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Repeated Physical Training and Environmental Enrichment Induce Neurogenesis and Synaptogenesis Following Neuronal Injury in an Inducible Mouse Model

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Abstract

Neuronal loss as a consequence of brain injury, stroke and neurodegenerative disorders causes functional impairments ranging from cognitive impairments to physical disabilities. Extensive rehabilitation and training may lead to neuroprotection and promote functional recovery, although little is known about the molecular and cellular mechanisms driving this event. To investigate the underlying mechanisms and levels of functional recovery elicited by repeated physical training or environmental enrichment, we generated an inducible mouse model of selective CA1 hippocampal neuronal loss. Following the CA1 neuronal injury, mice underwent one of the above mentioned conditions for 3 months. Exposure to either of these stimuli promoted functional cognitive recovery, which was associated with increased neurogenesis in the subgranular zone of dentate gyrus and enhanced synaptogenesis in the CA1 subfield. Notably, a significant correlation was found between the functional recovery and increased synaptogenesis among survived CA1 neurons. Collectively, these results support the utilization of cognitive and physical stimulation as approaches to promote recovery after neuronal loss and demonstrate the potential of this novel mouse model for the development of therapeutic strategies for various neurological disorders associated with focal neuronal loss.

Keywords: Hippocampus, Water Maze, Environmental Enrichment, Cognition, Head Injury

1. Introduction

Neuronal loss is the signature feature of numerous neurological conditions, including head injury, stroke, and neurodegenerative disorders such as Alzheimer disease. Depending on the brain regions impacted by the neuronal loss, individuals may experience physical disabilities and/or cognitive impairments. Currently, stroke is the most common single cause of disability, and Alzheimer disease is the leading cause of dementia among elderly, positioning these diseases as major medical concerns in our society [1,2]. Notably, no effective therapeutic strategies have been developed for these conditions so far. Experimentally, stem cell transplant has shown some potential in functional recovery in mouse models [3-5]. However, the technical hurdle implementing of stem cell-based therapeutic approaches in humans is still high and other issues including ethical and safety concerns need to be further evaluated.

Traditionally, rehabilitation has been practically applied to help recovery of lost or impaired functions caused by disease or injury. In the case of brain injuries, physical rehabilitation and social/environmental enrichment have been proposed to stimulate new neuronal connections and enhance neuronal plasticity among survived neurons, promoting functional recovery [6-8]. Endogenous neurogenesis stimulated by rehabilitation has been reported to play a critical role in functional recovery following brain damage [9-12]. However, the underlying mechanisms associated with the functional recovery have not yet been well understood. Therefore, we sought to examine the potential mechanisms and changes following rehabilitation in mice with defined neuronal injury.

To evaluate the effectiveness of rehabilitation after neuronal injury, we developed a novel genetic model of

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hippocampal neuronal loss that is mediated by the expression of diphtheria toxin A (DTA) and tightly regulated by tetracycline-inducible system (Tet-off). Utilization of the calmodulin kinase IIα (CaMKIIα) promoter allows the induction of DTA expression primarily in hippocampal CA1 pyramidal neurons followed by cortical neurons in a time-dependent manner. Of note, our previous studies have demonstrated that induction of this CaMKIIα/TetDTA genetic system for 20 - 25 days results in extensive neuronal loss specifically in the CA1, which causes severe cognitive decline in hippocam- pal-dependent tasks [5]. Using this model, mice underwent 3-months of repeated physical training within the Morris water maze or 3-months of environmental enrichment, mimicking a physical or social rehabilitation paradigm, respectively, following a 21-day induction of neuronal injury. Data presented indicates that both training protocols markedly increased BrdU-positive neurons in the subgranular zone (SGZ) of the dentate gyrus. Of great relevance, we also determined that repeated physical training and environmental enrichment rescues the neuronal loss-induced cognitive impairment as well as the synaptic density in the SGZ and CA1 in mice with neuronal injury. Taken together, our data indicates that rehabilitation following neuronal injury helps functional recovery through upregulation of neurogenesis in the SGZ and synaptogenesis within survived neurons.

2. Materials and Methods

2.1. Animals and Induction of Neuronal Injury

Double transgenic CaMKIIα/Tet-DTA mice or single transgenic Tet-DTA mice were maintained on a 12-hr light/dark cycle and freely accessible to food and water as previously described [5]. Doxycycline (2 mg/g of chow) containing food was given to all mice all the time. CA1 neuronal injury was induced in 6 - 9 month old CaMKIIα/-Tet-DTA mice by withdrawing doxycycline-containing food and replacing it with regular food for 21 days (referred to as lesioned mice). Doxycycline was also withdrawn in Tet-DTA mice for 21 days, but no neuronal loss was triggered due to a lack of CaMKIIα-TRE transgene (referred to as non-lesioned control mice). All procedures were performed in accordance with the regulations of the Institutional Animal Care and Use Committee of the University of California, Irvine.

