfat milk powder prepared in Tris-buffered saline containing 0.1% Tween 20 for 2 hours at room temperature, followed by incubation with primary antibodies overnight at 4°C. Anti-FAS (Abcam, Cambridge, UK), anti-FADD (Santa Cruz, Dallas, TX, USA), anti-caspase-8 (Cell Signaling Technology (CST), Danvers, MA, USA), anti-cleaved caspase-8 (CST, Danvers, MA, USA), anti-caspase-3 (CST, Danvers, MA, USA), and anti-cleaved caspase-3 (CST, Danvers, MA, USA) were the primary antibodies used at a 1:1,000 dilution. After several washes with Tris-buffered saline containing 0.1% Tween 20, the membranes were incubated with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (Abcam, Cambridge, UK) diluted 1:3,000 for 2 hours. The signals were detected by chemiluminescence with Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA). The same membrane was incubated with the β-actin antibody as an internal control.

Measurement of infarction volume

The animals were sacrificed and perfused with 4% paraformaldehyde. The harvested brain tissues were cryo-sectioned at 16-μm thickness along the coronal plane and stained with hematoxylin-eosin (H&E). H&E staining was performed with four sections from the frontal pole to the midbrain. A section from each of the segments above was stained with H&E to measure the infarct volume. The sections were photographed using a digital camera and analyzed using ImageJ program. The infarct volumes of the lesion were expressed as a percentage of the volume of the control hemisphere structures using the formula [(VC-VL)/VC] ×100%, where VC is the volume of control hemisphere and VL denotes the volume of non-infarcted tissue in the lesion hemisphere. The total infarct volume of each brain was calculated as the sum of the infarct volumes of the four brain slides.

Immunohistochemistry

The 16-μm sections were cut along the coronal and sagittal plane, and immunohistochemical staining of four sections was performed over a range of > 128 μm. Fluorometric terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay (Promega, Madison, WI, USA) was conducted to analyze the DNA fragmentation of brain cells in the cerebral cortex and hippocampus according to the manufacturer's protocol. Images of cell death were acquired via fluorescent microscopy (LSM700) and positive cell death (μm^2) with respect to DAPI area ($/\mu m^2$) was measured using ZEN Imaging Software version 2.1 (Blue edition; Zeiss). To validate Fas/FasL pathway-related apoptosis, endogenous expression of MAP-2 (1:400, Abcam) and FADD (1:400, Santa Cruz) were analyzed in the cerebral cortex and hippocampus. The sections were incubated with Alexa Fluor® 488 goat anti-rabbit (1:400, Invitrogen) and Alexa Fluor® 594 goat anti-mouse (1:400, Invitrogen) secondary antibodies, and covered with Vectashield® mounting medium with 4C, 6-diamidino-2-phenylindole (DAPI; Vector, Burlingame, CA, U.S.A.). Images of apoptotic and FADD-positive (FADD+) cells were taken using a fluorescent microscopy (LSM700), and positive apoptotic cells (μm^2) with respect to DAPI area ($/\mu m^2$), and FADD + cells (μm^2) with respect to MAP2-positive (MAP2+) area ($/\mu m^2$) were quantified using ZEN Imaging Software (Blue edition; Zeiss). Furthermore, the three-dimensional images of the apoptotic cells were acquired with ZEN Imaging Software version 2.1 (Blue edition; Zeiss).

Statistical Analysis

All data were expressed as means ± SEM. The variables between the two groups were compared using Student's t-test using the SPSS statistical software program (IBM, Armonk, NY; version 25.0). A p value < 0.05 was considered statistically significant. All graphical artworks were produced using GraphPad Prism version 9 (GraphPad Software Inc., San Diego, CA, USA).

Results

Early exposure to EE decreases hyperactivity, exerts anxiolytic effect, and improves fine motor function and cognitive function after HI brain injury

To investigate the motor, cognitive, and emotional functions, cylinder test, openfield test, ladder walking test, and Y-maze were conducted right after HI brain injury and after the EE treatment. In cylinder test, the number of rearing was significantly reduced in the EE group (Fig. 2a, $p < 0.001$), indicating that the hyperactivity derived from HI brain injury was significantly alleviated by early exposure to EE. In openfield test, the total distance traveled was significantly increased in the control group (Fig. 2b, $p < 0.05$) and significantly decreased in the EE group ($p < 0.001$) after the condition, indicating that HI brain injury-induced hyperactivity and anxiety were significantly alleviated by early exposure to EE. Moreover, the percentage of total slips was significantly reduced in the EE group (Fig. 2c, $p < 0.05$), indicating that fine motor impairment was significantly alleviated by early exposure to EE. In Y-maze test, the percentage of

alterations was significantly increased in the EE group (Fig. 2d, $p < 0.05$) after the condition, indicating that cognitive function was significantly improved by early exposure to EE.

Early exposure to EE downregulates the genes associated with cell death and apoptosis process

We selected DEGs based on greater than 2-fold change from all identified genes. The selected 1,691 DEGs (220 upregulated and 1,471 downregulated) in cortex and 2,942 DEGs (866 upregulated and 2,076 downregulated) in hippocampus in enriched mice were identified.

