Research report

Willed-movement training reduces brain damage and enhances synaptic plasticity related proteins synthesis after focal ischemia

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ABSTRACT

It has been widely accepted that willed movement(WM) training promotes neurological rehabilitation in patients with stroke. However, it was not clear whether the effect of WM is better than other forms of exercise. The purpose of this study is to assess different effects of WM and other forms of exercise on rats with focal ischemia. The subjects are all had right middle cerebral artery occlusion (MCAO) surgery and randomly allocated to three groups of training and one control group with no training. Infarct volume by 2,3,5-triphenyltetrazolium chloride (TTC) dye, expression of PICK1 and synaptophysin in cerebral cortex and striatum of injured side by western blotting and immunofluorescence performed are analyzed. Exercise has done respectively on rats in each group for 15 days and 30 days. Compared with the control group, the brain damage is reduced in other groups after 15 days exercise. The protein expressions levels of synaptophysin and PICK1 are upregulated after exercise. Concentration of PICK1 protein in WM is greater than other exercise groups, and the expression of synaptophysin in WM and SM groups are higher than EM groups. The number of PICK1 positive cells, synaptophysin and PICK1 co-positive cells are increased by exercise. Synaptophysin is widely distributed in cortex surrounding the injury area in WM and EM. It is indicated in our result that willed-movement training is the most effective intervention in enhancing the PICK1-mediated synaptic plasticity in the area adjacent to the damage region of ischemic rats.

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1. Introduction

Stroke is one of the leading causes of death and adult disability in the world. Rehabilitation plays a key role in therapeutic process in addition to drug interventions. The role of Motor skill training accompanied by perception training in rehabilitation is well established (Schweighofer et al., 2012; Summa et al., 2011), and there is considerable evidence of the value of voluntary motor training as a neuromotor intervention in stroke rehabilitation (Uysal et al., 2015; Ke et al., 2011a). Willed-movement (WM) training (Waterland, 1967) is defined as a task-oriented training with fully voluntary motor training and arouse the enthusiasm of patient.

Identification of functional molecules in the brain related to improvement of motor dysfunction and perception after stroke (Ploughman et al., 2009) will contribute to establish a new treatment strategy for stroke rehabilitation. Those molecules participate in promoting neuron growth, the processes of plasticity, and long-term memory formation after cerebral ischemia injury.

Synaptophysin(SYP) contributes to the structure and function of synapsis, and its upregulation might be involved in synaptic plasticity. Exercise could promote the expression of SYP in rats subjected to cerebral ischemia (Lan et al., 2014).

AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptors mediate most of the excitatory synaptic transmission in the brain. Therefore, alterations in AMPA receptors number or function at the synapse (Lee, 2011; Malinow and Malenka, 2002) might regulate synaptic strength. PICK1 (protein interacting with C-kinase 1) has been implicated in the regulation of AMPA receptors trafficking (Hanley, 2006; Jin et al., 2006) underlying several forms of synaptic plasticity. PICK1 was called a multi-talented modulator of AMPA receptor trafficking (Hanley, 2008). However, there were few report about the role of PICK1 in the recovery of cerebral ischemic model.

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Hence, the purpose of our study is to investigate the effect of WM training on brain damage. The expression patterns of SYP and PICK1 were assessed in rats with cerebral ischemia underwent exercises, and we speculate that WM might superior than other exercise after stroke and PICK1 might contribute to neuronal plasticity in rehabilitation process of focal ischemia rats.

2. Materials and methods

2.1. Subjects

One hundred and twenty-one male Sprague-Dawley rats weighing 250–280 g at the time of surgery were used in this study. Animals were housed in Plexiglas cages on a 12:12 h light: dark cycle at 23 ± 2 °C room temperature. All procedures were performed during the animals’ light phase. These experiments were performed in accordance with the guidelines for the care and use of animals approved by the animal ethics committees of Central South University. Rats were moderately food restricted (deprived of food for 12 h from 9 p.m. to 9 a.m. of the next day) to motivate performance on the reaching task.