2.2. Rehabilitation Strategies

1) Repeated physical training (Morris water maze, MWM) - Two weeks after the induction of neuronal injury, one group of mice underwent a repeated MWM experience as a physical training group, and another group of mice was not exposed to MWM at all (no training group, n = 11 - 13 per group). Mice in the training group received physical training in the water maze once a month for 3 months (please see below for detailed MWM procedure). During the 3-month training, both groups also received BrdU (50 mg/kg, ip) injections twice a week.

2) Environmental enrichment - After the induction of neuronal injury, mice were placed in environmentally enriched cages or regular cages for 3 months (n = 7 - 8 per group). Each environmentally-rich cage (45 × 30 × 30 cm) consisted of running wheels, various types of plastic or wooden shelters, tube maze, bells, plastic balls and other toys. 3 - 4 mice shared one cage to stimulate social interaction, and toys were cleaned weekly and rotated to different cages. Regular cages (30 × 15 × 20 cm) contained the same bedding but did not have any toys. All mice reeived BrdU (50 mg/kg, ip) injections every other day in the last 2 weeks of the experimental period.

2.3. Behavioral Tests

1) Morris water maze (MWM) test—The apparatus used for the water maze task was a circular aluminum tank (1.2 m diameter) painted white and filled with water maintained at 27˚C. The maze was located in a room containing several simple visual, extramaze cues. To reduce stress, mice were placed on the platform for 10 s prior to the first training trial. Mice were trained to swim to a 14 cm diameter circular clear Plexiglas platform submerged 1.5 cm beneath the surface of the water and invisible to the mice while swimming. On each trial, the mouse was placed into the tank at one of four designated start points in a pseudorandom order. If a mouse failed to find the platform within 60 s, it was manually guided to the platform and allowed to remain there for 10 s. After the trial, each mouse was placed into a holding cage under a warming lamp for 25 s until the start of the next trial. To ensure that memory differences were not due to lack of task learning, mice were given four trials a day for as many days as were required to reach criterion (<20 s mean escape latency before the first probe trial was run). To control for overtraining, probe trials were run for each group, both as soon as they reached group criterion and after all groups had reached criterion. Retention of the spatial training was assessed 24 h after the last training trial. Both probe trials consisted of a 60 s free swim in the pool without the platform. The parameters measured during the probe trial included (1) time spent in the platform quadrant, (2) latency to cross the platform location, and (3) number of platform location crosses.

2) Novel object recognition test—Each mouse was first habituated to an empty Plexiglass arena (45 × 25 ×

20 cm) for 3 consecutive days prior to the actual test. On the first day of testing, mice were exposed to two identical objects placed at opposite ends of the arena for 5 min. In the probe trial 24 h later, mice were presented for 5 min with one of the familiar objects and a novel object of similar dimensions. Exploration counted if the mouse's head was within one inch of the object with its neck extended and vibrissae moving. The recognition index represents the percentage of the time that mice spent exploring the novel object.

3) Contextual fear conditioning test—Each mouse was placed in the fear conditioning chamber (San Diego Instruments, San Diego, CA, USA) and allowed to explore for 2 min before receiving three electric foot-shocks (duration, 1 s; intensity, 0.2 mA; inter-shock interval, 2 min). The mouse was returned to the home cage 30 sec after the last foot-shock. Twenty-four hours later, the mouse was placed back in the chamber, and freezing behavior was recorded during a 5 min examination period.

2.4. Immunohistochemistry and Immunofluorescent Staining

Fixed brain halves were sliced on a vibratome at 50 μM thickness. Brain sections were mounted onto slides, and hematoxylin and eosin (H&E) staining was performed as previously described [5,13].

For BrdU double labeling, sections were first treated with 2 N HCl for 30 min at 37˚C, then neutralized by 0.1 M borate buffer (pH 8.5). Prior to overnight incubation with primary antibody in Tris-buffered saline (TBS) containing 3% serum and 2% BSA at 4˚C, sections were permeabilized with 0.1% Triton X-100 in TBS and blocked in solution containing 2% BSA. After the incubation with primary antibodies, sections were washed and incubated with the appropriate secondary antibody for 1 hr at ambient temperature. Primary antibodies used in this study were anti-BrdU antibody (1:500; Acculate Chemical, West- bury, NY), anti-Iba1 antibody (1:500; Wako, Richmond, VA), anti-GFAP antibody (1:1000; Dako, Glostrup, Denmark), anti-NG2 antibody (1:500; Chemicon, Temecula, CA), anti-NeuN antibody (1:000; Millipore, Billerica, MA), anti-PSD-95 antibody (1:500; Millipore), and anti-synaptophysin antibody (1:500; Sigma, St. Louis, MO). Secondary antibodies were anti-mouse or anti-rabbit conjugated with Alexafluor 488 or Alexafluor 555 (1:200; Invitrogen, Carlsbad, CA). All fluorescent images were captured using a Bio-Rad 2000 confocal microscopy (Bio-Rad Laboratories, Hercules, CA). Fluorescent intensity was quantified by averaging 3 - 5 random fields at 60× objective or higher in each section. Grayscale images were inverted and optical density was quantified using the Image J software. Pixel intensity from two sections per animal were averaged and compared.