The downregulated genes in the brain of enriched mice were categorized according to biological process, molecular function, cellular components and pathway using the DAVID software program. Based on the GO analysis, the top 10 biological processes of the downregulated genes in cerebral cortex and hippocampus were related to “cellular process” (GO:0009987), “biological regulation” (GO:0065007), “regulation of biological process” (GO:0050789), “response to stimulus” (GO:0050896), “regulation of cellular process” (GO:0050794), and “metabolic process” (GO:0008152). Genes, which were commonly downregulated in cerebral cortex and hippocampus, were also highly involved in “cell death” (GO:0008219), “apoptotic process” (GO:0006915), “regulation of cell death” (GO:0010941), “regulation of programmed cell death” (GO: 0043067), and “regulation of apoptotic process” (GO: 0042981). Further, in the KEGG pathway, the highlighted cluster in the downregulated genes significantly expressed the overall theme for apoptosis in cerebral cortex and hippocampus, respectively (Fig. 3a and 3b). Table 1 demonstrates apoptosis-related genes, which were significantly downregulated both in the cerebral cortex and hippocampus of mice in the hyperacute EE group. The GO and KEGG pathway enrichment analyses indicated the significant downregulation of TNFSF10, FAS, and CASP8 in both the cortex and hippocampus of the enriched mice, indicating that early exposure to EE can alter the expression of genes associated with extrinsic apoptotic pathway.

Table 1
List of genes commonly downregulated in the regions of mouse brain in HI early EE mice compared to early control mice

Gene symbol	Description	Fold change in cerebral cortex	Fold change in hippocampus
PIK3CG	phosphoinositide-3-kinase, catalytic, gamma polypeptide	-2.49	-4.12
TNFSF10	Tumor necrosis factor superfamily, member 10	-3.069	-3.46
CSF2RB2	Colony stimulating factor 2 receptor, beta 2, low-affinity (granulocyte-macrophage)	-12.81	-12.73
CASP12	caspase 12	-12.65	-10.24
CASP8	caspase 8	-2.45	-6.24
CSF2RB	Colony stimulating factor 2receptor, beta, low-affinity (granulocyte-macrophage)	-6.94	-20.10
FAS	Fas (TNF receptor superfamily member 6)	-3.79	-4.06
BIRC3	baculoviral IAP repeat-containing 3	-2.36	-4.35
IL3RA	Interleukin 3receptor, alpha chain	-2.81	-4.10

Early exposure to EE contributes to neuroprotection via regulating the expression of both extrinsic and intrinsic apoptosis-related proteins in cerebral cortex and hippocampus

To investigate the protein expression of extrinsic and intrinsic apoptosis-related proteins in cerebral cortex and hippocampus by early exposure to EE, western blot was conducted for FAS, FADD, cleaved caspase-8, total caspase-8, cleaved caspase-3, total caspase-3, Bax, Bcl-2 and β-Actin. The representative WB images in cerebral cortex and hippocampus are shown in Fig. 4a and Fig. 4c, respectively. The quantification thereof was shown in Fig. 4b and Fig. 4d. The extrinsic apoptotic pathway-related proteins, FAS ($p < 0.001$ and $p < 0.05$), FADD ($p < 0.01$ and $p < 0.05$), and cleaved caspase 8 ($p < 0.05$ and $p < 0.05$), were significantly decreased in the EE group compared to the control group in cerebral cortex and hippocampus, respectively. The intrinsic apoptotic pathway-related proteins, Bax ($p < 0.05$ and $p < 0.01$) and Bcl-2 ($p < 0.05$ and $p < 0.05$), were significantly regulated in the EE group compared to the control group in cerebral cortex and hippocampus, respectively. Consequently, the ratio of Bax to Bcl-2 was significantly decreased in the EE group ($p < 0.001$ and $p < 0.01$) compared to the control group in cerebral

cortex and hippocampus, respectively. These results indicated that early exposure to EE can induce neuroprotection via the regulation of both extrinsic and intrinsic apoptotic pathways.

Early exposure to EE decreases infarct size and apoptosis via the downregulation of FADD + expression

The representative H&E images are shown in Fig. 4a, and the quantification thereof is shown in Fig. 4b. The infarct volume was significantly decreased in the EE group ($p < 0.01$) compared to the control group, indicating that early exposure to EE induced neuroprotection. The representative TUNEL images are shown in Fig. 4c, and the quantification thereof is shown in Fig. 4d. The percentage of TUNEL + cells were significantly decreased in cerebral cortex and hippocampus of the EE group, respectively ($p < 0.01$ and $p < 0.05$), indicating that early exposure to EE alleviated apoptosis. To further investigate the extrinsic apoptotic pathway, the colocalization analysis of MAP-2 + and FADD + was conducted. The representative MAP-2 + FADD + images were shown in Fig. 4e, and the quantification thereof is shown in Fig. 4f. The colocalization of MAP-2 + FADD + was significantly decreased in cerebral cortex ($p < 0.01$) and hippocampus ($p < 0.05$) of the EE group. These results indicated that early exposure to EE can alleviate apoptosis via the downregulation of extrinsic apoptotic pathways.

Delayed exposure to EE decreases hyperactivity, exerts anxiolytic effect, and improves fine motor function and cognitive function after HI brain injury

To investigate the motor, cognitive, and emotional functions, cylinder test, openfield test, ladder walking test, and Y-maze were conducted right after HI brain injury and after the EE treatment. In cylinder test, the number of rearing was significantly reduced in the EE group (Fig. 6a, $p < 0.001$), indicating that the hyperactivity derived from HI brain injury was significantly alleviated by delayed exposure to EE. In openfield test, the total distance traveled was significantly decreased in the EE group (Fig. 6b, $p < 0.0001$) after the condition, indicating that HI brain injury-induced hyperactivity and anxiety were significantly alleviated by delayed exposure to EE. Moreover, the percentage of total slips was significantly reduced in the EE group (Fig. 6c,$p < 0.0001$), indicating that fine motor impairment was significantly alleviated by delayed exposure to EE. In Y-maze test, the percentage of alterations was significantly increased in the EE group (Fig. 6d, $p < 0.05$) after the condition, indicating that cognitive function was significantly improved by delayed exposure to EE.