2.2. Rehabilitation protocols

The training apparatus design with herringbone ladder was similar to Tang (Tang et al., 2007). Prior to infarct induction, animals were pre-trained in the apparatus over three consecutive days. According to Longa et al. (1989) and Tang et al. (2013), rats with a modified neurological deficit score (NDS) of 2 and 3, as evaluated at 2 h after MCAO surgery, were used in this study. Three days after subjected right middle cerebral artery occlusion surgery, ischemic animals were stratified randomly assigned to four training conditions according to neurological deficit score: 1) Willed-movement training (WM) rats need to climb the ladder or walls of the apparatus to reaching food and water, the exercise lasts for 30 min/day; 2) environmental modification (EM) rats can climb the ladder voluntarily but never need to climb in the training apparatus to get food and water; 3) forced swimming exercise (SE); rats are forced to swim for 15 min in a round glass tank (24 cm W × 44 cm H) filled to a depth of 30 cm with water (25 ± 1 °C). Control (no rehabilitation). Rehabilitation training lasts for 30 consecutive days.

2.3. Neurological and neurobehavioral assessments

Neurological examinations were performed on the exercise beginning day and then on days 3, 7, 15, and 30 post-exercise until sacrifice using the NDS as above. Investigators performing the outcome testing were kept blind to the group assignments. Only the subjects lasts 30 days of training were brought into statistical analysis, but rats with 15 days training before executed and dead on the training way were excluded from it.

2.4. TTC (2,3,5-triphenyltetrazolium chloride) staining

15 days after the training started, cerebral infarct volume was measured in 6 animals from each group. Rats were deeply anesthetized with sodium pentobarbital (65 mg/kg, intraperitoneally) and immediately decapitated, their whole brains were rapidly removed and frozen at −20°C for 20 min. Then cut into 62-mm-thick coronal sections, and sections were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC)(Sigma–Aldrich, USA) solution at 37°C for 30 min. Stained slides were immersed in 4% paraformaldehyde at 4°C overnight. The infarct area of each section was measured using Image-Pro Plus 6.0 software (Media Cybernetics Inc, Rockville, MD), and the total infarct volumes were calculated based on the formula which Swanson (Swanson et al., 1990) used.

2.5. Western blotting

Two sets of animals (n = 6 for each group) that performed until the 15th day and 30th day of intervention were used, and their brains get as above. For western blot analyses, the cortex and striatum adjacent to the injury brain were isolated for protein harvest. These tissue were extracted with RIPA lysis buffer (Beyotime, Shanghai, China) supplemented with PMSF. The concentration in the tissue samples was determined by using the Bradford method of using a BCA Protein Assay Kit (Beyotime, Shanghai, China). Protein lysates were separated by 10% SDS-PAGE and transferred to 0.45 um PVDF membranes (Millipore, USA); each sample was run at least twice. The membranes were blocked in 5% BSA overnight at 4°C and followed by incubation in primary antibody at 4°C overnight. Primary antibodies used were as follows: Rabbit anti-SYP (1:10000, Abcam, USA), Mouse anti-PICK1 (1:5000, Abcam, USA) and Mouse anti-beta-actin (1:5000, Abcam, USA). Blots were washed (3 × 10 min) in TBS containing 0.05% Tween (TBS-T, Sigma, USA), after incubation with HRP–labeled donkey secondary antibody (Anti-Rabbit, 1:2000, Anti–Rabbit, 1:2000) (Invitrogen, USA) and washed in TBS-T (3 × 10 min), protein bands were visualized using Amersham ECL Prime (GE Healthcare, NJ, USA) on GelDoc XR System (Bio-rad, USA) and quantified by NIH ImageJ software version 9.0. The intensities of the PICK1 and SYP protein bands were all normalized to β-actin.

