Effects of environmental enrichment on presynaptic active zone proteins in a mouse model of hypoxic-ischemic brain injury

Seok Young Song

Graduate Program of biomedical engineering

The Graduate School, Yonsei University

Effects of environmental enrichment on presynaptic active zone proteins in a mouse model of hypoxic-ischemic brain injury

Directed by Professor Sung-Rae Cho

The Doctoral Dissertation
submitted to the Graduate Program of biomedical engineering, the Graduate School, Yonsei University
in partial fulfillment of the requirements for the degree of
Master of Medical Science

Seok Young Song

December 2021

This certifies that the Doctoral Dissertation of
Seok Young Song is approved.

Thesis Supervisor: Sung-Rae Cho

Thesis Committee Member #1: Hyongbum Kim

Thesis Committee Member #2: Ha Young Shin

Thesis Committee Member #3: Yoonhee Jin

Thesis Committee Member #4: Hwunjae Lee

The Graduate School
Yonsei University

December 2021

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

Effects of environmental enrichment on presynaptic active zone proteins in a mouse model of hypoxic-ischemic brain injury

Seok Young Song

Graduate Program of biomedical engineering

The Graduate School, Yonsei University

(Directed by Professor Sung-Rae Cho)

Hypoxic–ischemic encephalopathy (HIE) is a devastating neonatal brain condition caused by lack of oxygen and limited blood flow. Environmental enrichment (EE) is a classic paradigm with a complex stimulation of physical, cognitive, and social components. EE can exert neuroplasticity and neuroprotective effects in immature brains. However, the exact mechanism of EE on the chronic condition of HIE remains unclear. HIE was induced by a permanent ligation of the right carotid artery, followed by an 8% O2 hypoxic condition for 1 h. At 6 weeks of age, HIE mice were randomly assigned to either standard cages or EE cages. In the behavioral assessments, EE mice showed significantly improved motor

performances in rotarod tests, ladder walking tests, and hanging wire tests, compared with HIE control mice. EE mice also significantly enhanced cognitive performances in Y-maze tests. Particularly, EE mice showed a significant increase in Cav 2.1 (P/Q type) and presynaptic proteins by molecular assessments, and a significant increase of Cav 2.1 in histological assessments of the cerebral cortex and hippocampus. These results indicate that EE can upregulate the expression of the Cav 2.1 channel and presynaptic proteins related to the synaptic vesicle cycle and neurotransmitter release, which may be responsible for motor and cognitive improvements in HIE.

Key words: Enriched environment, Synaptic plasticity, Synaptic vesicle, Hypoxic–ischemic encephathy, Cav 2.1 (P/Q type) Calcium channel

Effects of environmental enrichment on presynaptic active zone proteins in a mouse model of hypoxic-ischemic brain injury

Seok Young Song

Graduate Program of biomedical engineering

The Graduate School, Yonsei University

(Directed by Professor Sung-Rae Cho)

I. INTRODUCTION

Hypoxic–ischemic encephalopathy (HIE) is a brain condition that is caused by a lack of oxygen and limited blood flow in infants.[1] This kind of injury can cause neurological disabilities, including seizures, cerebral palsy, and cognitive and motor dysfunction in infants.[2] Due to their susceptibility, neurons can be permanently damaged when perfusion is halted for merely more than 5 min, ultimately leading to cell apoptosis .[3,4] The outcomes of HIE exist on a spectrum, and the only current therapy for HIE is hypothermia, which has to be initiated within the first 6 h of life, thus making it critical to identify and develop further therapeutic strategies to improve brain function.[1,5]

The mouse model for HIE has been developed to model human perinatal HIE, and it can be constructed by the permanent ligation of the common carotid artery (CCA) followed by exposure to a hypoxic condition for a short period of time.[6] This event can induce permanent synapse dysfunction and degeneration in various brain regions.[7,8] Moreover, significant cerebral infarction and malfunction in sensorimotor reflex performance, after HIE injury, were observed in neonatal mice.[9,10]

Previous studies have shown that neurons at the penumbra, which are conserved functionally and structurally, are responsible for functional recovery and presynaptic alterations.[11,12] Presynaptic dysfunctions, such as changes in the intracellular level of Ca2+ and improper synaptic vesicle cycling, further lead to synaptic failure.[13,14]

However, these dysfunctions can be partially rescued by various treatments.[15,16] Environmental enrichment (EE), which consists of complex combinations of physical, cognitive, and social stimuli, is a method of improving rodent welfare.[17,18] EE is also considered the modification of cages that mimics the human exercise/rehabilitation model.[19,20] The beneficial effects of EE on strength, sensorimotor, physiological, and psychological functions in neonatal hypoxic–ischemic (HI) animal models, have been highlighted in recent studies.[21-23]

Exposure to more enriched cages can induce neuroplasticity, with a higher expression of synaptic proteins, higher rates of synaptogenesis, and more complex dendrite arbors, by increasing physical and social stimuli.[18,24] Neuroplasticity is considered crucial for functional recovery from brain injury in developing brains.[25-27] Even for the chronic

phase of stroke, the beneficial effects of EE have been highlighted in both preclinical and clinical studies.[28-30]

Among many presynaptic active zone proteins, Rab3, Munc13, Munc18, SNAP25, syntaxin, VAMP2, and the calcium channel Cav 2.1, have been reported to affect synaptic plasticity. Rab3 can regulate neurotransmitter exocytosis via its GTP binding property,[31,32] and is considered as an essential component for regulating PKA-dependent LTP.[33] Munc13 can induce conformation change of syntaxin upon interaction with the 3a domain of the Munc18-syntaxin complex, resulting in the synthesis of the tetramer of Munc13, Munc18, syntaxin, VAMP2,[34,35] and, with the arrival of SNAP25, the full SNARE complex is assembled as Munc18 is released.[36] From this view, it can be inferred that Munc13 partici-pates in short-term presynaptic plasticity,[37] and Munc18 contributes to the improvement of synaptic function probability and plasticity.[38,39] SNAP25, syntaxin, and VAMP form a complex called the SNARE complex, which functions as the main machinery of membrane fusion.[40-42] Its role was implicated in the regulation of calcium channels,[13] and its effect on neuroregeneration has been identified.[43,44]

The P/Q type voltage-dependent calcium channel, Cav 2.1, is one of the major sources of calcium influx and is responsible for neurotransmitter exocytosis. Its upregulation is known to modify synaptic strength,[45] contribute to short-term plasticity,[46,47] and contribute to long-term plasticity.[46] These above proteins have noteworthy implica-tions for synaptic plasticity due to their major roles in synaptic transmission. Thus, I looked

for presynaptic active zone proteins and calcium channel Cav2.1, mentioned above, to verify whether EE on HIE models enhances neurobehavioral function via inducing neural plasticity.

More importantly, Cav2.1 could be the possible key players in EE-mediated neuroplasticity and Cav2.1 mediated presynaptic plasticity could be the mechanism behind this.[18,48]

The P/Q type voltage-dependent calcium channel, Cav 2.1, is one of the major sources of calcium influx and is responsible for neurotransmitter exocytosis. Its upregulation is known to modify synaptic strength,[45] contribute to short-term plasticity,[46,47] and contribute to long-term plasticity.[46] Also, it has been suggested that Cav 2.1 interacts with various active zone proteins that participate in the mechanism of neuroplasticity, including RIM1/2, RBP, and SNARE complex proteins.[49-52]

In this study, adding on to my previous research that Cav2.1 mediated presynaptic plasticity could be the mechanism behind EE-mediated neuroplasticity, I aimed to investigate whether blocking Cav2.1 could limit EE-mediated neuroplasticity and impair functional recovery by EE.

II. MATERIALS AND METHODS

1. Ethics Statement and Experimental Animals

All procedures were reviewed and approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) (2016) and the Institutional Animal Care and Use Committee (IACUC) of Yonsei University Health System (permit number: 2018-0110). All procedures were in accordance with the guidelines of the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. These regulations, notifications, and guidelines originated, and were modified, from the Animal Protection Law (2008), the Laboratory Animal Act (2008), and the Eighth Edition of the Guide for the Care and Use of Laboratory Animals (NRC 2011). Mice were provided food and water ad libitum under alternating 12-h light/dark cycles, according to animal protection regulations. They were sacrificed at 8 weeks after the housing conditions, under ketamine (100 mg/kg) and xylazine (10 mg/kg) anesthesia by intraperitoneal injection. All efforts were made to minimize animal suffering.

2. Construction of Hypoxic–Ischemic Encephalopathy (HIE) Model

At postnatal day 7, HI brain injury was induced by a permanent ligation of the unilateral right common carotid artery, right below where the external and internal carotid arteries branch out; acute exposure to hypoxic condition (8% O_2, 92% N_2) was then performed, as previously described.[6] With a visual microscopy, severity of a brain injury was assessed at

two weeks of age, and mice whose brain lesion size exceeded 20% of the cortical cavity, on the ipsilateral side of the brain, were excluded in this study.

3. ω-Agatoxin infusion using osmotic pump in the brain

After a recovery period, the brains of all the mice were injected with an osmotic pump (**Figure 7B).** In the HIE model, mice were continuously injected either with (1) PBS(solvent control) or (2) **ω-**Agatoxin (Sigma-Aldrich, Cat.MFCD0081294) in to the lateral ventricle using a micro-osmotic pump (Alzet 1003; 100ul volum, Cupertino,CA,USA) at a speed of 0.5ul/h(0.015ug/100ml 28day x 2). The infusion cannula (Brain Infusion Kit 3; 1-3mm; Durect Corp., Cupertino, CA, USA) was inserted into the lateral ventricle using stereotaxic coordinates (AP+0.3 mm from Bregma;ML – 0.7 mm from Bregma; DV – 2.0 mm from dura).In the HIE model, ω-Agatoxin was injected via one routes into the lateral ventricle using stereotaxic coordinates (AP + 0.4 mm from Bregma; ML -1.9 mm from Bregma; DV -3.3 mm from dura).

4. Experimental Procedures and Cage Condition

At 6 weeks of age, a total of 60 male HI ICR/CD-1 were randomly housed to either standard conditions (SC, n = 30) or an enriched environment (EE, n = 30) in this study. The condition lasted until 14 weeks of age. EE mice freely accessed novel objects and large-scale social interaction (12~15 mice/cage) (Figure 1A) relative to control mice (5 mice/cage) (**Figure 1B,C**). After the condition period, all mice were sacrificed for either molecular or

histological assessments at 14 weeks of age. The studied brain regions were dissected based on the mouse brain gross anatomy atlas, and the stereotaxic coordinates for the cerebral cortex, hippocampus, and striatum were (ML = −1.0, AP = 0.1, DV = 1.0), (ML = −1.0, AP = −2.0, DV = 2.0), and (ML = −1.0, AP = 0.1, DV = 2.5), respectively

5. Behavioral Assessment

A. Rotarod Performance

A rotarod (No. 47,600; UGO Basile, Comerio, VA, Italy) test was used to evaluate the motor coordination and balance of the experimental mice using an accelerating (4~80 RPM) speed paradigm and a constant (48 RPM) paradigm. After placing mice on the rotating rods, the time taken for the mice to fall from the rods was measured for 300 s. [18]

B. Ladder Walking Test

The ladder walking test can assess subtle disturbances of motor function through qualitative and quantitative analysis of walking.[6,53] This test was performed at five to six weeks of age as a baseline study. The ladder walking test was performed 8 weeks after intervention. In the ladder walking test, mice were required to walk a distance of 1 m, four times, on a horizontal ladder with metal rungs (Jeung Do Bio and Plant Co., Seoul, Korea) located at differing distances apart. The number of slips in each forelimb was measured using videotape analysis. The variance between the control and EE groups was calculated as 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 of the EE mice compared that of the controls.