Delayed exposure to EE contributes to neuroprotection via regulating the expression of intrinsic apoptosis-related

proteins but not extrinsic apoptosis-related proteins in cerebral cortex and hippocampus

To investigate the protein expression of extrinsic and intrinsic apoptosis-related proteins in cerebral cortex and hippocampus by delayed exposure to EE, western blot was conducted for FAS, FADD, cleaved caspase-8, total caspase-8, cleaved caspase-3, total caspase-3, Bax, Bcl-2 and β-Actin. The representative WB images in cerebral cortex and hippocampus are shown in Fig. 7a and Fig. 7c, respectively. The quantification thereof was shown in Fig. 7b and Fig. 4d. The extrinsic apoptotic pathway-related proteins, FAS and FADD were not significantly decreased in the EE group compared to the control group. Cleaved caspase 8 was significantly decreased in the EE group compared to the control group in cerebral cortex and hippocampus ($p < 0.01$ and $p < 0.05$). The intrinsic apoptotic pathway-related proteins, Bax ($p < 0.05$ and $p < 0.05$ and Bcl-2 ($p < 0.05$ and $p < 0.05$), were significantly regulated in the EE group compared to the control group in cerebral cortex and hippocampus, respectively. Consequently, the ratio of Bax to Bcl-2 was significantly decreased in the EE group ($p < 0.01$ and $p < 0.01$) compared to the control group in cerebral cortex and hippocampus, respectively. These results indicated that delayed exposure to EE can induce neuroprotection via the regulation of intrinsic apoptotic pathways but not by extrinsic apoptotic pathways.

Delayed exposure to EE decreases infarct size and apoptosis but not via the downregulation of FADD + expression

The representative H&E images are shown in Fig. 8a, and the quantification thereof is shown in Fig. 8b. The infarct volume was significantly decreased in the EE group ($p < 0.01$) compared to the control group, indicating that delayed exposure to EE induced neuroprotection. The representative TUNEL images are shown in Fig. 8c, and the quantification thereof is shown in Fig. 8d. The percentage of TUNEL + cells were significantly decreased in cerebral cortex and hippocampus of the EE group, respectively ($p < 0.05$ and $p < 0.05$), indicating that delayed exposure to EE alleviated apoptosis. To further investigate the extrinsic apoptotic pathway, the colocalization analysis of MAP-2 + and FADD + was conducted. The representative MAP-2 + FADD + images were shown in Fig. 8e, and the quantification thereof is shown in Fig. 8f. The colocalization of MAP-2 + FADD + was not significantly decreased both in the cerebral cortex and hippocampus of the EE group. These results indicated that early exposure to EE can alleviate apoptosis but not through the downregulation of extrinsic apoptotic pathways.

Discussion

Post-stroke early mobilization has been investigated extensively and is recommended in several international clinical practice guidelines because early initiation of exercise (24 to 72 h post-stroke) promotes functional recovery, prevents post-stroke complications, and facilitates return to society [34].

However, exposure to high doses at the early phase and forced exercise within 24 h post-stroke might adversely affect stroke outcomes. The effects of early exposure to EE on functional recovery and the underlying mechanism after stroke have yet to be fully elucidated.

In the current study, early exposure to EE significantly improved functional recovery and preserved neuronal survival. The neuroprotective effects were mediated via downregulation of both extrinsic and intrinsic signaling pathways of apoptosis. A significant downregulation of Fas/FasL-mediated apoptosis in cerebral cortex and hippocampus was detected in mice exposed to early EE.

Previous studies have shown that VE may improve motor and cognitive ability, and promoted anti-apoptotic as well as neuroprotective effects [57, 58]. Compared with forced exercise, VE is not associated with systemic stress and does not decrease the neuroprotective effect [58–60]. VE via exploratory movements such as EE may have greater benefit [61]. An early study demonstrated the beneficial effects of VE on hippocampal function, which were associated with suppression of cleaved caspase-3 expression, reduction of Bax expression and increased Bcl-2 expression in the hippocampus [62, 63]. Following stroke, VE may improve motor rehabilitation, enhance cognitive ability and hippocampal BDNF expression compared with involuntary muscle movement and forced exercise [60]. The effects of voluntary movements post-stroke on neuronal regeneration and repair were related to upregulation of the expression of growth-associated protein 43 and neurotrophin 3 [57]. A recent study reported that early commencement of VE post-stroke improved cerebral blood flow, vascular quality, and brain functions such as connectivity and motor abilities [64].

In terms of forced exercise post-stroke, mild-, moderate- and high-intensity exercise training initiated at various time points following stroke played a beneficial role [65–67]. Especially, mild- and moderate-intensity exercise enhanced neuroprotection compared with high-intensity exercise training [65, 66, 68, 69]. However, an early start of forced exercise post-stroke aggravated brain damage and apoptotic cell death, and triggered energy deficits along with generation of reactive oxygen species [41].