2.6. Immunofluorescence

After training for 15 days, rats were deeply anesthetized with sodium pentobartal and transcardially perfused with cold heparinized saline, followed by 4% paraformaldehyde in phosphate-buffered saline. Brains (each group n = 6) were removed from the skull, fixed in 4% paraformaldehyde in phosphate-buffered saline for 90 min at 4°C, and then transferred in turn to 20% and 30% sucrose at 4°C overnight. Thirty μm-thick coronal sections were cut with Leica CM1900 cryostat (Leica Microsystems, Wetzlar, Germany) beginning 5 mm from the anterior tip of the frontal lobe to 9 mm sections (Ashwal et al., 1998; Paxinos and Watson, 2007) were pretreated with 10 mM citrate buffer pH 6.1 for 15 min at 90°C in a steamer and then permeabilized with 0.1% TritonX-100 in PBS and blocked 30 min in PBS containing 5% normal Donkey Serum (Sigma–Aldrich, USA). After that, sections were incubated with Mouse anti-PICK1 (1:5000, Abcam, USA) and Rabbit anti-SYP (1:10000, Abcam, USA) ut supra at 4°C for 48 h. Fluorescent detection was obtained by secondary incubation with donkey antiserum specific against rabbit IgG coupled with AlexaFluor–488 (Invitrogen, 1:100, Germany) and donkey against mouse IgG coupled with Alexa Fluor 594 (Invitrogen, 1:100, Germany) respectively 2 h at 37°C. Sections were mounted on Polylysine slides in an Antifade Mounting Medium (Beyotime, Shanghai, China) before being examined. An inverted fluorescence microscope (Eclipse T1, Nikon, Melville, NY) and a confocal laser scanning microscope (LSM510, Zeiss; Jena, Germany) were used to examine immunofluorescence staining fluorescence micrographs. Specificity of the protein expression was assessed by counting the number of antibody-positive cells in each scanning field with Image-Pro Plus 6.0 (Media Cybernetics Inc, Rockville, MD).

2.7. Statistical analysis

SPSS software (version 18.0; SPSS Inc., Chicago, IL, USA) was used for data analysis. One-way ANOVA followed by LSD–t post hoc test was used to assess the differences of infarct volumes between
groups and result of immunofluorescence staining. Repeated measures ANOVA determined differences in NDS and targets protein expression among groups with time.

3. Result

Eighteen rats died during the experimental period and six rats were excluded from the study because of with either higher or lower neurological deficit score. Only 96 survived rats with neurological deficit score of 2 and 3 at 2 h after recirculation were used in the experience.

3.1. Neurological and neurobehavioral assessments

The animals subjected to middle cerebral artery occlusion showed the largest neurological deficits 3 days after ischemia (rehabilitation training starts on that day) compared to other time points. But, scores in four groups were all in a downward trend over time after rehabilitation begins, which fully indicates neurological deficits in cerebral ischemia rats would be slowly recovery whether with exercise or not (Fig. 1). Through repeated measurements of the variance of four groups in neurological deficit score, we find a significant effect of training on ameliorating neurological deficit. \( F(3,20) = 3.74, P < 0.05 \). Subsequent multiple comparisons showed that neurological deficits recovery in control group was more slowly than groups with exercise. (Student–Newman–Keuls test, \( P < 0.05 \)), but there were no difference between the effect of WM, EM and SM on rehabilitation of neurologic function (\( P > 0.05 \)).

3.2. Infarct volume

Cerebral infarctions were found in the hemisphere ipsilateral to the occlusion (right side) in animals that were exposed to MCAO surgery. Unstained areas were defined as infarcted tissue, and normal tissue was stained red. As shown in (Fig. 2A), the infarct area was in accordance with the distribution area of MCA in rats. Comparison of infarct volumes percentage showed a statistical difference between the 4 groups \( F(3,20) = 5.92, P < 0.05 \). Multiple comparisons showed that infarction volumes of control group \( (19.76 \pm 4.35\%, n = 6) \) were greater than all three exercise groups. The average infarct volumes of WM \( (12.84 \pm 3.79\%, n = 6) \) were not smaller than EM \( (13.66 \pm 5.14\%, n = 6) \) and SM \( (14.6 \pm 3.50\%, n = 6) \) groups after consecutive training 15 days \( (P > 0.05) \).

3.3. Effects of exercise regimens on brain levels of SYP and PICK1

In this study, the expression of two components in the lesion periphery areas of ischemia (Cortex and striatum) (each subgroup \( n = 6 \)) are characterized. By Western Blot analysis, the proteins (Fig. 3A) and compared expression levels among four groups are quantified to assess any differences in the training conditions. ANOVA revealed a significant effect of training \( \times \) time \( F(3,40) = 13.95, P < 0.05 \) on the ratio of SYP to β-actin, training factor \( F(3,40) = 89.89, P < 0.05 \), time factor \( F(1,40) = 114.07, P < 0.05 \). Subsequent multiple comparisons showed that rehabilitation had significantly elevated SYP levels compared with the control groups (Fig. 3B, Student–Newman–Keuls test, \( P < 0.05 \)). The expression of SYP in EM groups were slight higher than the control groups, but below the WM and SM groups \( (P < 0.05) \). However, there was no statistically significant difference in the level of SYP between WM and SM groups over time \( (P = 0.094) \). As time went by, SYP Protein levels increased in three training groups (paired-samples \( t \)-test, \( P < 0.05 \)), whereas there is no difference in the control groups at both time points (Fig. 3B).

