Constraint-induced movement therapy enhances AMPA receptor-dependent synaptic plasticity in the ipsilateral hemisphere following ischemic stroke

Abstract

Constraint-induced movement therapy (CIMT) can promote the recovery of motor function in injured upper limbs following stroke, which may be associated with upregulation of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) at synapses in the ipsilateral sensorimotor cortex in our previous study. However, AMPAR distribution is tightly regulated, and only AMPARs on the postsynaptic membrane can mediate synaptic transmission. We speculated that synaptic remodeling induced by movement-associated synaptic activity can promote functional recovery from stroke. To test this hypothesis, we compared AMPAR expression on the postsynaptic membrane surface in a rat model of ischemic stroke induced by middle cerebral artery occlusion (MCAO) with versus without CIMT, which consisted of daily running wheel training for 2 weeks starting on day 7 after MCAO. The results showed that CIMT increased the number of glutamate receptor (GluR)2-containing functional synapses in the ipsilateral sensorimotor cortex, and reduced non-GluR2 AMPARs in the ipsilateral sensorimotor cortex and hippocampal CA3 region. In addition, CIMT enhanced AMPAR expression on the surface of post-synaptic membrane in the ipsilateral sensorimotor cortex and hippocampus. Thus, CIMT promotes the recovery of motor function of injured upper limbs following stroke by enhancing AMPAR-mediated synaptic transmission in the ischemic hemisphere. These findings provide supporting evidence for the clinical value of CIMT for restoring limb movement in stroke patients. All experimental procedures and protocols were approved by the Department of Laboratory Animal Science of Fudan University, China (approval No. 201802173S) on March 3, 2018.

Key Words: brain; experiment; injury; plasticity; regeneration; repair; stroke; synapse

Introduction

Ischemic stroke is a leading cause of death and disability worldwide and constitutes a social and economic burden, with survivors often experiencing persistent sensorimotor and cognitive dysfunction (Writing Group Members et al., 2016; Benjamin et al., 2018). Rehabilitation training is the first-line intervention after ischemic stroke (Askim et al., 2009, 2010; Arya et al., 2011). Neural plasticity is critical for post-stroke recovery and rehabilitation (Wang et al., 2010; Carmichael, 2012; Alla et al., 2017); its efficacy has been linked to structural and functional reorganization in the damaged brain area, but also in the contralateral hemisphere (Bueteufisch, 2015; Jones and Adkins, 2015). Ischemic stroke can lead to synaptic dysfunction (Li et al., 2013; Hofmeijer et al., 2014; Park et al., 2015); the focus of post-stroke rehabilitation is to restore plasticity to the residual synapses (Nie and Yang, 2017; Xie et al., 2019).

Glutamate is the main excitatory neurotransmitter in the central nervous system (CNS). α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) and N-methyl-D-aspartic acid receptor (NR) are the main ionotropic glutamate receptors (GluRs) in the mammalian CNS; the former mediates most rapid excitatory synaptic transmission, and its regulation is critical for synaptic strength and efficacy (Carroll et al., 2001; Anggono and Huganir, 2012; Huganir and Nicoll, 2013). AMPARs in cortical and hippocampal pyramidal neurons are...
mainly composed of GluR1/2 and GluR2/3 heteromers (Geiger et al., 1995; Wenthold et al., 1996; Lu et al., 2009). Most AMPARs in the CNS are thought to contain a GluR2 subunit (Geiger et al., 2002), which determines the major biophysical properties of the receptors given its impermeability to calcium (Cull-Candy et al., 2006; Shimsheh et al., 2006; Isaac et al., 2007). Reduction of GluR2 and upregulation of Ca$^{2+}$-permeable AMPARs, which lack GluR2 but contain GluR1 or GluR3, was found to be associated with increased vulnerability of neurons to excitotoxicity in cerebral ischemia, which is known as the GluR2 hypothesis (Dixon et al., 2009; Wang et al., 2011, 2012; Zhai et al., 2013). Synaptic plasticity depends on changes in AMPAR number and composition (Malinow and Malenka, 2002). We speculated that such synaptic remodeling induced by movement-associated synaptic activity is the mechanism underlying recovery from stroke. To test this hypothesis, in this study, we investigated the effect of constraint-induced movement therapy (CIMT) on the expression of postsynaptic AMPARs in the sensorimotor cortex and hippocampus in a rat model of ischemic stroke.