C. Hanging Wire Test

The hanging wire test evaluated neuromuscular strength of the paws of the experimental mice.[54] To this end, mice were suspended on a horizontal rod (5 × 5 mm area, 35 cm long, between two 50 cm high poles), and the suspension latencies were measured for 5 min.

D. Y-Maze Test

The Y-maze test is used to evaluate cognition and short-term spatial memory.[55] This test was carried out in an enclosed "Y" shaped maze (Jeung Do B&P, Seoul, Korea). Normal mice tend to visit the arms of the maze one after the other. This behavior is called spontaneous alteration and is used to assess short-term spatial memory in a new environment. The number of each arm entries, spontaneous alteration, and percent alteration were recorded and determined for 8 min. The percent alteration was calculated as follows: [number of spontaneous alteration/(number of total arm entries − 2)] × 100. At the end of each trial, the maze was cleaned of urine and feces with 70% ethanol.

6. Molecular Assessments

A. Quantitative Real-Time PCR (qRT-PCR)

Total RNA was prepared in the studied brain tissue lysates using a TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. A nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to confirm the quality and quantity of extracted RNA. Differentially expressed genes of interest related to presynaptic scaffold proteins from the cerebral cortex and hippocampus were selected to be validated by a qRT-PCR. A ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) was used to synthesize cDNA with total RNA. Then, 2 μL of cDNA in a total volume of 20 μL was used in the following reaction. The qRT-PCR was performed in triplicate on a Light Cycler 480 (Roche Applied Science, Mannheim, Germany), using the Light Cycler 480 SYBR Green master mix (Roche), with thermocycler conditions as follows: amplifications were performed starting with a 300 s template preincubation step at 95 ◦C, followed by 45 cycles at 95 ◦C for 10 s, 60 ◦C for 10 s, and 72 ◦C for 10 s. The melting curve analysis began at 95 ◦C for 5 s, followed by 1 min at 60 ◦C. The specificity of the produced amplification product was confirmed by the examination of a melting curve analysis, and showed a distinct single sharp peak with the expected Tm for all samples. A distinct single peak indicates that a single DNA sequence was amplified during the qRT-PCR. The detail sequence of the primers is listed in Table S1. Primers were designed using the NCBI primer blast, with the parameters set to a product of 150–200 bp within the region surrounding the identified translocation. The expression of each gene of interest was obtained using the $2^{-\Delta\Delta Ct}$

method. The expression level of each gene of interest was obtained using the 2−ΔΔCt method. Target-gene expression was normalized relative to the expression of GAPDH and represented as fold change relative to the control.

B. Western Blot

To confirm the expression of Cav 2.1 and synaptic proteins in the cerebral cortex and hippocampus in the EE and control mice, 30 μg of total protein was extracted from all mice and dissolved in a sample buffer (60 mM Tris–HCl, pH 6.8, 14.4 mM b-mercaptoethanol, 25% glycerol, 2% SDS, and 0.1% bromophenol blue; Invitrogen), incubated for 10 min at 70 ◦C, and separated on a 10% SDS reducing polyacrylamide gel (Invitrogen). Protein samples were separated with SDS-polyacrylamide gel electrophoresis (PAGE) on a 4–12% gradient Bis-Tris gel and Tris-Acetate gel (Invitrogen, Carlsbad, CA, USA). The separated proteins were further transferred onto a 0.45 μm invitrolonTM polyvinylidene difluoride (PVDF) filter paper sandwich using an XCell IITM Blot Module (invitrogen, Life Technologies, Carlsbad, CA, USA). The membranes were blocked for one hour in Tris-buffered saline (TBS) (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) plus 0.05% Tween 20 (TBST) containing 5% non-fat dry milk (Bio-Rad, Hercules, CA, USA) at room temperature, washed three times with TBST, and incubated at 4 ◦C overnight with the following primary antibodies; anti-Munc13 (1:1000, Abcam), anti-Raphilin3A (1:1000, Synaptic Systems), anti-Munc18 (1:1000, Abcam), anti-VAMP2 (1:1000, Abcam), anti-SNAP25 (1:1000, Abcam), anti-Syntaxin (1:1000, Abcam), anti-Cav 2.1 (1:1000, Abcam),

and anti-ACTIN (1:5000, Santa Cruz). After washing the blots three times with TBST, the blots were incubated for one hour with horseradish peroxidase-conjugated secondary antibodies (1:5000; Santa Cruz, CA, USA) at room temperature. The proteins were further washed three times with TBST and visualized with an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Little Chalfont, UK). Using ImageQuant™ LAS 4000 software (GE Healthcare Life Science, Chicago, IL, USA), Western blot results were saved into TIFF image files, and then the images and the density of the band were analyzed and expressed as the ratio relative to the control band density using Multi-Gauge (Fuji Photo Film, version 3.0, Tokyo, Japan).

To normalize the values of all samples to account for band intensity, the average band intensity for each mouse group was first calculated. The samples were normalized to the group average of controls, and target protein expressions were normalized relative to the expression of ACTIN. The value of the control group was set to 1 and was divided by the value of each individual mouse.

7. Immunohistochemistry

The brain tissues were frozen in Surgipath FSC 22 clear frozen section compound (Leica Microsystems, Wetzlar, Germany) using dry ice and isopentane. The harvested brain tissues were cryosectioned at 16-μm thickness along the coronal plane, and immunohistochemistry staining was performed. At 8 weeks after EE, to confirm the endogenous expression of Cav 2.1 (1:100, Abcam) and MAP2 (1:400, Millipore,

Burlington, MA, USA), the brain sections of the cerebral cortex, hippocampus, and striatum were immunostained. 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, then covered with Vectashield® mounting medium with 4C, 6-diamidino-2-phenylindole (DAPI; Vector, Burlingame, CA, USA). The stained sections were analyzed using confocal microscopy (LSM700; Zeiss, Gottingen, Germany).

8. Statistical Analysis

Statistical analyses were performed using Statistical Package for Social Sciences software version 25.0 (IBM Corporation, Armonk, NY, USA). The continuous variables of molecular and histological assessments were compared between groups by a Mann–Whitney U test. A p value < 0.01 using a Bonferroni adjustment as a multiple pairwise comparison. For comparison among the three experimental groups in the other behavioral assessments, one-way ANOVA with least significant difference (LSD) for post-hoc comparison was conducted. All graphical artworks were produced using GraphPad Prism version 8.4.3 (GraphPad Software lnc., San Diego, CA, USA) version 8.4.3 (GraphPad Software lnc., San Diego, CA, USA)

III. RESULTS

1. EE Improves Motor Coordination and Strength in HIE Mice

HI mice were randomly allocated to either EE cages (**Figure 1A**) or standard cages (**Figure 1B,C**) at 6 weeks of age. Behavioral assessments were conducted based on the experimental scheme (**Figure 1D**).

The HI EE-treated group showed significant improvement and functional recovery in all examined motor function tests. A rotarod test in both accelerating (4–80 rpm, **Figure 2A**) and constant (48 rpm, **Figure 2B**) paradigms showed that NOR mice had significantly higher latency to fall than HI CON mice and HI EE mice right before the initiation of the housing condition, respectively (NOR v. HI CON, # $p < 0.01$, ## $p < 0.002$, ### $p < 0.0002$; NOR v. HI EE, $ $p < 0.01$, $$ $p < 0.002$, $$$ $p < 0.0002$). The differences between HI EE mice and NOR mice was not statistically significant throughout the condition period. The improved motor function of HI EE mice was maintained throughout the condition period in both accelerating and constant paradigms, compared to those of HI control mice (* $p < 0.01$, ** $p < 0.002$, *** $p < 0.0002$). Similarly, the ladder walking test showed that EE mice had a significant reduction in delta (post–pre) left limb slip rate compared to that of HI control mice, and NOR mice compared to that of HI control mice, respectively (* $p < 0.05$, ** $p < 0.01$, **Figure 2C**). HI EE mice had a significantly higher delta (post–pre) latency to fall compared to that of HI control mice, and NOR mice compared to that of HI control mice, respectively (* $p < 0.05$, *** $p < 0.001$, **Figure 2D**).

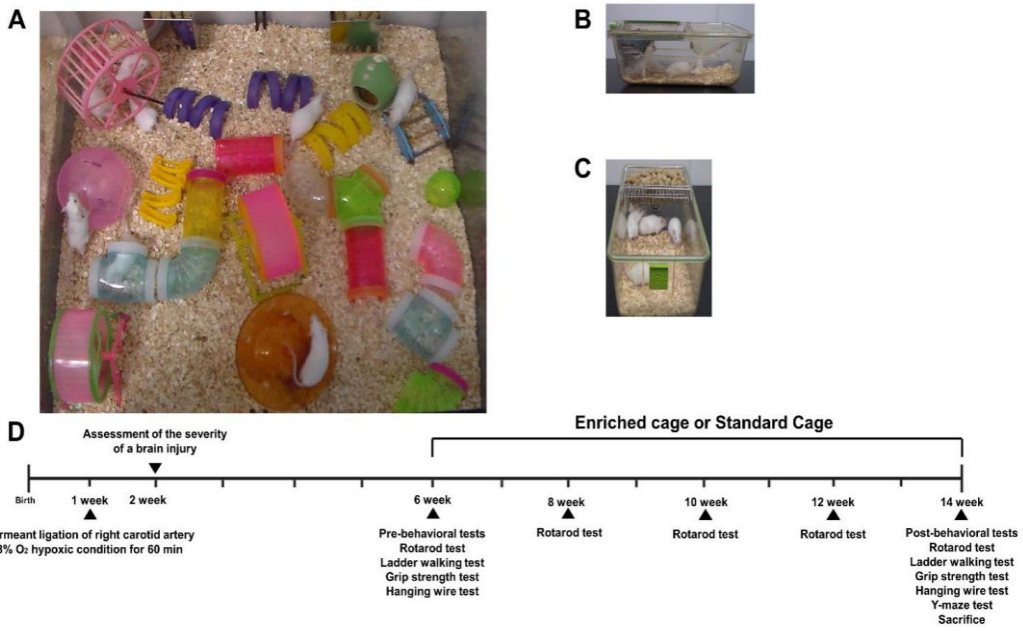


Figure 1. The experimental scheme of this study. (A) The representative picture of an environmental enrichment (EE) cage. (B,C) The representative pictures of standard (control) cages. (D) Schematic overview of the experimental design. A total of 60 mild HI mice were selected and randomly separated into 2 groups (control, N = 30; EE, N = 30), 7 days after surgery based on brain severity, and a total of 15 normal, intact mice were allocated to the standard cages. The location of EE objects was changed once every three days. At 14 weeks of age, all mice were sacrificed for molecular and histological analysis.