ANOVA revealed a significant effect of training \( \times \) time \( F(3,40) = 7.32, P < 0.05 \), the training factor \( F(3,40) = 133.25, P < 0.05 \), time factor \( F(1,40) = 155.15, P < 0.05 \). Subsequent multiple comparisons showed that the expression of PICK1 in WM groups was greater than other groups. The effect of SM was second, and EM groups had high levels of PICK1 levels compared to the control groups (Fig. 3C, Student–Newman–Keuls test, \( P < 0.05 \)). Finally, PICK1 Protein levels increased over time in all four groups (Fig. 3C paired-samples \( t \)-test, \( P < 0.05 \)).
3.4. Immunofluorescence

The expression of SYP in the motor cortex of penumbra area reflected the number of synaptic vesicle adjacent to the injury objectively. Exercise increased the expression of PICK1 and SYP, this was further corroborated by immunofluorescence with concentration of green fluorophore which anti-PICK1 antibody be marked with and red fluorophore which represented SYP distribution (Fig. 4A–D). Percentage of SYP positive cells in four groups were inconsistent ($F(3,20) = 29.62, p < 0.05$). Multiple comparison showed that SM and Control were smaller than EM ($p < 0.05$). Expression of SYP in WM was the most extensive ($p < 0.05$). Percentage of PICK1 positive cells in four groups were also inconsistent ($F(3,20) = 28.62, p < 0.05$). LSD-t test confirming that the Control displayed litter staining compared with rehabilitation groups ($p < 0.05$). Expression of PICK1 in WM group was extensive compared with EM and SM ($p < 0.05$). Nevertheless, confocal microscope analysis of fluorescent image revealed that part overlap between the expression of PICK1 and SYP in neuron cells (Fig. 4E), the two targets were obviously different in intracellular distribution position. SYP protein was mainly distributed on nerve neuron membrane, but PICK1 was localized in the cytoplasm of the cells largely. Percentage of SYP and PICK1 positive cells in WM group was not differ from other training groups, but greater than that in no rehabilitation control group (Table 1). ANOVA revealed that rehabilitation increased the number of SYP and PICK1 co-positive cells ($F(3,20) = 19.81, p < 0.05$). WM training enhanced the co-expression of SYP and PICK1 mostly in this study. ($p < 0.05$). Consistent with previous experiment, we detected the co-positive neuron radio in EM and SM was larger than that in the control group ($p < 0.05$).

4. Discussion

Increasing evidence suggests that damage to the adult brain could result in adaptive plasticity in regions adjacent to the injury, such as increased dendritic arbors and synaptogenesis (Jones et al., 1999; Bury and Jones, 2002). Drug therapy in ischemia stroke, which has been studied for decades, can protect the rat brain from ischemic damage caused by MCAO (Zhang et al., 2014). Meanwhile, the plastic changes may be modulated by post-injury rehabilitation training. Exercise is an effective way (Fang et al., 2010; Timmermans et al., 2010) to reduce damage in cerebral ischemia. It was reported that physical activity significantly reduced neurologic deficits and infarct volume (Li et al., 2004; Chang et al., 2007) in the frontoparietal cortex and the dorsolateral striatum of rats with cerebral ischemia. The results of our previous (Tang et al., 2013) and present study were in consistent with those findings. In the present study, we found that the infarction volumes of rats without exercise (control group) was larger than exercise groups (WM, EM, and SM groups) and NDS in control group was much higher than groups with exercise. An unexpected finding in our study, however, is the lack of significant variance among WM, EM and SM in infarction volumes. This is unexpected since Ke et al. (2011b) and Ploughman et al. (2005) have reported that the forced exercise was the least neuro-protective intervention with high-pressure exercise. SYP contributes to the structure and function of synapsis (Valforta et al., 2004; Thiel, 1993), and its upregulation might be involved in synaptic plasticity (Bai and Strong, 2014). Complex environment housing independently increased the number of synaptic mitochondria (Briones et al., 2005) in presynaptic terminals in the region adjacent to the site of the principal insult. In our study, we found SYP concentrations significantly higher in all exercise groups than control groups. And WM was more useful in formation of new synapses than EM, which was consistent with our inference. There