Apoptosis may contribute significantly to neuronal death following brain ischemia; however, the time window for effective stroke rehabilitation including VE and the underlying mechanisms are not fully understood. To our knowledge, the current study was the first of its kind to investigate the effect of hyperacute exposure to EE on both extrinsic and intrinsic pathways of apoptosis in an adult mouse model of HI brain injury. Our data reveal that hyperacute (within 3 hours) exposure to EE post-stroke suppresses both extrinsic and intrinsic pathways of apoptosis in cerebral cortex and hippocampus. This may be due to the higher sensitivity of these brain areas to therapies that suppress neuronal apoptosis [70, 71]. These results provide novel insights into the mechanisms underlying the neuroprotective effects of hyperacute EE treatment after ischemic stroke.

Previous studies highlighted the importance of suppressing the extrinsic pathway of apoptosis for favorable outcomes during the acute phase of ischemic stroke. Several molecules involved in the TNF/Fas family death receptor-mediated extrinsic pathway are increased particularly in the damaged penumbra and remained elevated in the stroke model [72]. The upregulated expression of Fas, FasL, and

TNF-related apoptosis-inducing ligands was observed within 12 hours after cerebral ischemia and peaked between 24 and 48 hours in the post-ischemic rat brain, which highly correlates with the time course of neuronal apoptosis [72, 73]. In patients with acute ischemic stroke, Fas-induced apoptosis in peripheral blood was activated in the first week after the onset, followed by a decrease towards the end of the acute period [74]. In a rodent model of ischemic stroke, acute treatment with edaravone was neuroprotective in transient focal ischemia, and the mechanism involved suppression of the Fas/FasL signaling pathway [23]. Recently, very early treatment with zonisamide decreased morbidity by suppressing the expression of caspase-3, caspase-8, and calpain-1, and inhibiting the apoptosis of neuronal cells after cerebral ischemia injury [12]. Further, intranasal administration of a Fas-blocking peptide 12 hours post-ischemic stroke attenuated Fas-mediated apoptosis, decreased infarct volumes, and reduced neurologic deficits.24 Importantly, a significant reduction in infarct volume occurred in hybrid mice expressing FasL dysfunction and in TNF knockout mice 24 hours after stroke[19, 73]. However, the mechanism of suppression of extrinsic apoptosis mediated by EE treatment in the hyperacute phase of ischemic stroke has yet to be reported. In the current study, we found that hyperacute exposure to EE significantly suppressed extrinsic apoptosis via downregulation of Fas/FasL-mediated signaling in both cerebral cortex and hippocampus; however, the delayed exposure to EE failed to inhibit the apoptosis execution pathway.

The Fas/FasL system plays an important role in apoptosis during the acute phase in other neurological disorders, and the current study findings were in accordance with previous studies [26, 28–30, 33]. In a mouse model of traumatic brain injury, the peaked expression of Fas was noticed in the cortex and hippocampus 24 hours after the injury [75]. Furthermore, Both the level of Fas and FasL in cortical neurons and astrocytes were sustained for up to 72 hours after injury [76]. Fas-mediated apoptosis of neurons occurred in mouse models of acute and subacute SCI and reduced apoptosis and neurological dysfunction were detected in Fas-deficient mice compared with control mice after SCI [27].

The extrinsic pathway of apoptosis plays a critical role in the induction of apoptosis in non-neuronal cells, especially in the acute phase. Previous studies demonstrated the effects of extrinsic pathway of apoptosis in acute injury of lung, liver, heart, and kidney, and acute parasite infection. Tiao et al. investigated the protective role of microRNA-29a in acute liver injury in a mouse model of obstructive jaundice, mediated at least partially by modulating the extrinsic rather than intrinsic pathway of apoptosis [28]. Furthermore, CD95 (AP0-1/Fas)-mediated apoptosis was shown to play an important role in the pathogenesis of fulminant hepatic failure in acute Wilson's disease [29]. FADD deletion attenuated cardiomyocyte death and improved cardiac function in acute myocardial ischemia/reperfusion injury [77]. Recent study reveals that the reduced Fas and FasL expression in CD4 + T cells was associated with let-7 microRNA expression, which significantly inhibited the apoptosis of these cells, and improved cell survival rates in patients with acute coronary syndrome [26]. Fas/FasL signaling mediated the pathogenesis of acute ischemic kidney injury via tubular apoptosis and necrosis, suggesting that the modulation of Fas/FasL system can be an effective therapeutic target in ischemic acute kidney injury [33].

While most of the studies elucidating the possible mechanisms underlying the neuroprotective effect of EE intervention focused on the early phase of stroke, the importance of neuronal survival in the relatively late phase has been neglected. The current study found that delayed exposure to EE reduces infarct volume and DNA damage in brain cells, and thereby improved neurobehavioral function. This result is consistent with a previous study, which demonstrated sustained functional recovery following exposure to EE 5 days after onset, resulting in significant survival of hippocampal newborn cells in a rat stroke model [78]. Moreover, exposure to EE for 10 days strongly rescued the diabetic brain from neurodegenerative progression [79]. However, our study demonstrated that delayed exposure to EE does not significantly suppress apoptosis.