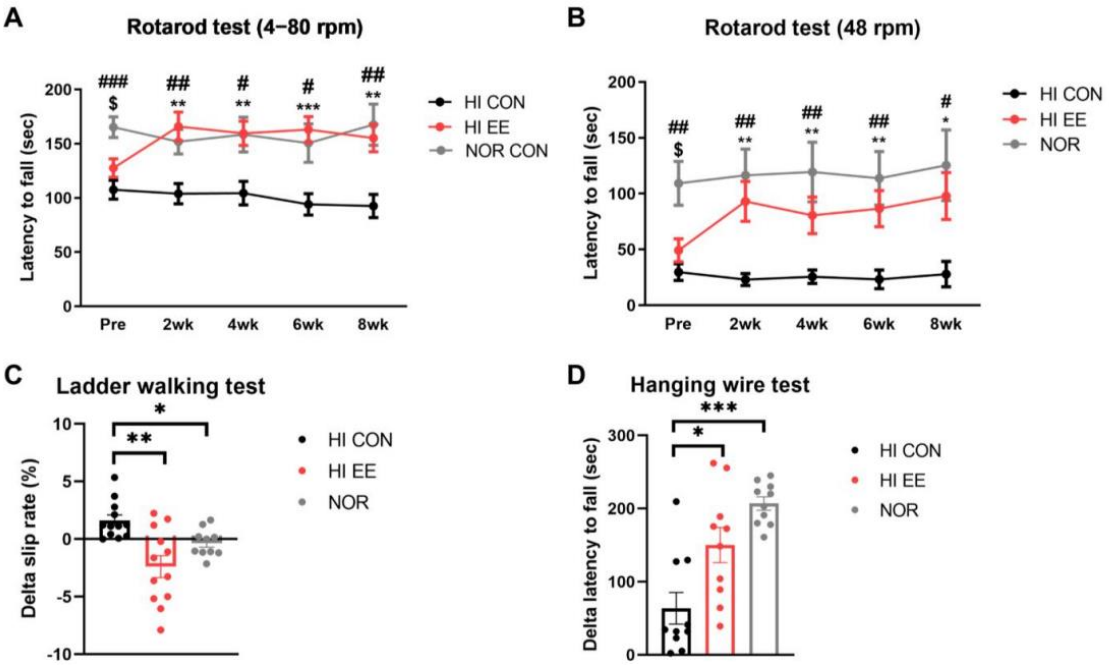


Figure 2. EE improves motor coordination and strength in HIE mice. (A) Accelerating rpm rotarod performance (4–80 rpm) at 2-week interval. The HI EE group and the NOR group significantly outperformed the HI control group throughout the condition period (Bonferroni multiple comparisons test). (B) Constant rpm rotarod performance (48 rpm) at 2-week interval (Bonferroni multiple comparisons test). The HI EE group and the NOR group significantly outperformed the HI control group throughout the study period. Data are expressed as mean ± SEM with 30 mice for the HI CON and EE groups, and 15 mice for the NOR group. The asterisk (*) indicates a significant difference between the HI CON group and the HI EE group (** $p < 0.002$, *** $p < 0.0002$). The pound sign (#) indicates a significant difference between the HI CON group and the NOR group (# $p < 0.01$, ## $p < 0.002$, ### $p < 0.0002$). The dollar sign ($) indicates a significant difference between the HI EE group and the NOR group ($ $p < 0.01$). (C) Ladder walking tests were performed at week 6 and week 14. Significant differences in delta left limb slip rate (post–pre) were observed between the HI CON group and the HI EE group, and in the HI CON group and

the NOR group, respectively, over the condition period (* $p < 0.05$, ** $p < 0.01$, the least significant difference test). Data are expressed as mean ± SEM with 12 mice for the HI groups and 10 mice for NOR group. (D) Hanging wire tests were performed at week 6 and week 14. Significant differences in delta latency to fall (post–pre) were observed between the HI control group and the HI EE group, and in the HI CON group and the NOR group, respectively (* $p < 0.05$, *** $p < 0.001$, the least significant difference test). Data are expressed as mean ± SEM with 10 mice for all groups. HI, hypoxic–ischemic; CON, control; NOR, normal intact.

2. EE Improves Short-Term Spatial Memory in HIE Mice

EE Improves Short-Term Spatial Memory in HIE Mice The HI EE-treated group showed significant improvement and functional recovery in cognitive function. Raw scores of the alternative behavior and number of entries are represented in Figure 3A,B, respectively. Although the raw scores were not significantly different among the three groups, the HI EE group and NOR intact group had significantly fewer total entries than the HI control group (** $p < 0.01$, * $p < 0.05$). This result is similar to the maze results of previous studies, and indicates that long-term exposure to EE may decrease levels of anxiety, as indicated by the significantly low number of total entries.[56,57] Overall, HI EE mice and NOR intact mice had a significantly higher alterative behavior percent compared to that of HI control mice (* $p < 0.05$, ** $p < 0.01$, **Figure 3C**). This result indicates that HI EE mice can retain fine working short-term memory after long-term exposure to EE.

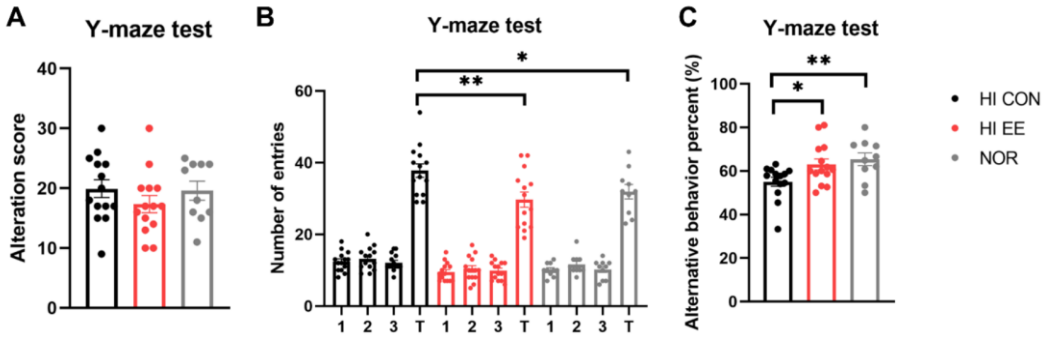


Figure 3. EE improves short-term spatial memory in HIE mice. (A) Raw alternation scores of HI control, EE, and NOR mice in the Y-maze. There was no significant difference among the groups in raw alternation scores. (B) Number of arm entries in the Y-maze. There was a significant difference in total entries of the HI CON group compared to the HI EE group, and in the HI CON group compared to the NOR group, respectively (** $p < 0.01$, * $p < 0.05$, the least significant difference test). (C) Alternation percent in the Y-maze test. A significant increase was observed in the HI EE group compared to the HI control group, and in the NOR group compared to the HI CON group, respectively (* $p < 0.05$, ** $p < 0.01$, the least significant difference test). Data are mean ± SEM with 14 mice for the HI groups and 10 mice for NOR group.

3. EE Upregulates the Expression of Cav 2.1 in the Cerebral Cortex and Hippocampus in HIE Mice

To examine EE-induced changes in gene expression in the synaptic proteins, a qRT-PCR was performed. In my qRT-PCR analysis, EE mice showed a significant increase in the mRNA expression of Cav 2.1 compared to that of HI control mice in the cerebral cortex (** $p < 0.01$, **Figure 4A**) and hippocampus (** $p < 0.01$, **Figure 4B**). To examine EE-induced changes in protein expression in the synaptic proteins, a Western blot (WB) was performed. The representative WB images of the Cav 2.1 protein are shown in Figure 4C. In WB analysis, EE mice showed a significant increase in the protein expression of Cav 2.1 compared to that of HI control mice in the cerebral cortex (* $p < 0.05$, **Figure 4D**) and hippocampus (** $p < 0.01$, **Figure 4E**).

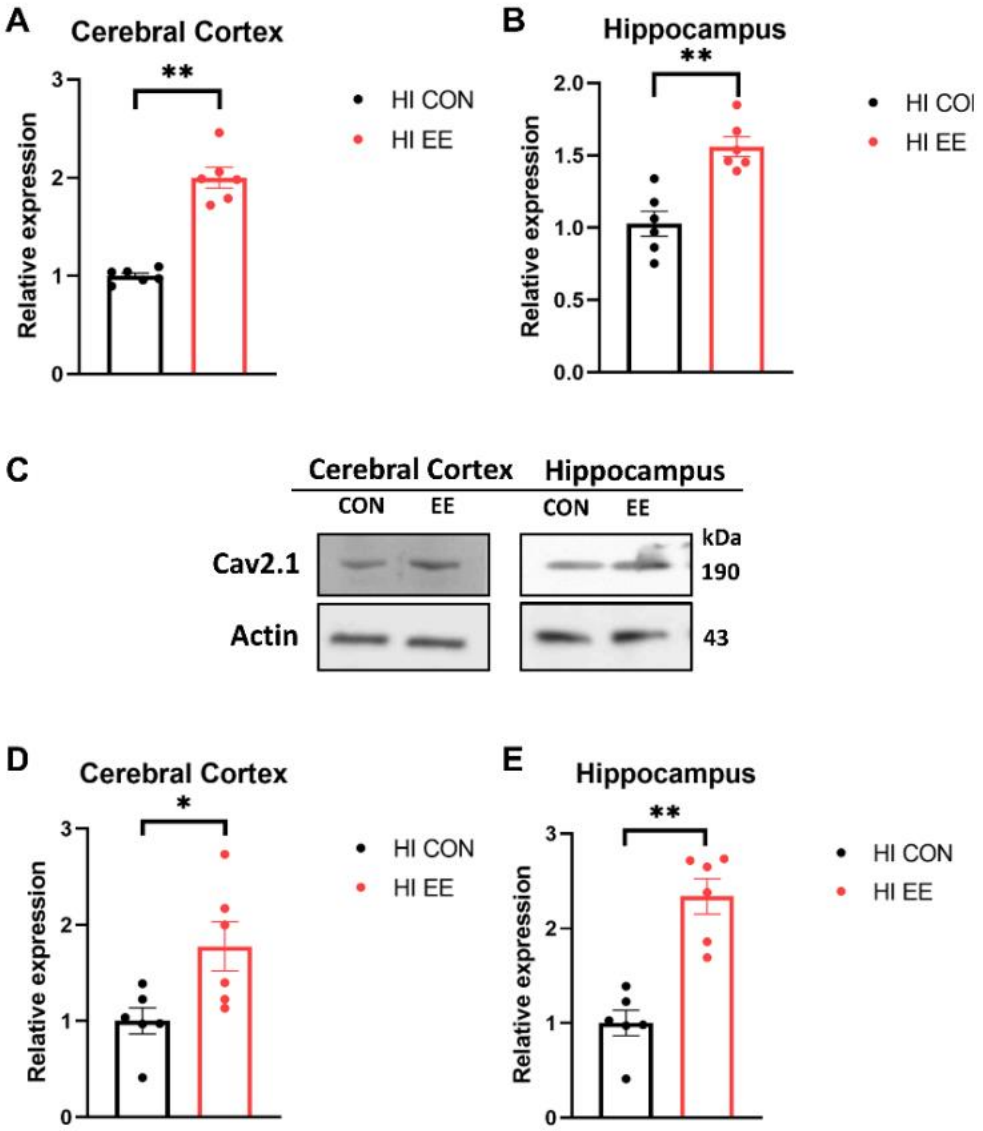
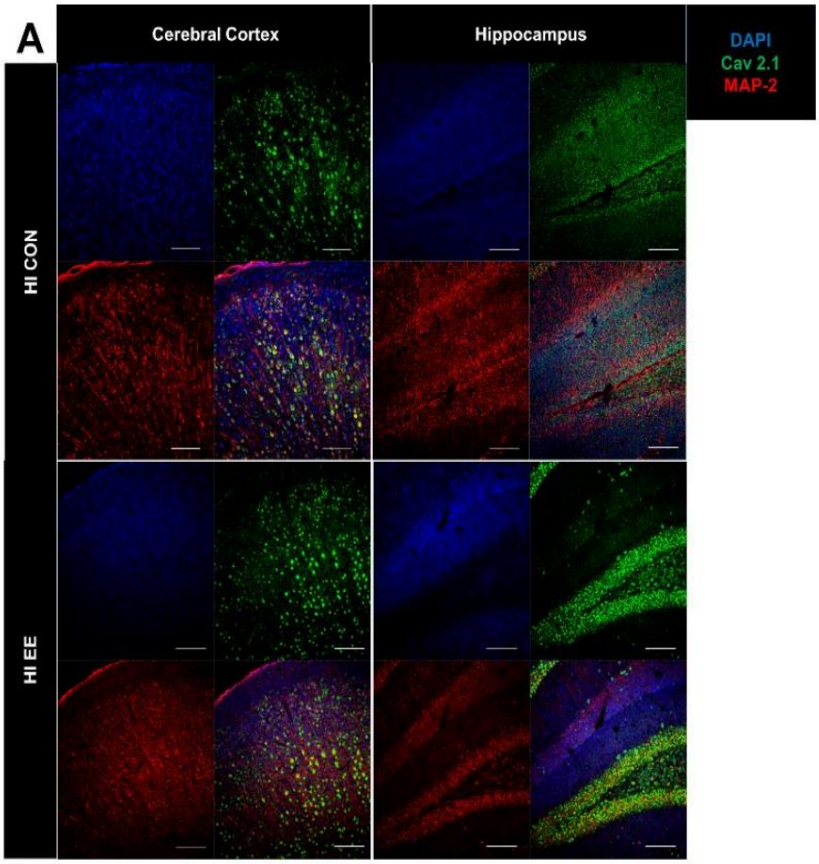


