Constraint-Induced Movement Therapy Overcomes the Intrinsic Axonal Growth–Inhibitory Signals in Stroke Rats

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Background and Purpose—Constraint-induced movement therapy (CIMT) improves functional outcome in patients with stroke possibly through structural plasticity. We hypothesized that CIMT could enhance axonal growth by overcoming the intrinsic growth–inhibitory signals, leading eventually to improved behavioral performance in stroke rats.

Methods—Focal cerebral ischemia was induced by intracerebral injection of endothelin-1. Adult Wistar rats were divided into a sham-operated group, an ischemic group, and an ischemic group treated with CIMT. CIMT started at postoperative day 7 and continued for 3 weeks. Biotinylated dextran amine was injected into the contralateral sensorimotor cortex at postoperative day 14 to trace crossing axons at the cervical spinal cord. The expressions of Nogo-A, Nogo receptor, RhoA, and Rho-associated kinase in the peri-infarct cortex, and the expressions of biotinylated dextran amine, growth associated protein-43, synaptophysin, vGlut1, and postsynaptic density-95 in the denervated spinal cord were measured by immunohistochemistry and Western blots. Behavioral recovery was analyzed at postoperative days 29 to 32.

Results—Infarct volumes were not different between groups after stroke. CIMT significantly increased the length and the number of midline crossings of contralateral corticospinal axons to the denervated cervical spinal cord. CIMT significantly decreased the expressions of Nogo-A/Nogo receptor and RhoA/Rho-associated kinase in the peri-infarct cortex, and increased the expressions of growth associated protein-43, synaptophysin, vGlut1, and postsynaptic density-95 in the denervated cervical spinal cord. Behavioral performances assessed by the beam-walking test and the water maze test were improved significantly by CIMT.

Conclusions—CIMT promoted poststroke synaptic plasticity and axonal growth at least partially by overcoming the intrinsic growth–inhibitory signaling, leading to improved behavioral outcome. (Stroke. 2013;44:1698-1705.)

Key Words: axonal growth ■ constraint-induced movement therapy ■ functional recovery ■ stroke

Effective treatments to improve functional outcome after stroke are currently limited. Treatments that enhance brain self-repair, such as axonal reorganization, may offer an attractive approach to restore the impaired functions after stroke attributable to an extended therapeutic time window.1 However, several intrinsic myelin–associated neurite growth inhibitors, including Nogo-A, myelin-associated glycoprotein, and oligodendrocyte-myelin glycoprotein, limit axonal growth and plasticity.2

Constraint-induced movement therapy (CIMT) has been extensively used for stroke rehabilitation.3 Recent studies suggest that CIMT might induce not only functional reorganization, but also structural plasticity after stroke. However, the underlying mechanism(s) of CIMT-induced structural plasticity remains to be studied.4

In the present study, we tested the hypothesis that CIMT would enhance axonal remodeling and functional recovery after focal cerebral ischemia. First, we measured the length and the number of new sprouting axons crossing from the intact corticospinal tract (CST) to the denervated side of the spinal cord in stroke rats. Next, we explored whether CIMT overcomes the intrinsic axonal growth–inhibitory pathways to facilitate plasticity at the cervical spinal cord. Finally, we assessed possible associations between altered structural plasticity and sensorimotor performance or cognitive outcome.

Materials and Methods

Animals
Forty-two male Wistar rats (200–250 g) were randomly assigned as sham-operated rats (SHAM; n=12), rats subjected to cerebral ischemia (ISC; n=15), and rats with cerebral ischemia that were later treated with CIMT (ISC+CIMT; n=15). All rats were housed under controlled temperature in a 12-hour light/dark cycle with easy access to food and water and assigned to groups with a minimum of 4 animals per enclosure. Protocols and procedures were approved by the Institutional Animal Care and Use Committee of China Medical University (permit No. SCXK [Liao] 2008-0005).