Limitation of this study is that the diverse potential mechanisms of neuronal cell death contributing to long-term neuroprotection and functional recovery after stroke were not investigated, given the complexity. Furthermore, EE positively contributes to improved behavioral recovery after stroke in young animals. A recent study reported that EE had a limited benefit on behavioral recovery of older rats compared with young rats [80]. Since stroke primarily affects mostly the elderly patients, the neuroprotective effects demonstrated in this study may not precisely be applicable to aged subjects. Additionally, ischemic stroke in humans occurs preferentially in patients of both sexes carrying multiple comorbidities requiring various treatments with complex interactions. In contrast, our study involved young, healthy, male inbred mice housed under ideal conditions. Therefore, it is highly desirable to investigate the effects of hyperacute exposure to EE in an aged animal model including both male and females with multiple comorbidities to demonstrate the clinical relevance to stroke rehabilitation. Although the mechanism of EE-induced inhibition of neuronal apoptosis requires further investigation, our study suggests that Fas/FasL-mediated apoptosis may be an important target underlying the neuroprotective effects of very early EE treatment after HI brain injury, contributing to improved emotional, cognitive, and locomotor performance post-stroke.

In summary, we demonstrated that early exposure to EE can induce greater improvement in behavioral outcomes, reduced infarct volume, and decreased neuronal death. It significantly downregulated Fas/FasL-mediated apoptosis and decreased expression of pro-apoptotic proteins in cerebral cortex and hippocampus. Overall, the results of this study demonstrates that very early exposure to EE is a promising neuroprotective candidate for therapeutic translation after stroke by effective inhibition of extrinsic as well as intrinsic apoptotic pathways.

Declarations

Acknowledgements

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Author Contributions

HYL, S-YS: study design data acquisition and analysis, manuscript draft and revision. JH, AB, SHK: data acquisition, analysis, and intellectual discussion. SP, S-RC: funding acquisition and study design, data interpretation, manuscript editing and final revision.

Compliance with Ethical Standards

Conflict of Interest

The authors declare no competing interests.

Ethical Approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

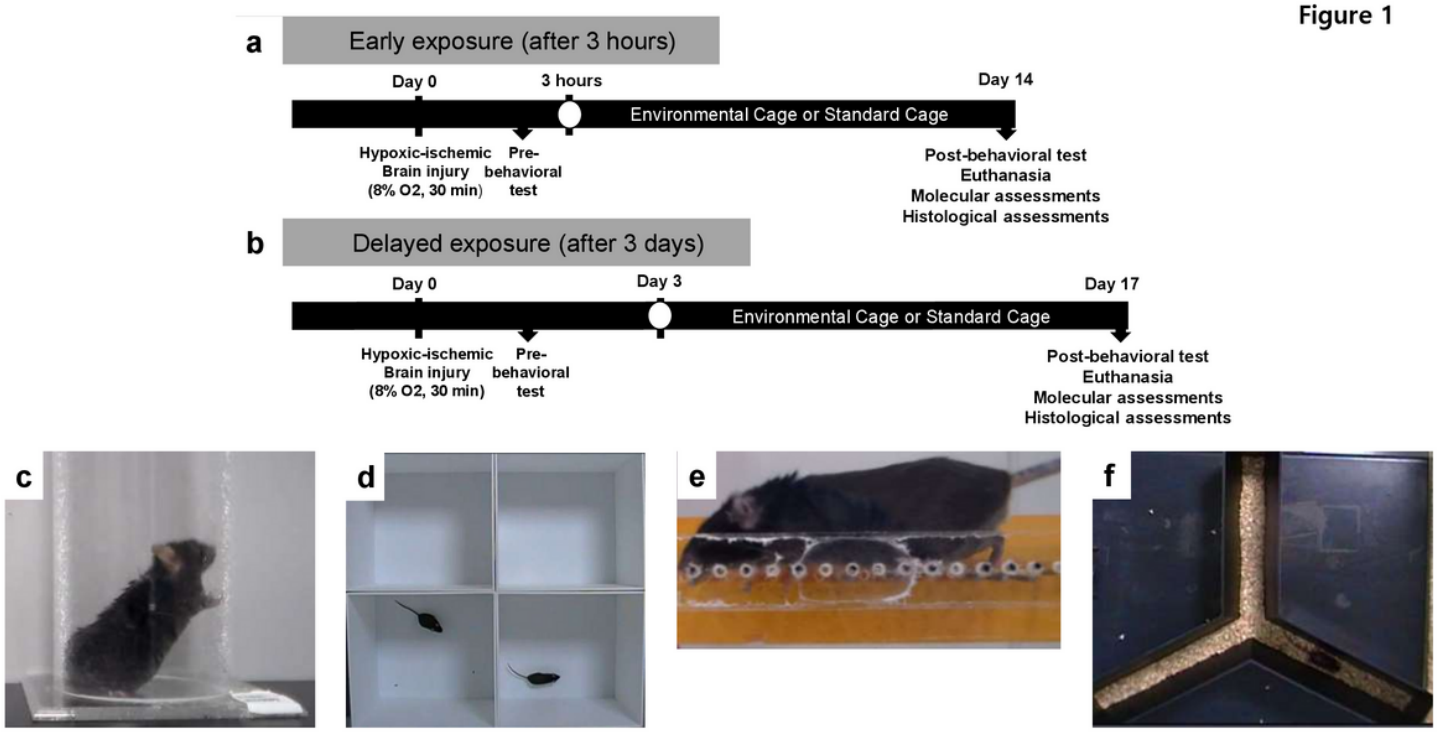

Figure 1

Experimental scheme for early (**a**) and delayed exposure (**b**) to EE. Behavioral assessments were conducted right after HI brain injury and after the EE treatment. Cylinder test (**c**) and open field test (**d**) were conducted for the assessment of anxiety and hyperactivity. Ladder walking test (**e**) was conducted for the assessment of fine motor function, and Y-maze test (**f**) was conducted for assessment of cognitive function.