Figure 4. EE significantly increases the expression of Cav 2.1 in the cerebral cortex and hippocampus in HIE mice. (A,B) The qRT-PCR results of Cav 2.1 in the cerebral cortex and hippocampus. A significant difference was observed between HI control mice and HI EE mice in the cerebral cortex and hippocampus. (C) The representative Western blot (WB) images of Cav 2.1 cerebral cortex and hippocampus. (D,E) A quantification of

Cav 2.1 protein expression in the cerebral cortex and hippocampus. A significant difference was observed between HI control mice and HI EE mice in the cerebral cortex and hippocampus. Molecular data are expressed as mean ± SEM with 6 mice per data are expressed as mean ± SEM with n the cerebral cortex and hippocampus. Molecular data are expressed as mean ± SEM with 6 mice per group (* $p < 0.05$, ** $p < 0.05$,** $p < 0.01$, Mann-Whithey U test).

4. EE Induces Presynaptic Plasticity through the Higher Colocalization of Cav2.1 with MAP2 in the Cerebral Cortex and Hippocampus in HIE Mice

To validate EE-induced changes in the expression of Cav 2.1 and colocalization with a specific neuronal subtype, immunohistochemistry was performed. The representative confocal images of the Cav 2.1 and MAP2 proteins are shown in Figure 5A. In the immunohistochemistry (IHC) analysis, EE mice had a significantly higher number of Cav 2.1 positive cells in the cerebral cortex (* $p < 0.05$, Figure 5B) and hippocampus (* $p < 0.05$, Figure 5C), and a higher area of Cav 2.1+MAP2+ cells compared to that of HI control mice in the cerebral cortex (** $p < 0.01$, Figure 5D) and hippocampus (** $p < 0.01$, Figure 5E).



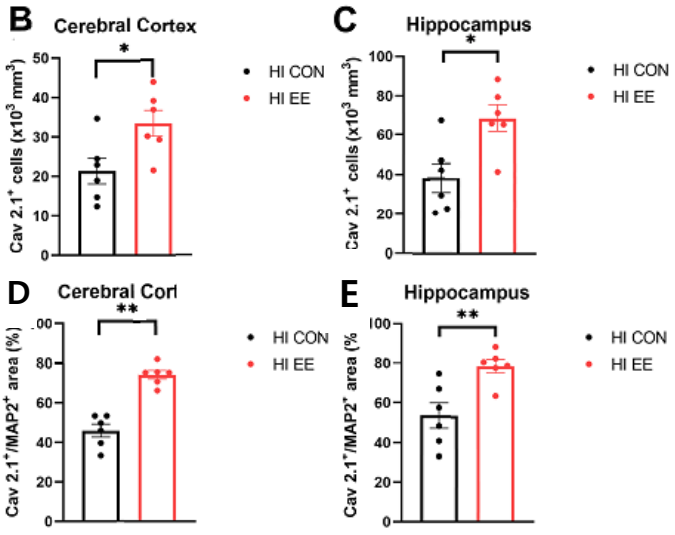


Figure 5. EE mediates presynaptic plasticity through higher colocalization of Cav2.1 with MAP2 in the cerebral cortex and hippocampus in HIE mice. (A) The representative confocal images of Cav 2.1 and MAP2 in the cerebral cortex and hippocampus. A white bar is 100 μm. (B,C) The number of Cav 2.1+ cells in the cerebral cortex and hippocampus was significantly different between HI control mice and HI EE mice. (D,E) A significant difference in the area of Cav 2.1+ MAP2+ was observed between HI control mice and HI EE mice in the cerebral cortex and hippocampus, respectively. Cav2.1, Cav2.1 P/Q voltage-dependent calcium channel; MAP2, microtubule associated protein 2, a mature neuronal marker; DAPI, 4′,6-diamidino-2-phenylindole, nuclear staining. Histological data are expressed as mean ± SEM with 6 mice per group (* $p < 0.05$, ** $p < 0.01$, Mann–Whitney U test).

5. EE Upregulates the Expression of Presynaptic Proteins in the Cerebral Cortex and Hippocampus in HIE Mice

The representative WB images of the synaptic protein are shown in Figure 6A. In WB analysis, EE mice showed a significant increase in the protein expression of Munc 13 (* $p < 0.05$), Rabphilin 3A (** $p < 0.01$), Munc 18 (** $p < 0.01$), VAMP2 (** $p < 0.01$), SNAP25 (* $p < 0.05$), and Syntaxin (* $p < 0.05$), compared to those of HI control mice in the cerebral cortex (**Figure 6B**). EE mice showed a significant increase in Munc 13 (** $p < 0.01$), Rabphilin 3A (** $p < 0.01$), Munc 18 (** $p < 0.01$), VAMP2 (** $p < 0.01$), SNAP25 (** $p < 0.01$), and Syntaxin (** $p < 0.01$), compared to those of HI control mice in the hippocampus (**Figure 6C**).

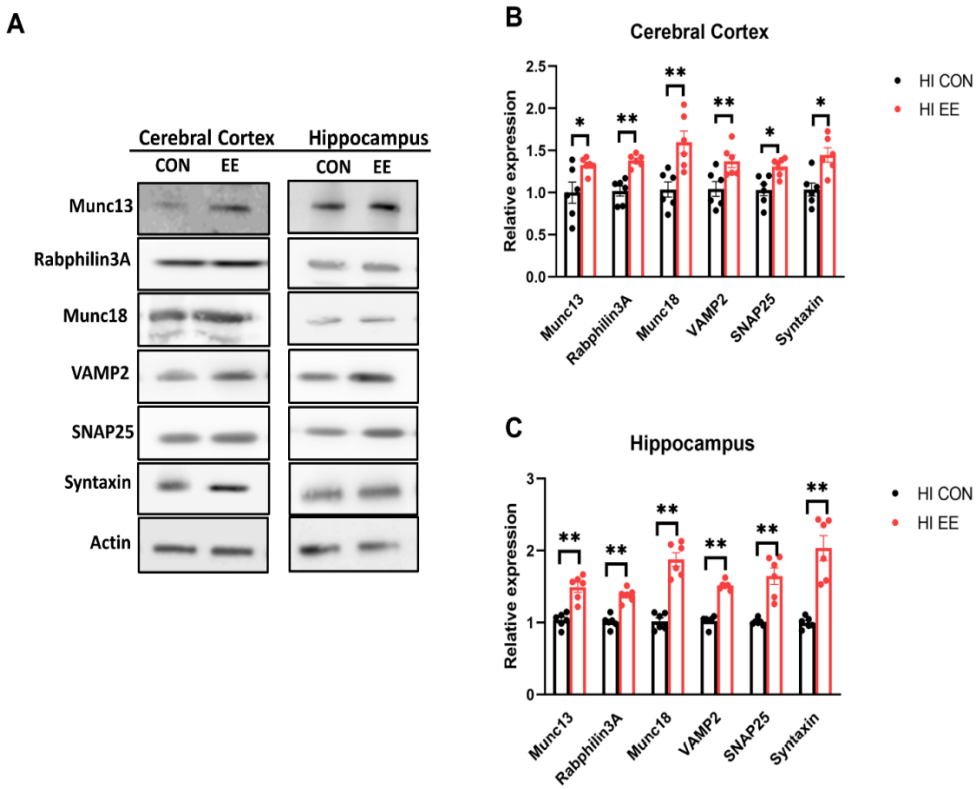


Figure 6. EE Upregulates the Expression of Presynaptic Proteins in the Cerebral Cortex and Hippocampus in HIE Mice. The representative WB images of the synaptic protein are shown in Figure 6A. In WB analysis, EE mice showed a significant increase in the protein expression of Munc 13 (* $p < 0.05$), Rabphilin 3A (** $p < 0.01$), Munc 18 (** $p < 0.01$), VAMP2 (** $p < 0.01$), SNAP25 (* $p < 0.05$), and Syntaxin (* $p < 0.05$), compared to those of HI control mice in the cerebral cortex (**Figure 6B**). EE mice showed a significant increase in Munc 13 (** $p < 0.01$), Rabphilin 3A (** $p < 0.01$), Munc 18 (** $p < 0.01$), VAMP2 (** $p < 0.01$), SNAP25 (** $p < 0.01$), and Syntaxin (** $p < 0.01$), compared to those of HI control mice in the hippocampus (**Figure 6C**). Figure 5. EE mediates presynaptic plasticity through higher colocalization of Cav2.1 with MAP2 in the cerebral cortex and hippocampus in HIE mice. (A) The representative confocal images of Cav 2.1 and MAP2 in the cerebral cortex and hippocampus. A white bar is 100 μm.

(B,C) The number of Cav 2.1+ cells in the cerebral cortex and hippocampus was significantly different between HI control mice and HI EE mice. (D,E) A significant difference in the area of Cav 2.1+ MAP2+ was observed between HI control mice and HI EE mice in the cerebral cortex and hippocampus, respectively. Cav 2.1, Cav 2.1 P/Q voltage-dependent calcium channel; MAP2, microtubule associated protein 2, a mature neuronal marker; DAPI, 40 ,6-diamidino-2-phenylindole, nuclear staining. Histological data are expressed as mean ± SEM with 6 mice per group (* $p < 0.05$, ** $p < 0.01$, Mann–Whitney U test).