2.5. Statistical Analysis

All data were analyzed using one-way ANOVA with post-test (Dunnett or Bonferroni post-test) when comparing three or more groups, or using unpaired t-test when comparing two groups. $p < 0.05$ or lower was considered to be statistically significant.

3. Results

3.1. Repeated Physical Training Improves Cognitive Function after Neuronal Injury

Twenty-one-day induction of DTA by withdrawing doxycycline significantly damaged neurons in CA1 hippocampus and dentate gyrus (DG) of CaMIIα/Tet-DTA (lesioned) mice (**Figures 1(a)** and **(b)**). In CA1 region, the fluorescent in- tensity of NeuN labeling reduced by 44% in lesioned mice (intensity 26.3 ± 0.8) compared to non-lesioned mice (intensity 47.1 ± 1.1), and the somatic layer of CA1 was clearly thinner due to neuronal loss (**Figure 1** and **Supplemental Figure 1**). Similarly, in the molecular layer of DG, the intensity of NeuN labeling reduced by 30% in lesioned mice (intensity 52.8 ± 2.2)

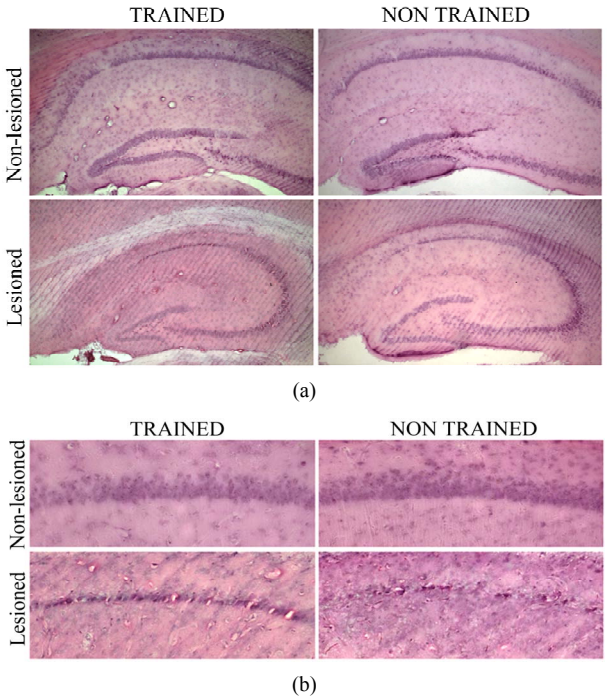

Figure 1. Twenty-one days of induction results in a focal CA1 neuronal lesioning in CaMKIIα/Tet-DTA transgenic mice. (a) Hematoxylin and eosin (H & E) staining clearly indicates thinning in CA1 pyramidal cell layer in lesioned mice, while other areas in hippocampus are not damaged. (b) Magnified images in CA1 pyramidal neuronal loss following the induction.

compared to non-lesioned mice (intensity 75.5 ± 1.9). However, the thickness of the molecular layer of DG was not as clear as that of CA1, suggesting that the neuronal loss in DG was not as robust as that in CA1 at 21-day induction (**Supplemental Figure 1**). The pattern and degree of neuronal injury were controlled and well-defined, consistent with previously reported observations [5]. All induced mice resulted in a similar degree of lesions, minimizing potential variability due to the severity of neuronal injury. After the completion of three repeated MWM training over the three-month period, we first examined cognitive function. Lesioned mice with repeated MWM training were indistinguishable from non-lesioned control mice with or without repeated training in the escape latency and number of platform crosses in MWM, whereas lesioned mice without repeated training showed significant impairments on both parameters (**Figures 2(a)** and **(b)**). The improvement of cognitive function of lesioned mice by the MWM test was not simply due to the repeated exposures to the MWM task as we found no difference in cognitive outcomes between no trained and repeatedly trained non-lesioned mice. To further rule out this possibility, we examined cognition using other tests that these mice had never been exposed to. Similarly, place-based object recognition as well as contextual fear conditioning, both hippocampus-dependent memory tasks, were markedly rescued by repeated training in lesioned mice (**Figures 2(c)** and **(d)**). On the other hand, context-based object recognition, which evaluates hippocampus-independent and corticaldependent memory, was not different in all four groups, and the recognition index was fairly consistent with our previous observation (**Supplemental Figure 2**).