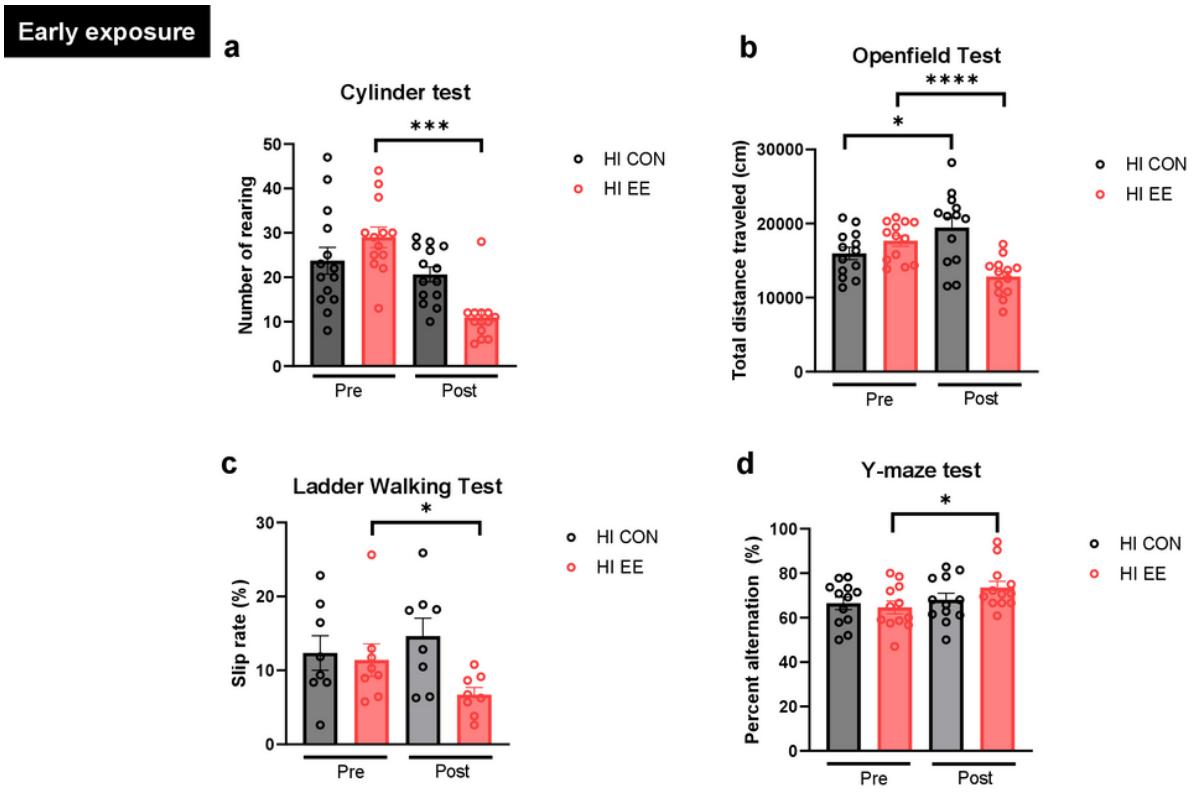

Figure 2

Figure 2

Early exposure to EE exposure exerts anxiolytic effect, and improves fine motor function and cognitive function in HI mice. The number of rearing was significantly decreased in the EE group in cylinder test (**a**) (n = 14 per group). The total distance traveled was significantly increased in the control group and significantly decreased in the EE group in openfield test (**b**) after the condition (n = 13 per group). The percentage of total slips was significantly reduced in the EE group in ladder walking test (**c**) after the condition (n = 8 per group). The percentage of alteration was significantly increased in the EE group in Y-maze test (**d**) after the condition (n = 12 per group). Student's t-test. Data represented are means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