6. Omega Agatoxin injection impairs EE-mediated Motor Coordination and Strength improvement in HIE Mice

HI brain injury was induced at 1 week of age and mice were randomly divided into four groups at 6 weeks of age: control + PBS injection, EE + PBS injection, control + Agatoxin injection, and EE + Agatoxin injection groups. Groups that received EE were assigned to EE cages (**Figure 1A**) and control groups were assigned to standard cages (**Figure 1B**). Schematic diagram of the experiment design and omega agatoxin injection is shown (**Figure 7A,B)**.

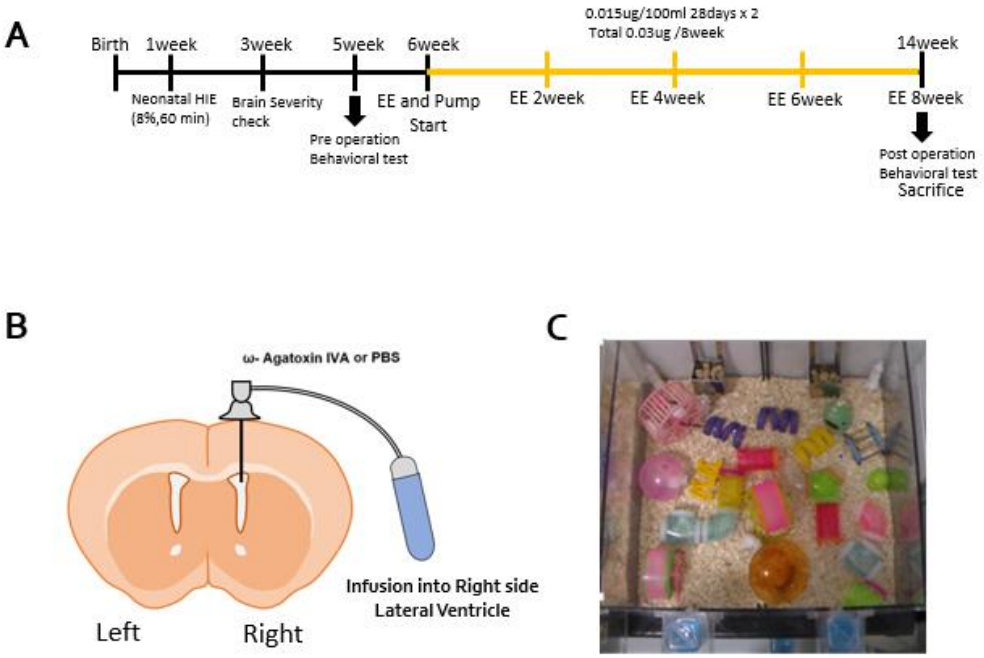


Figure 7. The experimental scheme of this study. (A) Schematic of the experimental design. A total of 32 mild HI mice were selected and randomly separated into 4 groups (Con+PBS, N = 8; EE+PBS, N = 8; Con+Agatoxin, N=8; EE+Agatoxin, N=8), 7 days after surgery based on brain severity, and a total of 16 mice(Con+PBS, Con+Agatoxin) were allocated to the standard cages. The location of EE objects was changed once every three days. At 14 weeks of age, all mice were sacrificed for molecular and histological analysis. (B) Schematic diagram. (C) The representative picture of an environmental enrichment (EE) cage.

To investigate whether Cav2.1 blockage affects EE-mediated functional recovery, we first examined motor functions with the rotarod test and the hanging wire test. We focused on the differences between (1) the EE+PBS group and the EE+Agatoxin group, and (2) the control+PBS group and the control Agatoxin group to figure out whether Cav2.1 inhibition specifically impairs EE-mediated functional recovery without harming the baseline functionality. After mice were exposed to either EE or control for 2 months, rotarod tests were performed every 2 weeks to determine if EE improved motor function at a constant speed of 48 rpm. At baseline, there were no significant difference in the results of rotarod test among the groups. A significant time x group interaction of the rotarod test was revealed [Wilk's Lambda = 0.142, $F_{(12, 53)} = 4.839$, $p = 0.001$]. For the main effect of time [Wilk's Lambda = 0.034, $F_{(4, 20)} = 142.172$, $p = 0.001$] was revealed by a two-way repeated measure ANOVA analysis(**Figure 8A)**. Similar results were shown in the hanging wire test (*p < 0.05, ** p < 0.01, **Figure 8B)**. Spatial short-term memory was assessed by Y-maze test. There are significant differences between Con+PBS group and EE+ Agatotoxin group and between EE+PBS group and EE + Agatotoxin group and there are higher number of entries in the Agatotoxin-treated groups. As a result, there are significant differences between EE+PBS group and the other groups in alteration percent. (* p < 0.05, ** p < 0.01, *** p < 0.001 **Figure 9**)

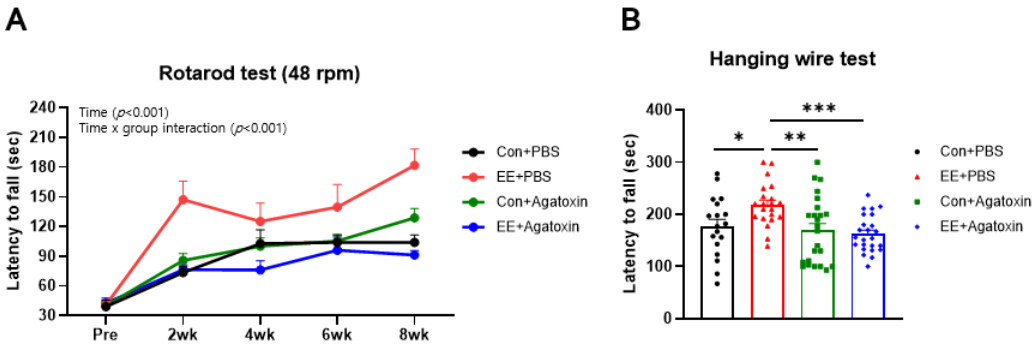


Figure 8. Omega Agatoxin inhibits EE-mediated motor coordination and strength in HIE mice. (A) Constant rpm rotarod performance (48 rpm) at 2-week interval. The EE+PBS group outperformed significantly than the Con+PBS group and EE+Agatoxin group at week2 and week 8. At baseline, there were no significant difference in the results of rotarod test among the groups. A significant time x group interaction of the rotarod test was revealed [Wilk's Lambda = 0.142, $F_{(12, 53)} = 4.839$, $p = 0.001$]. For the main effect of time [Wilk's Lambda = 0.034, $F_{(4, 20)} = 142.172$, $p = 0.001$] was revealed by a two-way repeated measure ANOVA analysis. (B) Hanging wire tests were performed at week 14. Differences in latency to fall (post) were significantly higher in EE+PBS group compared to Con+PBS, Con+Agatoxin and EE+Agatoxin group, respectively (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ least significant differences test). Data are mean ± SEM with 8 mice per group.

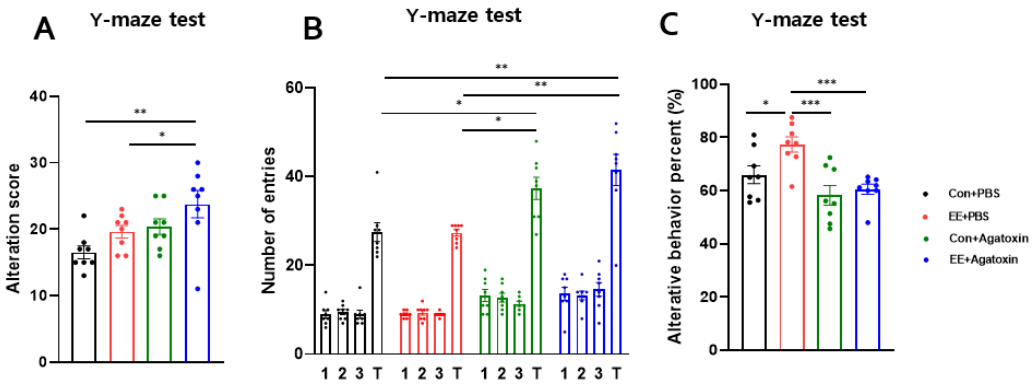


Figure 9. Omega Agatoxin inhibits the improvement in EE-mediated short-term spatial memory in HIE mice.

Y-maze test was performed to investigate short-term spatial memory. There are signficaint differencess between Con+PBS group and EE+Agatoxin group, and EE+PBS and EE+Agatotoxin group in raw alteration score. Reflecting the number of entries, there are significantly higher number of total entries in Con+Agatotoxin and EE+Agatotoxin compared to Con+PBS and EE+PBS. As a result, there are significantly higher alterative behavior percent in EE+PBS compared to the other groups. Omega Agatoxin inhibits the improvement in EE-mediated short-term spatial memory in HIE mice. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ least significant differences test) Data are mean ± SEM with 8 mice per group.

7. Inhibiting Cav 2.1 Decreases Active Zone Protein Expression in the Cerebral Cortex and Hippocampus in HIE Mice

Having established that motor and cognitive function improvement by EE is limited with Cav2.1 inhibition, we examined the mRNA expression levels of active zone proteins to investigate whether behavioral analyses match molecular analyses. qRT-PCR was performed to identify the effect of Cav2.1 inhibition on active zone protein gene expression in two regions: cerebral cortex and hippocampus.

Cerebral cortex active zone protein expression levels demonstrated a similar pattern. VAMP2 and SNAP25 showed significant differences when comparing EE+PBS group with EE+Agatoxin group while omega Agatoxin injection did not affect gene expression outcomes in the control groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$,**Figure 10A,11A**). Rabphilin 3A, Munc18, and Syntaxin mRNA levels remained steady among groups. Cav2.1 expression levels showed a distinct pattern with control+Agatoxin group showing significantly lower Cav2.1 gene expression than every other group.

In the hippocampus, Munc18, VAMP, SNAP25, and Syntaxin showed significant difference between the EE+PBS group and the EE+Agatoxin group, but with no significant difference among the control groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **Figure 10B,11B**). Munc13 and Rabphillin 3A did not show any significant differences among groups. Also, PBS groups that were assigned to EE cages demonstrated higher VAMP2, SNAP25, and Cav2.1 expression compared to control+PBS group mice.