**Materials and Methods**

**Animals**
Specific-pathogen-free adult male Sprague-Dawley rats (n = 36) weighing 260–280 g and aged 7–9 weeks were purchased from Shanghai SIPPR-BK LAB Animal Ltd. (Shanghai, China; license No. SCXK [Hu] 2018-0006). The rats were housed in cages at room temperature (23 ± 1°C) and exposed to light and dark cycles of 12–10 hours. Food and water were available ad libitum. Animals were maintained at 37°C using a heating pad. No occlusion of the middle cerebral artery. The rats' body temperature was maintained at 37°C using a heating pad.

**MCAO model establishment**
The MCAO model was established as previously described (Hu et al., 2019). Briefly, rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.36 ml/100 g). A thread (Beijing Xingong Technology Co., Beijing, China; product No. 2636-A5) with silicone covering 5–6 mm of the front head was inserted into the left middle cerebral artery of the rat. The thread had a length of 45 mm and a diameter of 0.26 mm, with a head diameter of 0.36 ± 0.02 mm. Ischemia time was 90 minutes. To ensure that the middle cerebral artery was blocked, the suture was inserted into the internal carotid artery at a depth of 18–6 mm of the front head. Ischemia time was 90 minutes. The foot-fault test was performed at 7, 14, and 21 days after surgery. The ipsilateral sensorimotor cortex and hippocampus were rapidly removed and weighed, and ten volumes (1 g:10 mL) of Syn-PER reagent containing protease inhibitors were prepared. Rats in the sham and MCAO groups were anesthetized by intraperitoneal injection of 10% chloral hydrate and transcardially perfused with phosphate-buffered saline (PBS) and 4% paraformaldehyde. The brain was removed and fixed overnight in 4% paraformaldehyde with 0.2% sodium metaperiodate. After dehydration in a graded ethanol series and embedding in paraffin, 30 μm sections were cut and stained with Hematoxylin and Eosin (H&E) for histological analysis.

**CIMT**
CIMT was initiated on day 7 after the surgery. The forelimb on the healthy (left) side of the rat was fixed with plaster lined on the inside with cotton pads to prevent direct contact between the plaster and skin. Rats in the CIMT group were forced to use their impaired forelimbs for daily activities such as eating and drinking, and 20 minutes of running wheel training was added every day for 2 weeks. The running wheel had a diameter of 28 cm, width of 9.5 cm, and spoke interval of 1.5 cm. Before each test, with a camera recording the entire process. The total number of steps and number of empty steps on the affected side of the rats were used to calculate the foot fault ratio. The results of three trials were averaged.

