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Research report

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

Jingjing Nie[a], Xiaosu Yang[a,*], Qingping Tang[b], Qin Shen[c], Simin Li[d]

[a] Department of Neurology, Xiang Ya hospital, Central South University, Xiang Ya Road 87, 410008 Changsha, Hunan, China
[b] Department of Neurology, Brain Hospital of Hunan Province, Hunan University of Chinese Medicine, Fu Rong Road 427, Changsha 410007 Hunan, China
[c] Department of Radiology, The Second Xiangya hospital of Central South University, People Road 139, 410011 Changsha, Hunan, China
[d] Department of research and education office, XiangYa Stomatological Hospital, Central South University, Xiang Ya Road 72, 410008 Changsha, Hunan, China

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ABSTRACT

It has been wildly 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.


## 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.

* Corresponding author. Fax: +86 7314327332.
*E-mail address:* sjnk.xy@aliyun.com (X. Yang).

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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 assess 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 from9 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) 4. 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. And then cut into 6 2-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-Mouse, 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 pentobarbital and transcardially perfused with cold heparinized saline, followed by 4% paraformaldehyde in phosphatebuffered 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 antisera 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 Polysine 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