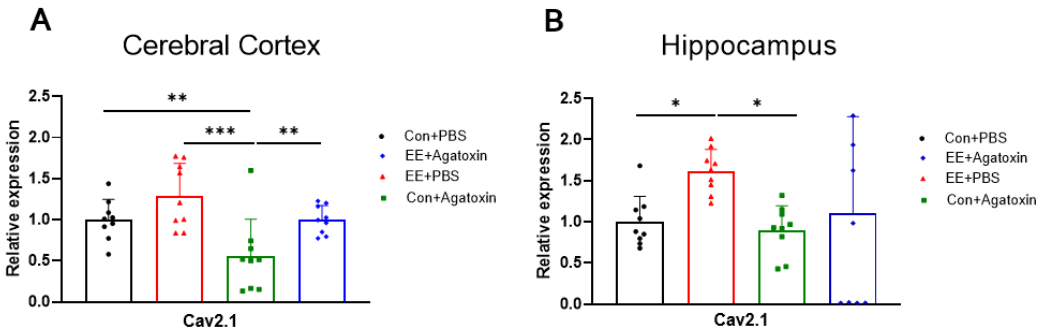


Figure 10. Cav 2.1 inhibition downregulates Cav2.1 gene expression in the cerebral cortex and hippocampus. (A) qRT-PCR results of Cav2.1 expression in the cerebral cortex. There were significant differences in Cav2.1 levels (EE +PBS vs Con+Agatoxin, EE+PBS vs EE+Agatoxin). (B) qRT-PCR results of Cav2.1 expression in the hippocampus. Significant differences were observed in Cav2.1 levels (Con+PBS vs Con+Agatoxin). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, least significant differences test). Data are mean ± SEM with 8 mice per group.

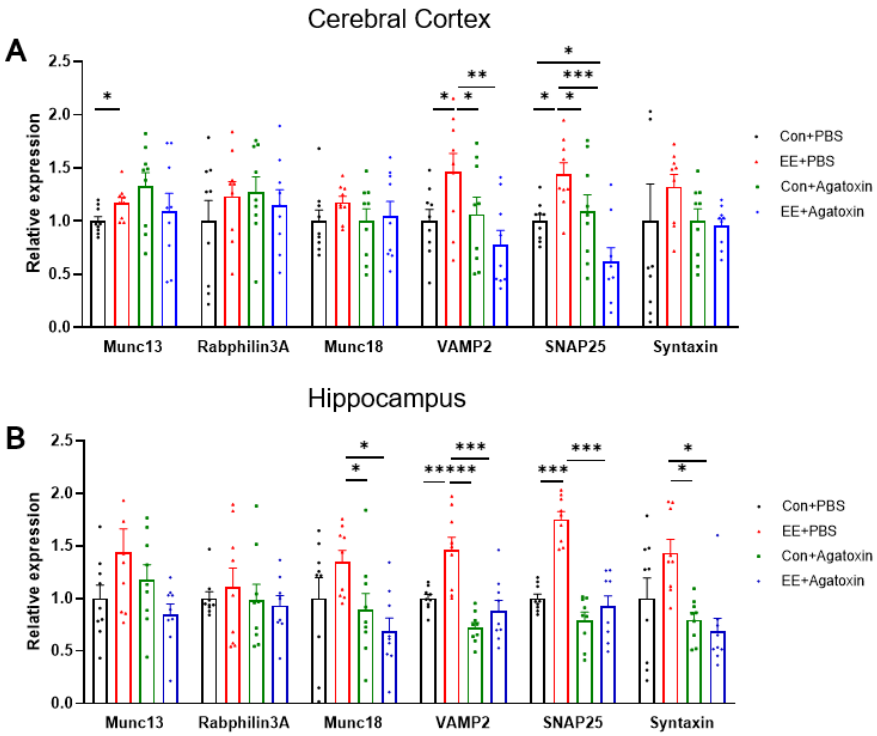


Figure 11. Cav 2.1 inhibition downregulates active zone protein gene expression in the cerebral cortex and hippocampus. (A) qRT-PCR results of active zone protein expression in the cerebral cortex. There were significant differences in Munc18 levels (EE +PBS vs Con+Agatoxin, EE+PBS vs EE+Agatoxin), VAMP2 levels (Con+PBS vs EE+PBS, EE+PBS vs Con+Agatoxin, EE+PBS vs EE+Agatoxin), SNAP25 (Con+PBS vs EE+PBS, EE+PBS vs EE+Agatoxin), and Syntaxin levels (EE+PBS vs Con+Agatoxin, EE +PBS vs EE+Agatoxin). (B) qRT-PCR results of active zone protein expression in the hippocampus. Significant differences were observed in Munc13 levels (Con+PBS vs Con+Agatoxin), VAMP2 levels (Con+PBS vs EE+PBS, EE+PBS vs Con+Agatoxin, EE+PBS vs EE +Agatoxin), and SNAP25 (Con+PBS vs EE+PBS, Con+PBS vs EE+Agatoxin, EE+PBS vs Con+Agatoxin, EE+PBS vs EE+Agatoxin). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, least significant differences test). Data are mean ± SEM with 8 mice per group.

IV. DISCUSSION

HI brain damage in the perinatal period remains one of the main causes of permanent neurodevelopmental impairments and mortality.[1] Present study provided evidence that exposure to EE, starting 35 days after an HI brain injury, can still improve motor and cognitive deficits to the extent of normal intact mice. Moreover, the molecular and histological analysis also revealed that EE upregulates Ca_v 2.1 expression and the presynaptic related proteins in various brain regions, such as the cerebral cortex and hippocampus in HI mice. In addition to these brain regions, I also noticed a significant increase of Ca_v 2.1 and a higher area of $Ca_v 2.1^+MAP2^+$ cells in the striatum of EE mice compared to that of HI control mice.

WB analysis indicated that the significant upregulation of synaptic proteins is prominent in the hippocampal and the neocortical regions. This may be due to the fact that these areas tend to be more sensitive to treatments and stressors, such as oxidative stress, which has more potential to affect brain plasticity.[58-60] HI injury can induce more damage to these brain regions,[61] and this injury may be neuroprotected and more neuroplastic by long-term exposure to EE.

Upregulation in the Ca_v 2.1 expression and the presynaptic related proteins may contribute to behavioral improvements in stroke. Previous studies have shown that exposure to EE can improve behavioral functions through synaptic plasticity in intact and stroke models.[18,24,62-64] However, despite recent data showing that synaptic plasticity is associated with exercise and behavioral improvement, there are only a few basic studies

focusing on the effect of EE on the expression of synaptic proteins in stroke models.[18,65]

Voltage-gated Ca^{2+} (Ca_v) channels play an important role as the primary mediator of membrane depolarization.[66,67] Massive calcium entry through Ca_v channels triggers neuronal firing and neurotransmitter release from synaptic vesicles, which are highly dependent on the physical distance between Ca_v 2.1 and synaptic vesicle-related proteins.[68-70] Ca_v channels can transduce electrical activity into the flow of Ca^{2+} ions that initiate the vesicular release of neurotransmitters at synapses, interacting directly or indirectly with a variety of synaptic proteins in a presynaptic terminal.[45,71-73]

Studies have shown that the functional disruption of Ca_v channels and synaptic loss is accompanied by stroke, and partly reversed by motor rehabilitation with the increased expression of synaptic proteins in the peri-infarct region.[74,75] These previous studies are consistent with my results, in that EE mediated the increased expression of synaptic proteins in the peri-infarct region of the cerebral cortex and hippocampus.[75] Moreover, motor function recovery and motor cortical reorganization can occur at a later stage of stroke through rehabilitative training.[76-78] Therefore, boosting this recovery process and enhancing residual brain synapses and networks are critical for better outcomes of stroke patients.

Previous studies have demonstrated the close relationship between motor improvement, synaptic plasticity, and the altered expression of synaptic proteins.[79-81] Motor improvement is associated with the increase in the expression of presynaptic proteins.[82,83]

Therefore, I aimed to test the role of calcium channel Cav2.1 in the EE-mediated functional recovery of HI brain damage by blocking Cav2.1 with omega Agatoxin. Inhibition of Cav2.1 led to lower functional recovery in terms of cognition and motor function, while the baseline functionality was not harmed significantly with the injection rate that was used. Moreover, molecular and histological analyses matched the functional outcomes that Cav2.1 inhibition hindered the EE-mediated upregulation of active zone proteins in cerebral cortex and hippocampus. Taken together, our study suggests the importance of Cav2.1 mediated neuroplasticity in the mechanism of EE induced functional recovery after HI brain damage.

Also, I previously reported that EE leads to Cav2.1 and presynaptic protein upregulation, thus resulting in functional improvement31. Current findings extend this concept that Cav2.1 mediated neuroplasticity contributes to EE induced functional recovery.

Molecular and histological analyses indicate that EE upregulates active zone proteins but this is impaired with Cav2.1 inhibition. However, this varied among active zone proteins and brain regions. SNARE complex proteins (VAMP, SNAP25, and syntaxin) mostly followed this trend but synaptic vesicle associated protein Rabphilin 3A relatively remained unchanged with omega Agatoxin injection. Between hippocampus and cerebral cortex, active zone proteins showed more fluctuation in the hippocampus compared to cerebral cortex with EE and omega Agatoxin injection. Hippocampal region having relatively high baseline metabolic activity and being most sensitive to hypoxic

conditions[84,85]could have caused better responsiveness to EE.

There are some limitations to this study. First, EE is a complex process that cannot be explained with a single mechanism.[86] Although our findings suggest Cav2.1 as a major player in EE-mediated recovery after HI brain injury, Cav2.1 alone cannot explain EE as a whole, thus requiring further studies that could explain other mechanisms and their complex interplay. Also, strict criterion on subject selection was used. Mild HI mice (less than 20% of cortical cavity) were only included in this study to obtain visible tissues of the cerebral cortex and hippocampus.

V. CONCLUSION

Taken together, EE improves cognitive and motor functions in mice with chronic HI brain injuries that mimic the pathophysiology of human HIE. These beneficial effects of EE may be due to the increased expression of Cav 2.1 in neurons and the upregulation of presynaptic proteins that are related to the synaptic vesicle cycle and neurotransmitter release in the cerebral cortex and hippocampus. Moreover, this study demonstrates that (1) inhibition of Cav2.1 with omega Agatoxin significantly impairs EE-mediated neuroplasticity and that (2) EE mediates presynaptic plasticity through increasing expression of Cav2.1 and colocalization of Cav2.1 with MAP2 in cerebral cortex and hippocampus. Thus, these results suggest that Cav2.1 mediated neuroplasticity could be the mechanism behind cognitive and motor function improvement of EE.

REFERENCE

1. Allen KA, Brandon DH. Hypoxic ischemic encephalopathy: pathophysiology and experimental treatments. Newborn and Infant Nursing Reviews 2011;11:125-33.
2. Robertson CM, Perlman M. Follow-up of the term infant after hypoxic-ischemic encephalopathy. Paediatrics & child health 2006;11:278-82.
3. Thornton C, Leaw B, Mallard C, Nair S, Jinnai M, Hagberg H. Cell death in the developing brain after hypoxia-ischemia. Frontiers in cellular neuroscience 2017;11:248.
4. Northington FJ, Chavez-Valdez R, Martin LJ. Neuronal cell death in neonatal hypoxia-ischemia. Annals of neurology 2011;69:743-58.
5. Reiss J, Sinha M, Gold J, Bykowski J, Lawrence SM. Outcomes of infants with mild hypoxic ischemic encephalopathy who did not receive therapeutic hypothermia. Biomedicine hub 2019;4:1-9.
6. Kim M, Yu JH, Seo JH, Shin Y-K, Wi S, Baek A, et al. Neurobehavioral assessments in a mouse model of neonatal hypoxic-ischemic brain injury. Journal of visualized experiments: JoVE 2017.
7. Hofmeijer J, van Putten MJ. Ischemic cerebral damage: an appraisal of synaptic failure. Stroke 2012;43:607-15.
8. Liu N, Tong X, Huang W, Fu J, Xue X. Synaptic injury in the thalamus accompanies white matter injury in hypoxia/ischemia-mediated brain injury in neonatal rats. BioMed research international 2019;2019.
9. Ten VS, Bradley-Moore M, Gingrich JA, Stark RI, Pinsky DJ. Brain injury and neurofunctional deficit in neonatal mice with hypoxic-ischemic encephalopathy. Behavioural brain research 2003;145:209-19.
10. Muntsant A, Shrivastava K, Recasens M, Giménez-Llort L. Severe perinatal hypoxic-ischemic brain injury induces long-term sensorimotor deficits, anxiety-like behaviors and cognitive impairment in a sex-, age-and task-selective manner in c57bl/6 mice but can be modulated by neonatal handling. Frontiers in behavioral neuroscience 2019;13:7.
11. Berger D, Varriale E, van Kessenich LM, Herrmann HJ, de Arcangelis L. Three cooperative mechanisms required for recovery after brain damage. Scientific reports 2019;9:1-8.
12. Calabresi P, Centonze D, Pisani A, Cupini LM, Bernardi G. Synaptic plasticity in the ischaemic brain. The Lancet Neurology 2003;2:622-9.
13. Catterall WA, Few AP. Calcium channel regulation and presynaptic plasticity. Neuron 2008;59:882-901.