**Surface biotinylation of GluR1 and GluR2 in synaptosomes**
Synaptosome preparations (500 μg of protein/tube) were incubated with PBS/Ca$^{2+}$/Mg$^{2+}$ buffer containing 0.5 mg/mL EZ-Link Sulfo-NHS-SS-Biotin (Thermo Fisher Scientific; Cat# EZ-Link Sulfo-NHS-SS-Biotin (Thermo Fisher Scientific; Cat# P0012)).
21945) for 1 hour at 4°C with gentle shaking. Glycine-containing PBS/Ca\(^{2+}\)/Mg\(^{2+}\) buffer (100 mM; 1 mL) was then added and incubated on ice for 10 minutes to terminate the biotinylation reaction, and the sample was then centrifuged at 4°C and 8000 \(\times\) g for 4 minutes. The supernatant was discarded, and the pellet was resuspended in 1 mL of glycine-containing PBS/Ca\(^{2+}\)/Mg\(^{2+}\) buffer (100 mM) followed by centrifugation at 4°C and 8000 \(\times\) g for 4 minutes. This step was repeated, and the pellet was resuspended in 1 mL of 100 mM glycine-containing PBS/Ca\(^{2+}\)/Mg\(^{2+}\) buffer, followed by incubation at 4°C for 30 minutes with gentle shaking and centrifugation at 4°C and 8000 \(\times\) g for 4 minutes. The pellet was resuspended in 1 mL of PBS/Ca\(^{2+}\)/Mg\(^{2+}\) buffer and centrifuged at 4°C and 8000 \(\times\) g for 4 minutes. This step was repeated 2 more times, and the final pellet was incubated in 250 \(\mu\)L of 1% Triton X-100 buffer containing protease inhibitor at 4°C for 30 minutes with shaking, then centrifuged at 4°C and 20,000 \(\times\) g for 30 minutes. One-third of the supernatant was taken as the total synaptosome fraction. The remaining supernatant was mixed with 200 \(\mu\)L NeutrAvidin agarose resin (Thermo Fisher Scientific; Cat# 29200) and incubated overnight at 4°C with gentle shaking. The samples were centrifuged at 4°C and 17,700 \(\times\) g for 4 minutes. The biotinylated protein/avidin pellets were resuspended in 1 mL of 1% Triton X-100 buffer and centrifuged at 4°C and 17,700 \(\times\) g for 4 minutes. This step was repeated 2 more times, and 100 \(\mu\)L Laemmeli buffer (Beyotime; Cat# P0015) was added; the mixture was incubated at room temperature for 20 minutes with gentle shaking to elute the biotinylated protein. The samples were centrifuged at 4°C and 17,700 \(\times\) g for 4 minutes and the supernatant containing the biotinylated synaptic membrane surface protein was collected. Total samples were prepared for western blot analysis by adding sample loading buffer. Both total and biotinylated samples were heated at 95°C for 5 minutes and stored at −20°C.

**Western blot analysis**

Total and biotinylated protein samples were electrophoretically separated on 7.5% polyacrylamide gels. The proteins were transferred to a polyvinylidene difluoride membrane (Merck KGaA; Cat# IPVH00010). After blocking with 3% bovine serum agarose resin (Thermo Fisher Scientific; Cat# 29200) and incubated overnight at 4°C with gentle shaking. The samples were biotinylated and avidin pellets were resuspended in 1 mL of 1% Triton X-100 buffer and centrifuged at 4°C and 17,700 \(\times\) g for 4 minutes. The remaining supernatant was mixed with 200 \(\mu\)L NeutrAvidin agarose resin (Thermo Fisher Scientific; Cat# 29200) and incubated overnight at 4°C with gentle shaking. The samples were centrifuged at 4°C and 17,700 \(\times\) g for 4 minutes and the supernatant containing the biotinylated synaptic membrane surface protein was collected. Total samples were prepared for western blot analysis by adding sample loading buffer. Both total and biotinylated samples were heated at 95°C for 5 minutes and stored at −20°C.

**Figure 1** CIDM improves motor function in rats with cerebral ischemia. (A) Flow chart of the experimental design. (B) Foot fault ratio of the right forelimb. Data are expressed as the mean ± SD. ***P < 0.001, ****P < 0.0001 (two-way analysis of variance and Tukey’s multiple comparisons test).