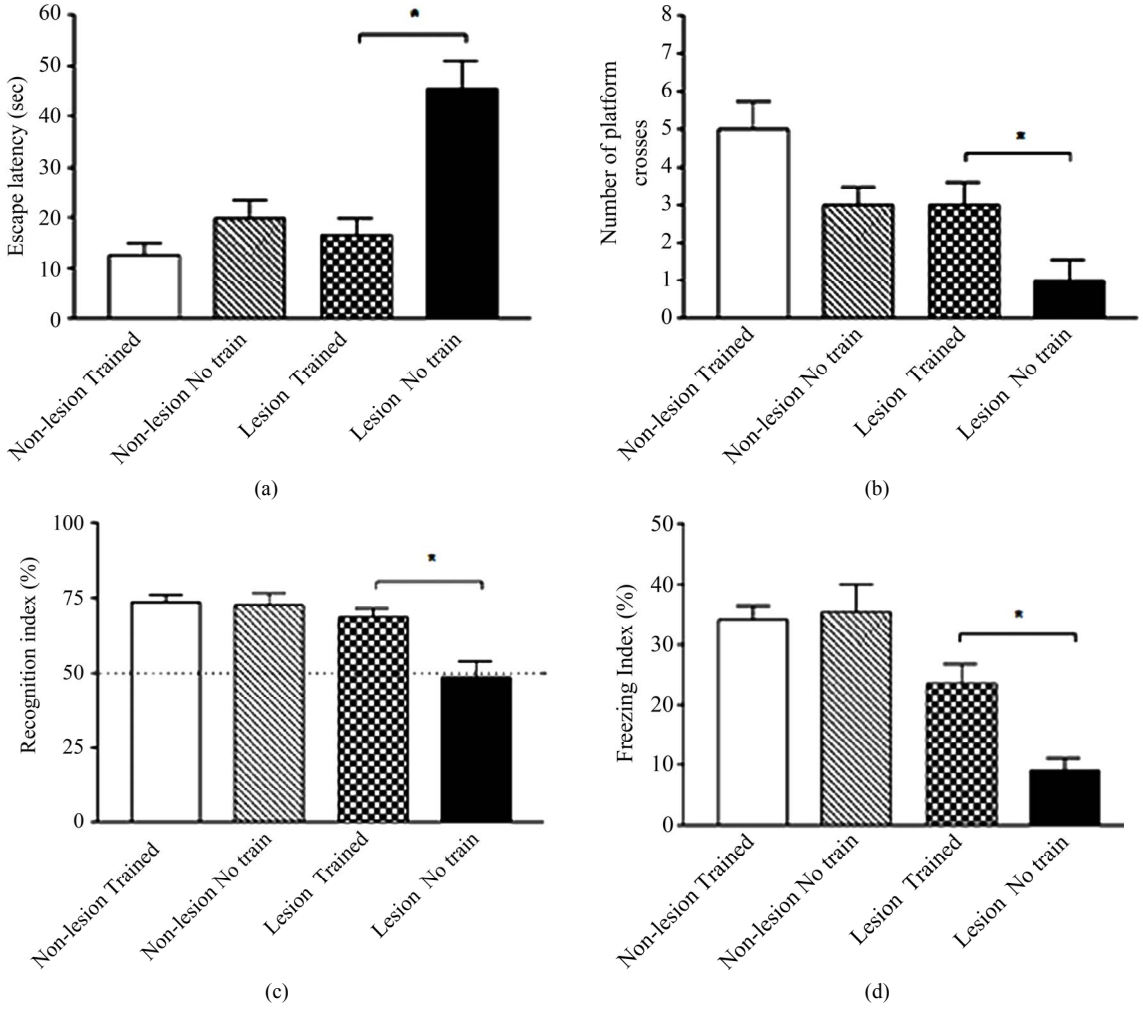

Figure 2. A repeated physical training helps to recover the CA1 lesion-induced cognitive impairment. After 3 months of physical training, all mice were tested with a battery of behavioral tests. (a) Escape latency and (b) number of platform crosses for a 24-hr probe trial of MWM show that a repeated physical training promotes a functional recovery in lesioned mice. (c) Place recognition test and (d) contextual fear conditioning test also detect a marked recovery of hippocampus- dependent cognitive function in lesioned mice. Each graph represents mean ± S.E.M. for n = 11 - 13, and $^{*}p < 0.05$.

3.2. Repeated Physical Training Increases Neurogenesis in Dentate Gyrus

We next examined molecular changes in the brain after the repeated MWM training to explain observed restora- tion of cognition. The overall neurogenesis in subgranular zone/dentate gyrus (SGZ/DG) was significantly increased in mice with physical training regardless of neuronal injury as detected by BrdU and NeuN double labeling (**Figures 3(a)** and **(b)**), suggesting the repeated MWM training itself stimulated neurogenesis over the period of 3 months, consistent with previous findings [11,12]. Notably, it appeared that neuronal injury itself also stimulated neuron-genesis in SGZ as lesioned mice without physical trainingexhibited a marked increase of neurogenesis compared to non-lesioned control mice without physical training (**Figures 3(a)** and **(b)**). The proliferation of other cell types; microglia and astrocytes, was also quantitatively analyzed by counting BrdU/Iba1 (microglia) and BrdU/ GFAP (astrocytes) positive cells in SGZ/DG and CA1 hippocampus. Although neuronal injury significantly increased proliferation of microglia, the repeated MWM training itself did not alter proliferation of both cell types (**Figures 3(c)** and **(d)**).