14. Bezprozvanny I, Hiesinger PR. The synaptic maintenance problem: membrane recycling, Ca 2+ homeostasis and late onset degeneration. Molecular neurodegeneration 2013;8:1-14.
15. Liu R, Yuan H, Yuan F, Yang S-H. Neuroprotection targeting ischemic penumbra and beyond for the treatment of ischemic stroke. Neurological research 2012;34:331-7.
16. Nie J, Yang X. Modulation of synaptic plasticity by exercise training as a basis for ischemic stroke rehabilitation. Cellular and molecular neurobiology 2017;37:5-16.
17. Van Praag H, Kempermann G, Gage FH. Neural consequences of enviromental enrichment. Nature Reviews Neuroscience 2000;1:191-8.
18. Song S-Y, Chae M, Yu JH, Lee MY, Pyo S, Shin Y-K, et al. Environmental enrichment upregulates striatal synaptic vesicle-associated proteins and improves motor function. Frontiers in neurology 2018;9:465.
19. Rosbergen IC, Grimley RS, Hayward KS, Brauer SG. The impact of environmental enrichment in an acute stroke unit on how and when patients undertake activities. Clinical rehabilitation 2019;33:784-95.
20. McDonald MW, Hayward KS, Rosbergen I, Jeffers MS, Corbett D. Is environmental enrichment ready for clinical application in human post-stroke rehabilitation? Frontiers in behavioral neuroscience 2018;12:135.
21. Diaz R, Miguel PM, Deniz BF, Confortim HD, Barbosa S, Mendonça MCP, et al. Environmental enrichment attenuates the blood brain barrier dysfunction induced by the neonatal hypoxia-ischemia. International Journal of Developmental Neuroscience 2016;53:35-45.
22. Cechetti F, Worm PV, Lovatel G, Moysés F, Siqueira IR, Netto CA. Environmental enrichment prevents behavioral deficits and oxidative stress caused by chronic cerebral hypoperfusion in the rat. Life sciences 2012;91:29-36.
23. Rojas J, Deniz B, Schuch C, Carletti J, Deckmann I, Diaz R, et al. Environmental stimulation improves performance in the ox-maze task and recovers Na+, K+-ATPase activity in the hippocampus of hypoxic–ischemic rats. Neuroscience 2015;291:118-27.
24. Chae SY, Jang JH, Im GH, Jeong J-H, Jung W-B, Ko S, et al. Physical exercise enhances adult cortical plasticity in a neonatal rat model of hypoxic-ischemic injury: Evidence from BOLD-fMRI and electrophysiological recordings. NeuroImage 2019;188:335-46.
25. Nudo RJ. Recovery after brain injury: mechanisms and principles. Frontiers in human neuroscience 2013;7:887.

26. Jung W-B, Im GH, Chung JJ, Ahn S-Y, Jeon TY, Chang YS, et al. Neuroplasticity for spontaneous functional recovery after neonatal hypoxic ischemic brain injury in rats observed by functional MRI and diffusion tensor imaging. Neuroimage 2016;126:140-50.
27. Rocha-Ferreira E, Hristova M. Plasticity in the neonatal brain following hypoxic-ischaemic injury. Neural plasticity 2016.
28. Vive S, Bunketorp-Käll L, Carlsson G. Experience of enriched rehabilitation in the chronic phase of stroke. Disability and Rehabilitation 2020:1-8.
29. de Boer A, Storm A, Gomez-Soler M, Smolders S, Rué L, Poppe L, et al. Environmental enrichment during the chronic phase after experimental stroke promotes functional recovery without synergistic effects of EphA4 targeted therapy. Human molecular genetics 2020;29:605-17.
30. Vive S, Jean-Luc af Geijerstam HG, Kuhn LB-K. Enriched, task-specific therapy in the chronic phase after stroke: an exploratory study. Journal of Neurologic Physical Therapy 2020;44:145.
31. Südhof TC. The synaptic vesicle cycle. Annu. Rev. Neurosci. 2004;27:509-47.
32. Sakane A, Manabe S, Ishizaki H, Tanaka-Okamoto M, Kiyokage E, Toida K, et al. Rab3 GTPase-activating protein regulates synaptic transmission and plasticity through the inactivation of Rab3. Proceedings of the National Academy of Sciences 2006;103:10029-34.
33. Kaeser PS, Kwon H-B, Blundell J, Chevaleyre V, Morishita W, Malenka RC, et al. RIM1α phosphorylation at serine-413 by protein kinase A is not required for presynaptic long-term plasticity or learning. Proceedings of the National Academy of Sciences 2008;105:14680-5.
34. Wang X, Gong J, Zhu L, Wang S, Yang X, Xu Y, et al. Munc13 activates the Munc18-1/syntaxin-1 complex and enables Munc18-1 to prime SNARE assembly. The EMBO Journal 2020;39:e103631.
35. Yang X, Wang S, Sheng Y, Zhang M, Zou W, Wu L, et al. Syntaxin opening by the MUN domain underlies the function of Munc13 in synaptic-vesicle priming. Nature structural & molecular biology 2015;22:547-54.
36. Shu T, Jin H, Rothman JE, Zhang Y. Munc13-1 MUN domain and Munc18-1 cooperatively chaperone SNARE assembly through a tetrameric complex. Proceedings of the National Academy of Sciences 2020;117:1036-41.
37. Lipstein N, Sakaba T, Cooper BH, Lin K-H, Strenzke N, Ashery U, et al. Dynamic control of synaptic vesicle replenishment and short-term plasticity by Ca2+-calmodulin-Munc13-1 signaling. Neuron 2013;79:82-96.

38. Guillén J, Ferrer-Orta C, Buxaderas M, Pérez-Sánchez D, Guerrero-Valero M, Luengo-Gil G, et al. Structural insights into the Ca2+ and PI (4, 5) P2 binding modes of the C2 domains of rabphilin 3A and synaptotagmin 1. Proceedings of the National Academy of Sciences 2013;110:20503-8.
39. Chang W-P, Südhof TC. SV2 renders primed synaptic vesicles competent for Ca2+-induced exocytosis. Journal of Neuroscience 2009;29:883-97.
40. Lazzell DR, Belizaire R, Thakur P, Sherry DM, Janz R. SV2B regulates synaptotagmin 1 by direct interaction. Journal of Biological Chemistry 2004;279:52124-31.
41. Morgans CW, Kensel-Hammes P, Hurley JB, Burton K, Idzerda R, McKnight GS, et al. Loss of the Synaptic Vesicle Protein SV2B results in reduced neurotransmission and altered synaptic vesicle protein expression in the retina. PLoS One 2009;4:e5230.
42. Gerachshenko T, Schwartz E, Bleckert A, Photowala H, Seymour A, Alford S. Presynaptic G-protein-coupled receptors dynamically modify vesicle fusion, synaptic cleft glutamate concentrations, and motor behavior. Journal of Neuroscience 2009;29:10221-33.
43. Genç Ö, Kochubey O, Toonen RF, Verhage M, Schneggenburger R. Munc18-1 is a dynamically regulated PKC target during short-term enhancement of transmitter release. Elife 2014;3:e01715.
44. Toonen RF, Wierda K, De Wit H, Cornelisse LN, Brussaard A, Plomp JJ, et al. Munc18-1 expression levels control synapse recovery by regulating readily releasable pool size. Proceedings of the National Academy of Sciences 2006;103:18332-7.
45. Lübbert M, Goral RO, Keine C, Thomas C, Guerrero-Given D, Putzke T, et al. CaV2. 1 α1 subunit expression regulates presynaptic CaV2. 1 abundance and synaptic strength at a central synapse. Neuron 2019;101:260-73. e6.
46. Nanou E, Scheuer T, Catterall WA. Calcium sensor regulation of the CaV2. 1 Ca2+ channel contributes to long-term potentiation and spatial learning. Proceedings of the National Academy of Sciences 2016;113:13209-14.
47. Yan J, Leal K, Magupalli VG, Nanou E, Martinez GQ, Scheuer T, et al. Modulation of CaV2. 1 channels by neuronal calcium sensor-1 induces short-term synaptic facilitation. Molecular and Cellular Neuroscience 2014;63:124-31.
48. Song S-Y, Pyo S, Choi S, Oh HS, Seo JH, Yu JH, et al. Environmental Enrichment Enhances Cav 2.1 Channel-Mediated Presynaptic Plasticity in Hypoxic–Ischemic Encephalopathy. 2021;22:3414.
49. Lübbert M, Goral RO, Satterfield R, Putzke T, van den Maagdenberg AM, Kamasawa N, et al. A novel region in the Ca(V)2.1 α(1) subunit C-terminus

regulates fast synaptic vesicle fusion and vesicle docking at the mammalian presynaptic active zone. Elife 2017;6.
50. Han Y, Kaeser PS, Südhof TC, Schneggenburger R. RIM Determines Ca²⁺ Channel Density and Vesicle Docking at the Presynaptic Active Zone. Neuron 2011;69:304-16.
51. Acuna C, Liu X, Gonzalez A, Südhof Thomas C. RIM-BPs Mediate Tight Coupling of Action Potentials to Ca²⁺-Triggered Neurotransmitter Release. Neuron 2015;87:1234-47.
52. Taverna E, Saba E, Rowe J, Francolini M, Clementi F, Rosa P. Role of Lipid Microdomains in P/Q-type Calcium Channel (Ca_v2.1) Clustering and Function in Presynaptic Membranes *. Journal of Biological Chemistry 2004;279:5127-34.
53. Metz GA, Whishaw IQ. The ladder rung walking task: a scoring system and its practical application. JoVE (Journal of Visualized Experiments) 2009:e1204.
54. Hoffman E, Winder SJ. A modified wire hanging apparatus for small animal muscle function testing. PLoS currents 2016;8.
55. Sarnyai Z, Sibille EL, Pavlides C, Fenster RJ, McEwen BS, Tóth M. Impaired hippocampal-dependent learning and functional abnormalities in the hippocampus in mice lacking serotonin1A receptors. Proceedings of the National Academy of Sciences 2000;97:14731-6.
56. Anderson BJ, Rapp DN, Baek DH, McCloskey DP, Coburn-Litvak PS, Robinson JK. Exercise influences spatial learning in the radial arm maze. Physiol Behav 2000;70:425-9.
57. McQuaid RJ, Dunn R, Jacobson-Pick S, Anisman H, Audet M-C. Post-weaning environmental enrichment in male CD-1 mice: impact on social behaviors, corticosterone levels and prefrontal cytokine expression in adulthood. Frontiers in behavioral neuroscience 2018;12:145.
58. Leuner B, Gould E. Structural plasticity and hippocampal function. Annual review of psychology 2010;61:111-40.
59. Kitabatake Y, Sailor KA, Ming G-l, Song H. Adult neurogenesis and hippocampal memory function: new cells, more plasticity, new memories? Neurosurgery Clinics 2007;18:105-13.
60. Wang X, Michaelis EK. Selective neuronal vulnerability to oxidative stress in the brain. Frontiers in aging neuroscience 2010;2:12.
61. Nikonenko AG, Radenovic L, Andjus PR, Skibo GG. Structural features of ischemic damage in the hippocampus. The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology: Advances in Integrative Anatomy and Evolutionary Biology 2009;292:1914-21.