**Results**

**CIMT improves motor function in rats with cerebral ischemia**

The foot-fault test was performed on days 7, 14, and 21 post-stroke. At 7 days, foot fault ratios of the right forelimb were significantly higher in the CIMT and MCAO groups than in the sham group (P < 0.0001), with no significant difference between CIMT and MCAO groups (P = 0.9999). After 1 week of CIMT, an obvious deficit still existed in the CIMT group and there was no change in the MCAO group (P = 0.9838). However, after 2 weeks of CIMT, the foot fault ratio of the right forelimb was significantly lower in the CIMT group than in the MCAO group (P < 0.0001). Additionally, the ratio in the MCAO group was decreased on day 14 but not on day 21, while that in the CIMT group continued to decrease during these 2 weeks (Figure 1B). Thus, although rats in the CIMT group still showed right forelimb dysfunction compared to the sham group after 2 weeks of CIMT, these data indicate that 2 weeks of CIMT can improve motor function in rats following ischemic stroke.

**CIMT increases GluR2-containing synapses while reducing AMPARs lacking GluR2 in the ipsilateral sensorimotor cortex**

In the ipsilateral sensorimotor cortex, colocalization of NR1 with GluR2 was higher (P < 0.0001; Figure 2A and B) whereas colocalization of NR1 with GluR1 was lower (P = 0.0017; Figure 2A and B) in the MCAO group than in the other two groups (sham vs. MCAO, P = 0.1248; Figure 2A and B). These results indicate that AMPARs at functional synapses of the MCAO group are mainly GluR2/GluR3 heteromeric receptors. In the ipsilateral CA3 region, colocalization of NR1 with GluR1 was higher than in the MCAO group and in the other two groups (sham vs. MCAO, P = 0.0017; CIMT vs. MCAO, P = 0.0340; Figure 2D). There was no significant difference in the colocalization of NR1 with GluR2 between groups (sham vs. CIMT, P = 0.9924; sham vs. MCAO, P = 0.8603; CIMT vs. MCAO, P = 0.7530; Figure 2C). Thus,
CIMT abrogated the increase in AMPARs lacking GluR2 that was observed following stroke.

CIMT increases total synaptic and surface AMPAR expression in the ipsilateral sensorimotor cortex and hippocampus

In the ipsilateral sensorimotor cortex, total GluR1 and total GluR2 expression in synaptosomes was higher in the CIMT group than in the MCAO group (total GluR1: P = 0.0088; total GluR2: P = 0.0384; **Figure 4A-a, b**). Moreover, compared to the MCAO group, surface GluR1 expression was elevated in the CIMT group (P = 0.0420, **Figure 4A-c**). In contrast, surface GluR2 levels did not differ between groups (sham vs. CIMT, P = 0.8253; sham vs. MCAO, P = 0.8746; CIMT vs. MCAO, P = 0.9893; **Figure 4A-d**). In the ipsilateral hippocampus, surface GluR1 and GluR2 expression was higher in the CIMT group than in the MCAO group (surface GluR1: P = 0.0225; surface GluR2: P = 0.0330; **Figure 4B-c, d**). However, total GluR1 and GluR2 levels in the synaptosome protein fraction were similar across groups (total GluR1: sham vs. CIMT, P = 0.7622; sham vs. MCAO, P = 0.4107; total GluR2: sham vs. CIMT, P = 0.1261; total GluR2: sham vs. MCAO, P = 0.6705; **Figure 4B-a, b**). These data indicate that CIMT not only increases the expression of synaptic AMPARs but also that of AMPARs on the synaptic membrane in ipsilateral brain areas. Representative results of the western blot analysis are shown in **Figure 4C**.