Fig. 3. (A) Example of western blot bands in SYP, PICK1 and internal reference β-actin levels in ischemic penumbra of brain in 8 subgroups. (B) Relative quantitative analysis show statistical differences of SYP levels in 4 groups. (C) Relative quantitative analysis show statistical differences of PICK1 levels in 4 groups. Values are expressed as mean ± S.D. (*VS other groups Student–Newman–Keuls test, $p < 0.05$)
Fig. 4. Immunofluorescence of SYP and PICK1 in the ischemic penumbra of WM, SM, EM and control rats. (A–D) images represented the SYP (red) and PICK1 (green) cells at the adjacent fields close to injury of the cortical sections in the SM, EM, WM and Control rats. (E) images displayed colocalization (Orange) of SYP (red) and PICK1 (green) in neurones in the ischemic penumbra of WM rat at high power Confocal microscope. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
was no difference in the level of SYP in WM and SM, which suggests that the willing training is not the main factor to affect structure and function of synapsis in the process of recovery after brain damage. When concerning the alteration of SYP between 15 and 30 days after exercise initiate, we found that level of SYP increased as exercise went on. The result indicates that the duration of training is an independent factor conductive to synapse formation and strengthen the synaptic plasticity.

PICK1 has increasingly been recognized as a key regulator of AMPA receptor trafficking. Different from simply regulating the number of AMPARs at the synapse, recent reports (Cull-Candy et al., 2006; Isaac et al., 2007) suggest that PICK1 is involved in both plasticity and pathological mechanisms after cerebral ischemia. Previous studies (Tang et al., 2013) had reported WM exercise result in an increase of PICK1 protein and a recovery of motor function in ischemic rats. Compared to other groups, our data also shows that WM training can lead to the increasing expression of PICK1 and reduce brain damage significantly. The level of PICK1 protein in EM and SM also increased compared to the control. We conclude that PICK1 might contribute to neuroprotection and neuronal plasticity in rehabilitation process of focal ischemia rats.

Recent randomized controlled trials (Lan et al., 2014; Tang et al., 2013; Qian et al., 2015; Zhao et al., 2013) point out that SYP is widely distributed in cortex and hippocampus of ischemic peripheral in rehabilitation process and PICK1 is abundantly expressed in the cortex, neural nuclei below the putamen and thalamus, but much less expressed in the hippocampus. Our study also showed SYP and PICK1 was expressed in abundance in motor cortex area adjacent to damage. SYP and PICK1 positive cell radio following WM training is significantly higher than the other two types of rehabilitation, which suggested that exercise with will, purpose and no stress might be more appropriate for the growth of new branches on the neurons and synaptic plasticity related proteins synthesis in rats with cerebral ischemia.

All in all, our findings demonstrate that WM training results in a decreased brain damage and an increase of synaptic number morphologically, an increase of PICK1 protein in the IP. It is concluded that WM training was the most effective intervention in enhancing the PICK1-mediated synaptic plasticity in the IP region of ischemic rats. Further study is needed to figure out which forms of synaptic plasticity PICK1-mediated from the view of electrophysiology and molecular mechanisms of that following WM training after the ischemic events with application of translational electrophysiological and neuroimaging methods, which illustrate ischemia area and damage degree (Moyanova and Dijkhuizen, 2014) more accurately than the methods in this paper.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.brainresbull.2015.11.004.

**References**


**Table 1**

| Percentage of SYP, PICK1 positive cells and SYP co-expression with PICK1 (mean ± SD, n = 6). |
|---------------------------------|----------------------------------|----------------------------------|
| Control | SM | EM | WM |
| SYP | 60.51 ± 4.78 | 58.07 ± 5.34 | 76.14 ± 5.09b | 82.67 ± 6.21b,y |
| PICK1 | 49.89 ± 6.08 | 69.82 ± 5.10a | 72.95 ± 6.68a | 80.27 ± 5.31b,y |
| SYP′/PICK1 | 42.85 ± 6.71 | 56.62 ± 6.45a | 62.44 ± 4.83a | 66.55 ± 4.42a,y |

VS Control.

p < 0.05 VS SM.

p < 0.05 VS EM.

p < 0.05.