3.3. Repeated Physical Training Stimulates SynaptoGenesis in Survived Neurons

We further examined whether repeated MWM training modulated synaptic plasticity in remaining neurons in the

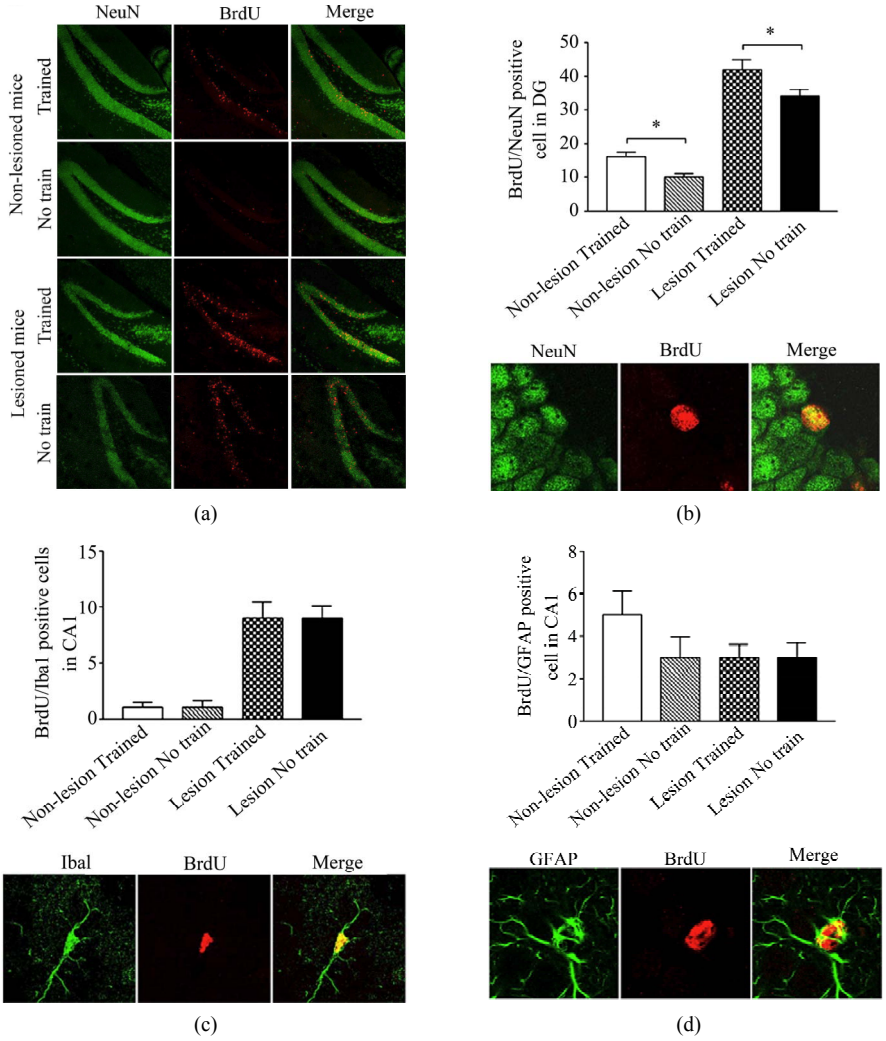

Figure 3. A repeated physical training increases neurogenesis in SGZ. (a) Representative low-magnification images of double immunofluorescent staining of NeuN (green) and BrdU (red) in SGZ. (b) Quantitative analysis of NeuN/BrdU positive newborn neurons in SGZ and representative high-magnification images of NeuN/BrdU double labeled neurons in SGZ. (c, d) Quantitative analyses of Iba1/BrdU (c) and GFAP/BrdU (d) and representative high-magnification images of double labeled cells. Each graph represents mean ± S.E.M. for n = 11 - 13, and $^{*}p < 0.05$.

hippocampus, and subsequently contributed to functional recovery. In this regard, we measured densities of synaptophysin, a pre-synaptic protein, and PSD-95, a post-synaptic protein, in DG and CA1 hippocampus. Notably, synaptophysin in CA1 hippocampus was restored in mice with repeated MWM training, and the levels were almost equivalent to those in non-lesioned control mice (**Figure 4(a)**). Similarly, PSD-95 levels in CA1 were also signifycantly increased following repeated MWM training in lesioned mice (**Figure 4(b)**). Interestingly, neither synaptophysin (**Figure 4(c)**) nor PSD-95 (data not shown) levels in DG were significantly different among four groups.

3.4. Environmental Enrichment Restores Cognition and Stimulates Neurogenesis and Synaptogenesis in Mice with Neuronal Injury

Repeated physical training represented by monthly MWM is a relatively stressful procedure for mice. Acute or chronic stress has been reported to suppress learning and memory function [14-16]. To rule out any negative impacts of stress that may interfere with the beneficial function of rehabilitation, we next evaluated whether less stressful rehabilitation similarly promoted functional recovery and neuronal changes in mice with neuronal injury. In this regard,