**Discussion**

CIMT involves the forced use of the impaired limb while restricting that of the contralateral limb in daily activities. This method is widely used in post-stroke rehabilitation to improve the motor function of impaired upper limbs (Zhao et al., 2009; Kwakkel et al., 2015; Qu et al., 2015). Accordingly, in the present study as well as our previous work, we found that CIMT improved motor function in rats with ischemic stroke (Hu et al., 2019; Liu et al., 2019). The mechanisms underlying the effects of CIMT may be related to functional reorganization and structural plasticity in the brain (Yoon et al., 2014; Qu et al., 2015). It was previously reported that CIMT promoted post-stroke synaptic plasticity and improved behavioral outcome by inducing the formation of synapses and enhancing synaptophysin and postsynaptic density-95 expression (Zhao et al., 2009, 2013). Activity-dependent AMPAR trafficking has been well-studied in the context of synaptic plasticity and remodeling (Henley, 2003; Sheng and Hyoung Lee, 2003). The distribution of AMPARs in the postsynaptic membrane is tightly regulated through lateral diffusion, endocytosis, and exocytosis (Lussier et al., 2012). We therefore measured the surface expression of AMPARs in the present study and found that CIMT enhanced AMPAR-dependent synaptic plasticity in the ipsilateral sensorimotor cortex and hippocampus following stroke.

CIMT increased the colocalization of NR1 with GluR2 while decreasing that of NR1 with GluR1 in the ipsilateral sensorimotor cortex. These data suggest that CIMT increased GluR2-containing functional synapses. There are two possible explanations for these results. GluR2-positive synapses in the CIMT group mostly comprised GluR2/3 heteromers rather than GluR1/2 heteromers. Alternatively, it is possible that more AMPARs lacking GluR2 existed in the MCAO group. The latter is the more likely explanation because GluR2/3 heteromers are rare in the cortex and hippocampus. Our results are consistent with the GluR2 hypothesis following stroke, which suggests that delayed neuronal death following cerebral ischemia could be due to reduced surface expression of GluR2 and upregulation of AMPARs lacking GluR2, resulting in an abnormal increase in calcium influx (Pellegrini-Giampietro et al., 1997; Wang et al., 2011) given that the latter are calcium-permeable and exhibit a high single-channel conductance (Bowie and Mayer, 1995; Geiger et al., 1995). Inhibiting GluR2 internalization or the decreases in GluR2 mRNA and protein levels after ischemic injury is thought to have neuroprotective effects (Oxon et al., 2009; Montori et al., 2010; Wang et al., 2011; Zhai et al., 2013; Chen et al., 2014). Another study found that redistribution of GluR1 receptors on the synaptic membrane surface enhanced neuronal death following oxygen glucose deprivation; conversely, preventing the oxygen...
However, surface GluR1 and GluR2 levels were higher in the CIMT group than in the MCAO group, but this was non-significant (d). (B) In the ipsilateral hippocampus, total synaptic GluR1 and GluR2 levels did not differ between groups (a, b). However, surface GluR1 and GluR2 levels were higher in the CIMT group than in the MCAO group (c, d). (C) Tubulin, which is not present in the membrane fraction, served as a reference to test the purity of the extracted membrane proteins. Data are expressed as the mean ± SD. *P < 0.05, **P < 0.01 (one-way analysis of variance followed by Tukey’s multiple comparisons test). AMPAR: a-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor; CIMT: constraint-induced movement therapy; GluR: glutamate receptor; MCAO: middle cerebral artery occlusion; S: membrane surface protein; T: synaptic total protein.

In conclusion, CIMT not only increased the number of GluR2-containing functional synapses in the ipsilateral sensorimotor cortex, but also decreased the abundance of AMPARs lacking GluR2 in the ipsilateral sensorimotor cortex and hippocampal CA3 region. It also enhanced the expression of synaptic AMPARs in the ipsilateral sensorimotor cortex and surface expression of synaptic AMPARs in the ipsilateral sensorimotor cortex and hippocampus. Thus, CIMT improves motor function following ischemic stroke by strengthening synaptic transmission in the sensorimotor cortex and hippocampus, highlighting its clinical value for restoring limb movement in stroke patients.
GLuR1 in ipsilateral hippocampal CA1 region (double immunofluorescence staining).

Additional Figure 3: Effect of CIMT on the colocalization of NR1 with GLuR2 in ipsilateral hippocampal CA3 region (double immunofluorescence staining).