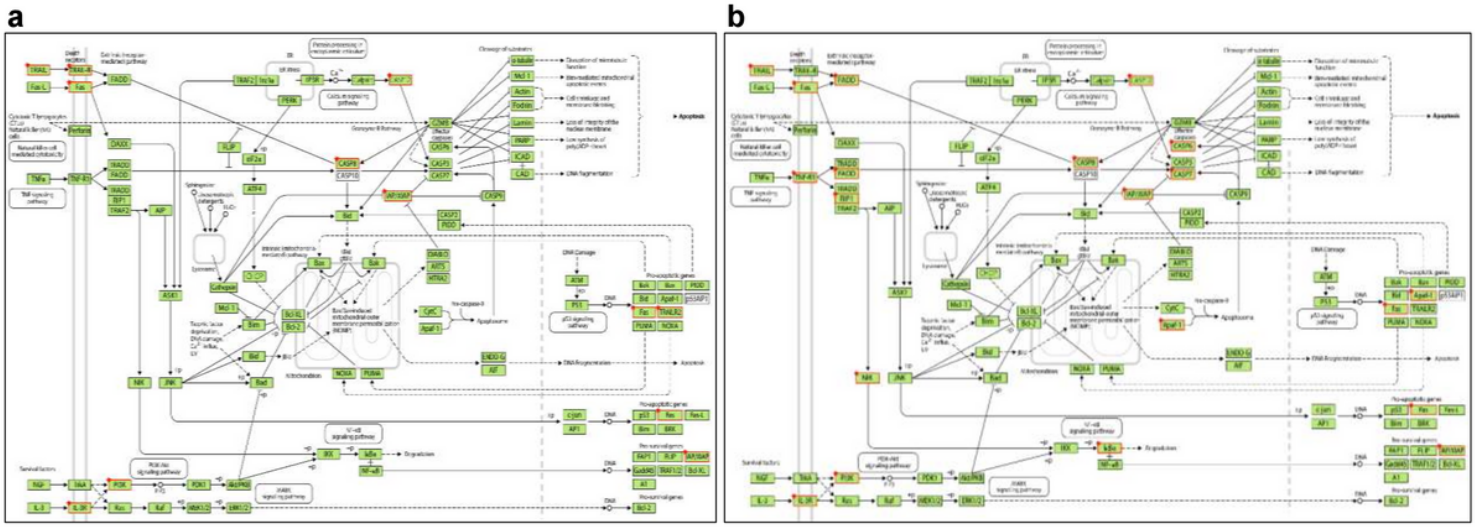

Figure 3

The GO and KEGG pathway enrichment analyses. Early exposure to EE significantly downregulates extrinsic apoptotic pathways in cerebral cortex (**a**) and hippocampus (**b**) The network diagram was sourced from KEGG database. The red rectangle indicates the significantly decreased expression of the protein in the EE group compared to the control group.

Figure 4

WB analysis of the early exposure groups. Early exposure to EE contributes to neuroprotection via regulating the expression of both extrinsic and intrinsic apoptosis-related proteins in cerebral cortex and hippocampus. The representative WB images of cerebral cortex and hippocampus were shown in (**a**) and (**c**), respectively. The quantifications thereof were shown in (**b**) and (**d**), respectively (n = 6 per group). Student's t-test. Data represented are means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

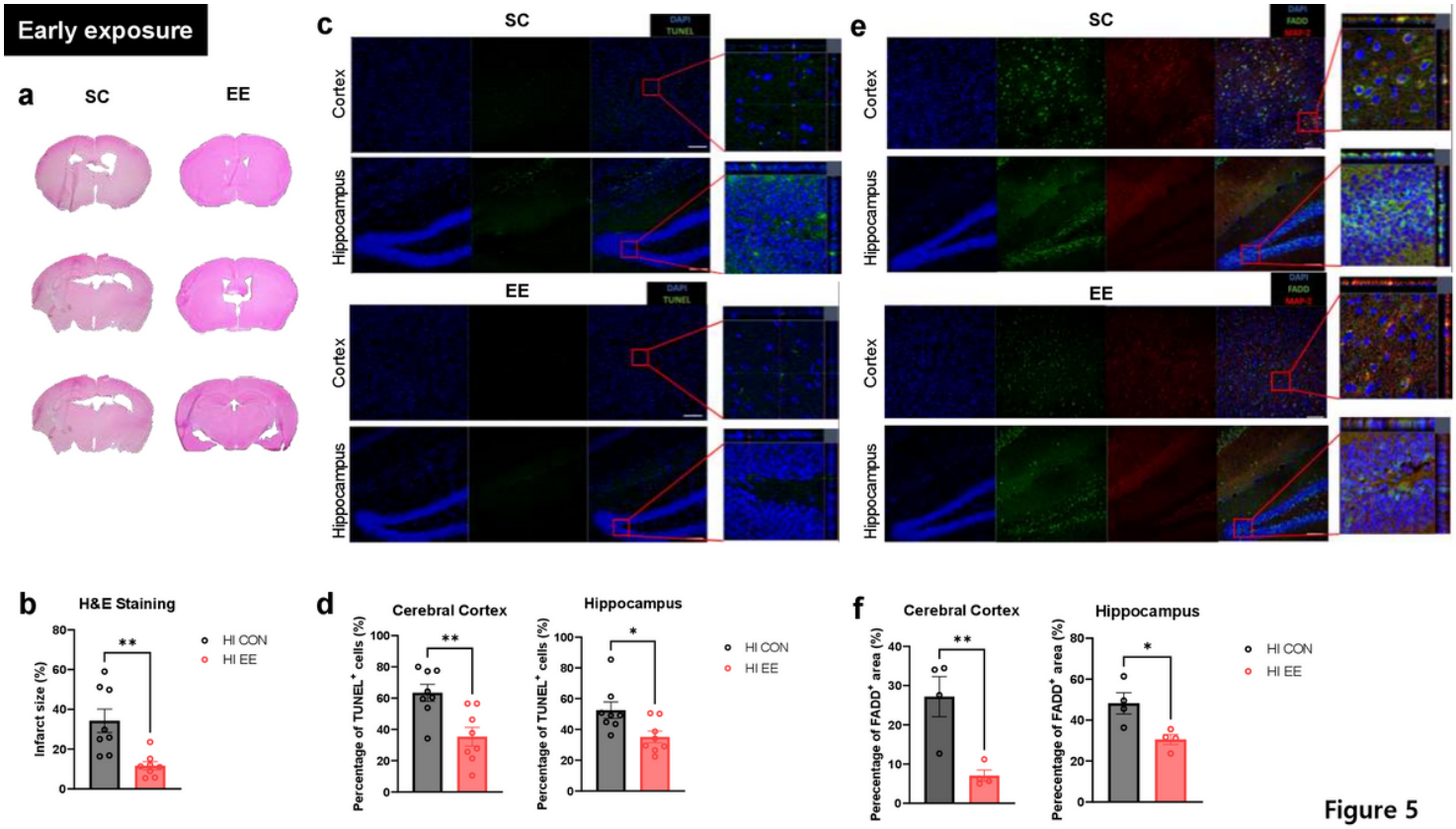

Figure 5

Histological assessments of the early exposure groups. The representative H&E images were shown in (**a**), and the quantification thereof was shown in (**b**) (n = 8 per group). The representative TUNEL images were shown in (**c**), and the quantification thereof was shown in (**d**) (n = 8 per group). The representative MAP-2+FADD+ images were shown in (**e**), and the quantification thereof was shown in (**f**) (n = 4 per group). Student's t-test. Data represented are means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