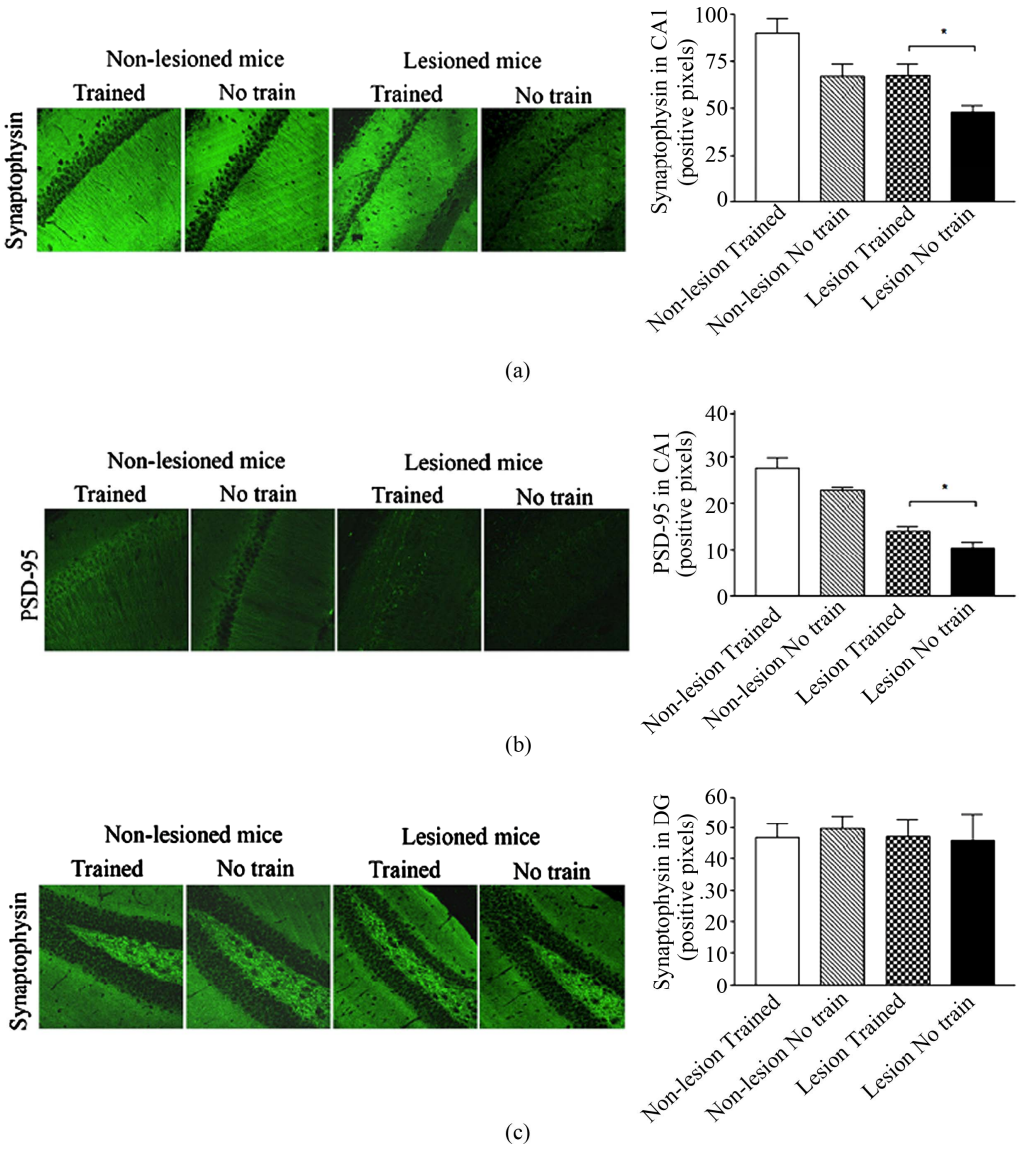

Figure 4. Synaptogenesis in CA1 hippocampus is upregulated by a repeated physical training in lesioned mice. (a) The fluorescent intensity of synaptophysin in CA1 hippocampal region was quantified. (b) The fluorescent intensity of PSD-95 in apical dendrites of CA1 pyramidal neurons was quantified. (c) The fluorescent intensity of synaptophysin in DG was not different among the four groups. Each graph represents mean ± S.E.M. for n = 11 - 13, and $^{*}p < 0.05$.

lesioned mice were housed in environmentally enriched cages. This approach mimicked rehabilitation associated with social and environmental interactions [17]. Three months of environmental enrichment partially improved hippocampus-associated cognitive function as determined by place recognition test. Lesioned mice with environmental enrichment explored novel object significantly more than chance level (50%) while lesioned mice singly housed in a regular cage did not distinguish novel object from familiar object (**Figure 5(a)**). This functional recovery was not due to a recovery of CA1 neurons as both groups showed similar levels of CA1 neuronal thinning or loss (**Figure 5(b)**). Rather, it correlated well with the increased synaptogenesis in survived CA1 neurons (**Figures 5(c)** and **(d)**). Neurogenesis was also markedly increased in mice under the environmental enrichment as previously described in various studies (data not shown) [12,18,19]. Taken together, both repeated physical training and environmental enrichment stimulated neurogenesis in SGZ and synaptic plasticity among survived CA1 neurons and promoted functional recovery after neuronal injury.