Additional Figure 4: Effect of CIMT on the colocalization of NR1 with GLuR2 in ipsilateral hippocampal CA3 region (double immunofluorescence staining).

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Additional Figure 1 Effect of CIMT on the colocalization of NR1 with GluR2 in ipsilateral hippocampal CA1 region (double immunofluorescence staining).

Double-immunolabeled confocal images for NR1 (red, stained by Cy3) and GluR2 (green, stained by Alexa Fluor 488) in CA1 area. Selecting areas other than neuron nuclei (with DAPI staining) were for colocalization analysis. The right image is a scatter plot of the correlation between NR1 and GluR2 distributions. The x-axis is the red fluorescence channel and the y-axis is the green fluorescence channel. The colocalization of NR1 with GluR2 in MCAO group was significantly higher than that in other two groups. Scale bars: 50 μm. CIMT: Constraint-induced movement therapy; DAPI: 4′,6-diamidino-2-phenylindole; GluR2: glutamate receptor 2; MCAO: middle cerebral artery occlusion; NR1: glutamate receptor N-methyl-D-aspartic acid receptor 1.
Additional Figure 2 Effect of CIMT on the colocalization of NR1 with GluR1 in ipsilateral hippocampal CA1 region (double immunofluorescence staining).

Double-immunolabeled confocal images for NR1 (red, stained by Cy3) and GluR1 (green, stained by Alexa Fluor 488) in CA1 area. Selecting areas other than neuron nuclei (with DAPI staining) were for colocalization analysis. The right image is a scatter plot of the correlation between NR1 and GluR1 distributions. The x-axis is the red fluorescence channel and the y-axis is the green fluorescence channel. There was no significant difference in colocalization of NR1 with GluR1 between groups. Scale bars: 50 μm. CIMT: Constraint-induced movement therapy; DAPI: 4′,6-diamidino-2-phenylindole; GluR1: glutamate receptor 1; MCAO: middle cerebral artery occlusion; NR1: glutamate receptor N-methyl-D-aspartic acid receptor 1.
Additional Figure 3 Effect of CIMT on the colocalization of NR1 with GluR2 in ipsilateral hippocampal CA3 region (double immunofluorescence staining).

Double-immunolabeled confocal images for NR1 (red, stained by Cy3) and GluR2 (green, stained by Alexa Fluor 488) in CA3 area. Selecting areas other than neuron nuclei (with DAPI staining) were for colocalization analysis. The right image is a scatter plot of the correlation between NR1 and GluR2 distributions. The x-axis is the red fluorescence channel and the y-axis is the green fluorescence channel. There was no significant difference in colocalization of NR1 with GluR2 between groups. Scale bars: 50 μm. CIMT: Constraint-induced movement therapy; DAPI: 4′,6-diamidino-2-phenylindole; GluR2: glutamate receptor 2; MCAO: middle cerebral artery occlusion; NR1: glutamate receptor N-methyl-D-aspartic acid receptor 1.
Additional Figure 4 Effect of CIMT on the colocalization of NR1 with GluR1 in ipsilateral hippocampal CA3 region (double immunofluorescence staining).

Double-immunolabeled confocal images for NR1 (red, stained by Cy3) and GluR1 (green, stained by Alexa Fluor 488) in CA3 area. Selecting areas other than neuron nuclei (with DAPI staining) were for colocalization analysis. The right image is a scatter plot of the correlation between NR1 and GluR1 distributions. The x-axis is the red fluorescence channel and the y-axis is the green fluorescence channel. The colocalization of NR1 with GluR1 in MCAO group was significantly higher than that in other two groups. Scale bars: 50 μm. CIMT: Constraint-induced movement therapy; DAPI: 4′,6-diamidino-2-phenylindole; GluR1: glutamate receptor 1; MCAO: middle cerebral artery occlusion; NR1: glutamate receptor N-methyl-D-aspartic acid receptor 1.