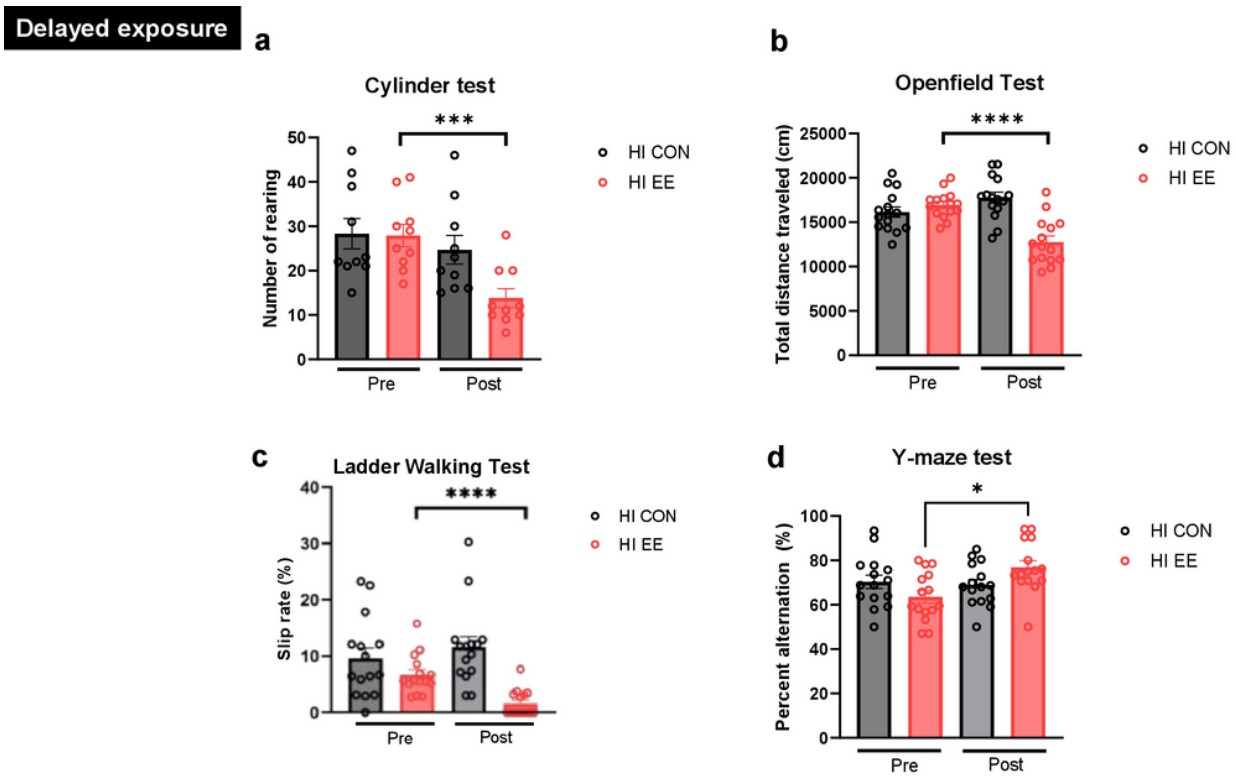

Figure 6

Figure 6

Delayed exposure to EE exposure exerts anxiolytic effect, and improves fine motor function and cognitive function in HI mice. The number of rearing was significantly decreased in the EE group in cylinder test (**a**) (n = 15 per group). The total distance traveled was significantly decreased in the EE group in openfield test (**b**) after the condition (n = 15 per group). The percentage of total slips was significantly reduced in the EE group in ladder walking test (**c**) after the condition (n = 15 per group). The percentage of alteration was significantly increased in the EE group in Y-maze test (**d**) after the condition (n = 15 per group). Student's t-test. Data represented are means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

Figure 7

WB analysis of the delayed exposure groups. Delayed exposure to EE contributes to neuroprotection via regulating the expression of intrinsic apoptosis-related proteins in cerebral cortex and hippocampus. The representative WB images of cerebral cortex and hippocampus were shown in (**a**) and (**c**), respectively. The quantifications thereof were shown in (**b**) and (**d**), respectively (n = 6 per group). Student's t-test. Data represented are means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

Figure 8

Histological assessments of the delayed exposure groups. The representative H&E images were shown in (**a**), and the quantification thereof was shown in (**b**) (n = 4 per group). The representative TUNEL images were shown in (**c**), and the quantification thereof was shown in (**d**) (n = 4 per group). The representative MAP-2+FADD+ images were shown in (**e**), and the quantification thereof was shown in (**f**) (n = 4 per group). Student's t-test. Data represented are means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

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