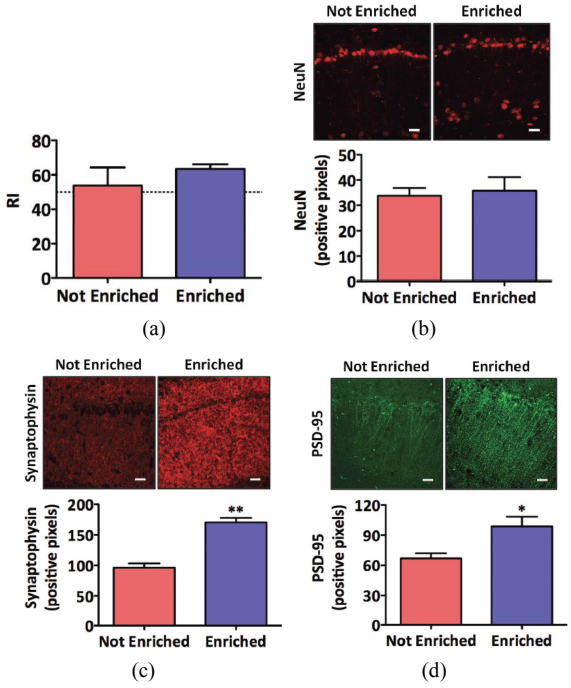

Figure 5. Environmental enrichment promotes functional recovery and synaptogenesis in lesioned mice. (a) Place recognition test was performed after the 3-month environmental enrichment in mice with neuronal injury. Each graph represents mean ± S.E.M. (n = 7 - 8), and *p < 0.05 from the chance (50%) level. (b) Immunofluorescent staining of NeuN in CA1 hippocampus region. (c) Synaptophysin immunofluorescent staining in CA1 region and (d) PSD-95 immunofluorescent staining in CA1 region. Fluorescent intensity was quantified by averaging 3 - 5 random fields at 63x in each section and expressed as mean ± S.E.M. in the graph. *p < 0.05 or **p < 0.01 compared to non-enriched group. Scale bar = 25 μm.

4. Discussion

Functional impairments of the brain are serious medical conditions that adversely affect a patient's life in many ways. Types and severity of impairments are highly dependent on the areas and extent of the damage in the brain. Various diseases and conditions cause brain damage and functional impairments. For example, one-time stroke, hypoxia or traumatic brain injuries result in focal damage and neuronal loss in the brain. These are often non-progressive, and the damaged area is limited. On the other hand, Alzheimer disease (AD), Lewy body dementia or frontotemporal dementia are progressive neurodegenerative diseases, and the damaged areas spread over time together with progressive cognitive and functional impairments. Notably, the incidence of brain injuries and neurodegenerative disorders are increasing every year, and effective treatments and medical support are in immediate demand.

Rehabilitation is one of the most important post-operative care strategies for patients with brain injuries. In order to better understand the therapeutic properties of rehabilitation, we examined the some of the mechanisms associated with the restoration of brain function induced by physical and social/environmental stimulation following neuronal injury in mice. Importantly, our mouse model develops a very defined and temporal pattern of neuronal loss following the induction of DTA by withdrawing doxycycline from the diet. Significant pyramidal neuronal loss in CA1 region is first detected at 15 - 20 days of induction, followed by a neuronal loss in DG and cortecies at 25 - 30 days of induction. We have chosen the 21-day induction, which gave us a defined neuronal loss in CA1 and DG with minimum loss of neurons in cortecies. Subsequently, this induction paradigm impaired predominantly hippocampal-based, but not cortical-based, cognitive function [5]. Notably, it has been well documented that CA1 pyramidal neurons are selectively damaged during global cerebral ischemia [20-22]. Therefore, we utilized this model as a focal neuronal injury model and examined whether repeated physical and social/environmental stimulations rescued clinical phenotypes. We applied repeated Morris water maze as physical training-based stimulation and group-housed environmental enrichment as a social and environmental stimulation for neuronal injury mouse model. A repeated water maze training paradigm has been shown to ameliorate post-seizure-induced cognitive impairments in rats [23]. Both paradigms markedly restored injury-associated cognitive function in our mouse model. Such improvement in the cognitive performance was clearly

associated with increased synaptogenesis among survived neurons in CA1 as well as neurogenesis in SGZ. Similar findings have been reported in different rodent models of brain injuries [6,9-12].

Neurogenesis in SGZ has been reported to play a critical role in memory formation and long-term memory consolidation [24,25]. In various rodent stroke models, increased neurogenesis in SGZ is associated with functional recovery following skilled physical activity [11,22]. Our study showed that the neurogenesis was significantly upregulated not only by repeated physical training but also by CA1 neuronal injury. Interestingly, the increased neurogenesis in SGZ was not sufficient to facilitate functional recovery in our neuronal lesioned mouse model. Therefore, it is speculated that neurogenesis together with increased synaptogenesis among survived neurons in CA1 ameliorate the lesion-induced cognitive impairments. This idea is supported by our recent findings on neural stem cell transplant in a mouse model of AD [26]. We have demonstrated that brain-derived neurotrophic factor (BDNF) secreted from transplanted neuronal stem cells promotes synaptogenesis in hippocampal neurons and rescues AD-associated cognitive impairments without attenuating AD neuropathologies [26]. The relationship between synaptogenesis and BDNF is further supported by multiple studies demonstrating that treatment with neurotrophic factors including BDNF, glial cell-derived neurotrophic factor (GDNF) or insulin-like growth factor-1 (IGF-1) show neuroprotective effects against brain injuries in rodent models [27-29]. Environmental enrichment or exercise has been shown to upregulate BDNF and restore cognitive function in rats with chronic hypoperfusion or ischemia and blockade of BDNF production significantly negated its beneficial effects in rats with ischemia [30-32] Interestingly, BDNF also enhances neurogenesis after the stroke [33]. Promoting synaptogenesis in post-stroke or brain injuries may be a potential therapeutic strategy. In stroke and ischemia models, increased synaptogenesis by pharmacological agents or skilled training facilitates functional recovery [6,34,35]. In this context, the repeated physical and social/environmental stimulations in our paradigm could promote the release of neurotrophic factors, which in turn contribute to the induction of synaptogenesis and functional recovery. However, additional studies are necessary to confirm this hypothesis.