62. Wang C-J, Wu Y, Zhang Q, Yu K-W, Wang Y-Y. An enriched environment promotes synaptic plasticity and cognitive recovery after permanent middle cerebral artery occlusion in mice. Neural regeneration research 2019;14:462.
63. Lee M-Y, Yu JH, Kim JY, Seo JH, Park ES, Kim CH, et al. Alteration of synaptic activity–regulating genes underlying functional improvement by long-term exposure to an enriched environment in the adult brain. Neurorehabilitation and neural repair 2013;27:561-74.
64. Tang Y, Li M-Y, Zhang X, Jin X, Liu J, Wei P-H. Delayed exposure to environmental enrichment improves functional outcome after stroke. Journal of pharmacological sciences 2019;140:137-43.
65. Hong M, Kim M, Kim T-W, Park S-S, Kim M-K, Park YH, et al. Treadmill exercise improves motor function and short-term memory by enhancing synaptic plasticity and neurogenesis in photothrombotic stroke mice. International Neurourology Journal 2020;24:S28.
66. Catterall WA. Voltage-gated calcium channels. Cold Spring Harbor perspectives in biology 2011;3:a003947.
67. Simms BA, Zamponi GW. Neuronal voltage-gated calcium channels: structure, function, and dysfunction. Neuron 2014;82:24-45.
68. Lübbert M, Goral RO, Satterfield R, Putzke T, van den Maagdenberg AM, Kamasawa N, et al. A novel region in the CaV2. 1 α1 subunit C-terminus regulates fast synaptic vesicle fusion and vesicle docking at the mammalian presynaptic active zone. Elife 2017;6:e28412.
69. Heck J, Parutto P, Ciuraszkiewicz A, Bikbaev A, Freund R, Mitlöhner J, et al. Transient confinement of cav2. 1 ca2+-channel splice variants shapes synaptic short-term plasticity. Neuron 2019;103:66-79. e12.
70. Leal K, Mochida S, Scheuer T, Catterall WA. Fine-tuning synaptic plasticity by modulation of CaV2. 1 channels with Ca2+ sensor proteins. Proceedings of the National Academy of Sciences 2012;109:17069-74.
71. Cohen-Kutner M, Nachmanni D, Atlas D. CaV2. 1 (P/Q channel) interaction with synaptic proteins is essential for depolarization-evoked release. Channels 2010;4:266-77.
72. Gandini MA, Souza IA, Fan J, Li K, Wang D, Zamponi GW. Interactions of Rabconnectin-3 with Cav2 calcium channels. Molecular brain 2019;12:1-7.
73. Dolphin AC. Functions of presynaptic voltage-gated calcium channels. Function 2021;2:zqaa027.
74. Chung J-W, Ryu W-S, Kim BJ, Yoon B-W. Elevated calcium after acute ischemic stroke: association with a poor short-term outcome and long-term mortality. Journal of stroke 2015;17:54.

75. Kim SY, Hsu JE, Husbands LC, Kleim JA, Jones TA. Coordinated plasticity of synapses and astrocytes underlies practice-driven functional vicariation in peri-infarct motor cortex. Journal of Neuroscience 2018;38:93-107.
76. Jones TA, Adkins DL. Motor system reorganization after stroke: stimulating and training toward perfection. Physiology 2015;30:358-70.
77. Hatem SM, Saussez G, Della Faille M, Prist V, Zhang X, Dispa D, et al. Rehabilitation of motor function after stroke: a multiple systematic review focused on techniques to stimulate upper extremity recovery. Frontiers in human neuroscience 2016;10:442.
78. Ballester BR, Maier M, Duff A, Cameirão M, Bermúdez S, Duarte E, et al. A critical time window for recovery extends beyond one-year post-stroke. Journal of neurophysiology 2019.
79. Park H-YL, Kim SW, Kim JH, Park CK. Increased levels of synaptic proteins involved in synaptic plasticity after chronic intraocular pressure elevation and modulation by brain-derived neurotrophic factor in a glaucoma animal model. Disease models & mechanisms 2019;12:dmm037184.
80. Rosenberg T, Gal-Ben-Ari S, Dieterich DC, Kreutz MR, Ziv NE, Gundelfinger ED, et al. The roles of protein expression in synaptic plasticity and memory consolidation. Frontiers in molecular neuroscience 2014;7:86.
81. Torres DMC, Cardenas FP. Synaptic plasticity in Alzheimer's disease and healthy aging. Reviews in the Neurosciences 2020;31:245-68.
82. Gutierrez RMS, Real CC, Scaranzi CR, Garcia PC, Oliveira DL, Britto LR, et al. Motor improvement requires an increase in presynaptic protein expression and depends on exercise type and age. Experimental gerontology 2018;113:18-28.
83. Grant SG. Synapse molecular complexity and the plasticity behaviour problem. Brain and neuroscience advances 2018;2:2398212818810685.
84. Payabvash S, Souza LCS, Wang Y, Schaefer PW, Furie KL, Halpern EF, et al. Regional Ischemic Vulnerability of the Brain to Hypoperfusion. 2011;42:1255-60.
85. Cervós-Navarro J, Diemer NH. Selective vulnerability in brain hypoxia. Crit Rev Neurobiol 1991;6:149-82.
86. Kempermann G. Environmental enrichment, new neurons and the neurobiology of individuality. Nature Reviews Neuroscience 2019;20:235-45.

ABSTRACT (IN KOREAN)

저산소성 허혈성 뇌 손상의 마우스 모델에서 재활환경이 시냅스활성 영역 단백질에 미치는 영향

<지도교수 조 성 래>

연세대학교 대학원 생체공학

송 석 영

EE(Environmental Enrichment)는 물리적, 인지적, 사회적 구성 요소를 복합적으로 자극하는 고전적인 패러다임이다. EE 는 미성숙한 뇌의 생화학 및 형태학적 변화를 유도하고 동물모델에서 운동 및 인지기능의 향상을 촉진 할수 있어 신경가소성과 신경보호 효과를 발휘할 수 있다. 저산소성 허혈성 뇌병증(HIE)은 산소 부족과 제한된 혈류로 인해 발생하는 치명적인 신생아 뇌 질환이다. 그러나 HIE 의 만성상태에 대한 EE 의 정확한 기전은 불분명하다.

본 연구에서는 신생아 저산소성 허혈성 뇌손상 마우스 모델과 비손상 마우스 모델에서 재활환경이 전시냅스 활성영역 단백 발현에 미치는 영향을 분석하였고 게다가 Cav2.1 칼슘채널을 억제 하였을때의 영향도 확인 하였다. 이를위해 생후 7 일된 마우스를 편측 경동맥 결찰 후 저산소 환경 (8% O2)에 60 분간 노출 하였다. 6 주령에 HIE 마우스를 표준 사육환경(대조군) 또는 EE 케이지에 무작위로 배정하였다. 신경기능을 평가 하기 위해 로타로드 테스트, 사다리 걷기 테스트 및 행잉 와이어 테스트, Y-미로 테스트에서 유의하게 개선된 운동 성능과 편측마비 모델에서 비대칭성을

개선하였고 인지 능력이 향상되었음을 보여주었다. 특히, EE 마우스는 분자 생물학 평가에서 Cav 2.1(P/Q 유형) 및 시냅스전 단백질의 유의한 증가를 보였고, 대뇌 피질 및 해마의 조직학적 평가에서 Cav 2.1 의 유의한 증가를 보였다.

이러한 결과는 EE 가 HIE 의 운동 및 인지 개선을 담당할 수 있는 시냅스 소포 주기 및 신경전달물질 방출과 관련된 Cav 2.1 채널 및 시냅스전 단백질의 발현을 상향조절 할 수 있음을 나타냈다. 게다가 이 연구는 오메가 아가톡신으로 Cav2.1 을 억제하면 EE 매개 신경 가소성을 크게 손상시키고, EE 가 대뇌 피질과 해마에서 Cav2.1 의 발현과 신경기능 향상을 통해 시냅스 전 가소성을 매개한다는 것을 보여준다. 따라서 이러한 결과는 Cav2.1 매개 신경가소성이 EE 의 인지 및 운동 기능 개선의 이면에 있는 메커니즘이 될 수 있음을 시사한다.

핵심 되는말 : 뇌성마비, 재활환경, 시냅스가소성, 시냅스전 활성영역 단백, Cav 2.1칼슘채널

PUBLICATION LIST

1. **Song SY**, Pyo S, Choi S, Oh HS, Seo JH, Yu JH, Baek AR, Shin YK, Lee Hy, Choi JY, Cho SR. Environmental Enrichment Enhances Cav 2.1 Channel-Mediated Presynaptic Plasticity in Hypoxic–Ischemic Encephalopathy.
International Journal of Molecular Sciences 2021;22(7):3414.

2. **Song SY**, Chae M, Yu JH, Lee MY, Pyo Soonil, Shin YK, Baek AR, Park JW, Park ES, Choi JY, Cho SR. Environmental Enrichment Upregulates Striatal Synaptic Vesicle-Associated Proteins and Improves Motor Function.
Frontiers 2018;16;9:465.

3. Kim MG, Yu JH, Seo JH, Shin YK, Wi SH, Back AR, **Song SY**, Cho SR. Neurobehavioral assessments in a mouse model of neonatal hypoxic-ischemic brain injury. *J Vis Exp 2018;71:89-93.*

4. **Song SY**, Jung YY, Hwang CH, Lee HP, Sok CH, Kim JH, Lee SM, Seo HO, Hyun BK, Choi DY, Han SB, Ham YW, Hwang BY, Hong JT. Inhibitory effect of ent-Sauchinone on amyloidogenesis via inhibition of STAT3-mediated NF-κB activation in cultured astrocytes and microglial BV-2 cells.
Journal of Neuroinflammation. 2014;2;11:118.

5.Oh SB, Hwang CH, **Song SY**, Jung YY, Yun HM, Sok CH, Sung HC, Yi JM, Park DH, Ham YW, Han SB, Hwang BY, Hong JT. Anti-cancer effect of tectochrysin in NSCLC cells through overexpression of death receptor and inactivation of STAT3. *Cancer Latter. 2014;10;353(1):95-103.*