The current results provide functional and molecular evidence indicating that physical and social/environmental stimulations after neuronal injury are ways to effectively facilitate recovery without any invasive methods. Such approaches may have great potential as therapeutic alternatives for neurological disorders associated with neuronal loss.

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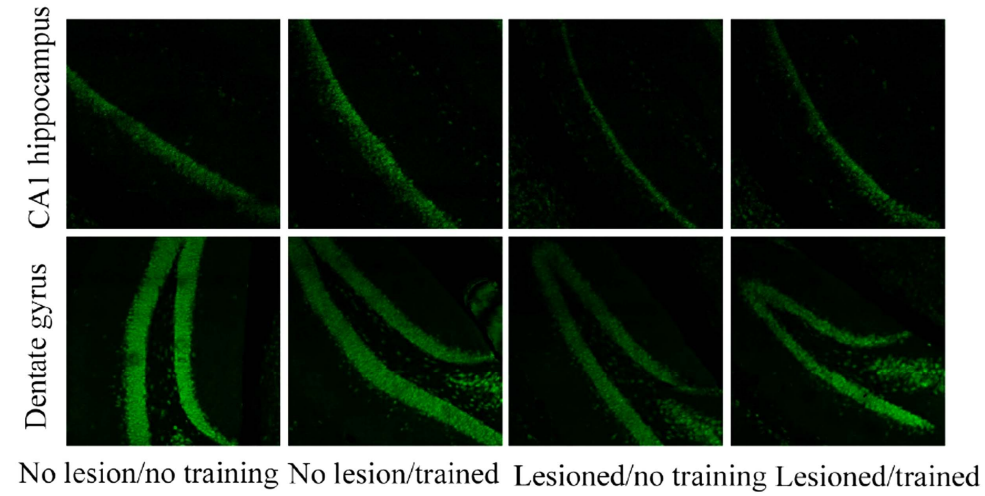
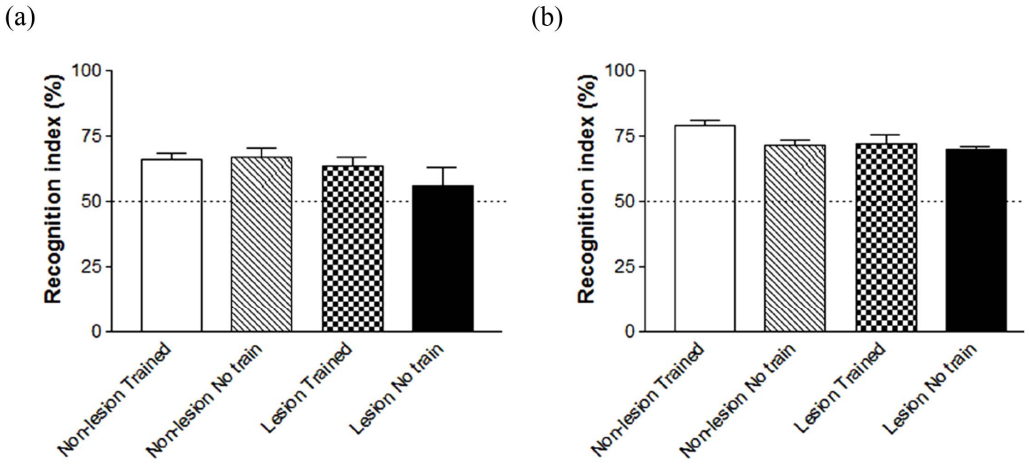
Supplemental Figures



Supplemental Figure 1. Twenty-one days of induction causes neuronal loss in CA1 hippocampus and dentate gyrus in CaMKIIα/Tet-DTA transgenic mice. NeuN immunofluorescent staining shows significant reductions of the intensity in CA1 and dentate gyrus of lesioned mice. Graphs represent changes in the NeuN intensity between the two groups (p < 0.01). No significant changes are observed between no training and trained groups.**



Supplemental Figure 2. Cortical-dependent cognitive function is minimally affected in lesioned mice. Cortical-dependent memory tasks, (a) context recognition and (a) object recognition tests, are also evaluated after the repeated physical training and found no difference among the 4 groups, indicating that neuronal damages are minimal in cortex areas (n = 11 - 13).