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Experimental Design

The experimental protocol is summarized in Figure 1. The rats were pretrained in the beam-walking test for 3 days before ischemia. Cerebral ischemia was induced in the left cerebral motor cortex and underlying striatum by the vasoconstrictive peptide endothelin-1 (ET-1). As described previously, ISC+CIMT rats were wrapped with plaster of Paris strips around the ipsilateral forelimbs and upper torsos for 21 days after the ischemia.5 Biotinylated dextran amine (BDA) was used as an anterograde tracer at postoperative day 14. The animals were tested with behavioral tests at postoperative days 29 to 32.

ET-1 Stroke Model

ET-1 (Sigma) was dissolved in sterile saline at a concentration of 0.5 μL/mL and injected using a Hamilton microsyringe at postoperative day 14. The injection was given intracerebroventricularly at a rate of 0.5 μL/min by an infusion pump, and the needle remained in place for 3 minutes after completion of each injection. The dose of ET-1 for each injection site was 2 μL. Sham-operated rats were injected with saline instead of ET-1.

BDA Tracing

BDA (Molecular Probes, 10,000 MW, 10% wt/vol solution in 0.01 mol/L PBS) was pressure injected stereotactically into the contralesional (right) motor cortex to trace the CST fibers crossing the midline into the denervated spinal gray matter (Figure 2C) at depths of 1.5 mm and 2.0 mm below the cortical surface with the following stereotaxic coordinates: AP +1.0 mm, ML −2.0 mm; AP 0 mm, ML −1.5 mm; AP −1.0 mm, ML −1.4 mm; and AP −2.0 mm, ML −1.4 mm. The volume for each injection site was 1 μL, and the syringe remained in place for 3 minutes after completion of each injection.

Tissue Preparation

Thirty-three days after stroke, animals were transcardially perfused, and the brains and the cervical spinal cords were dissected and postfixed. A series of 25-μm-thick sections were cut in coronal plane from forebrain blocks for immunostaining of axonal growth inhibitors and infarct volume measurement. The cervical spinal cord segments of C6 to C8 were cut into 50-μm-thick coronal sections for BDA immunostaining and 25-μm-thick sections for immunostaining of the synaptic markers.

Infarct Volume Measurement

Forebrain coronal sections (25 μm thick) were selected from +4.5 mm to −7.5 mm relative to the bregma at 1-mm intervals for measurement of the infarct volume. Frozen sections were mounted onto slides in 0.01 mol/L PBS and were stained with cresyl violet acetate (Sigma). Contralateral and ipsilateral hemispheric areas were measured by a blinded observer using NIH ImageJ. Infarct volumes were calculated by subtracting the area of the injured hemisphere from the area of the normal hemisphere in each section, and areas were multiplied by the distance between sections to obtain the respective volumes.3

Immunohistochemistry

Brain sections were stained with the following primary antibodies: rabbit anti–Nogo-A (1:400, Abcam), rabbit anti–Nogo receptor (NgR: 1:400, Abcam), rabbit anti-RhoA (1:500, Abcam), and rabbit anti–Rho-associated kinase (ROCK; 1:1000, Abcam). Spinal sections were stained with the following primary antibodies: rabbit anti–growth associated protein-43 (1:500, Abcam), rabbit anti–v-Clut (1:100, Abcam), rabbit anti–post-synaptic density-95 (1:800, Abcam), and mouse anti–synaptophysin (1:400, Millipore) at 4°C overnight. For axonal growth inhibitor staining, sections were incubated with goat anti–rabbit (1:400, Abcam), rabbit anti–RhoA (1:500, Abcam), and rabbit anti–Nogo receptor (NgR; 1:400, Abcam), rabbit anti–Nogo-A (1:400, Abcam), and rabbit anti–Rho-associated kinase (ROCK; 1:1000, Abcam), and mouse anti–synaptophysin (1:400, Millipore) at 4°C overnight. For axonal growth inhibitor staining, sections were incubated with goat anti–rabbit (1:200, Alexa Fluor 488 and Alexa Fluor 594 IgG, Invitrogen) secondary antibodies, and for BDA and double staining, sections were incubated with conjugate streptavidin secondary antibody (1:200, Alexa Fluor 594, Invitrogen) or mixture with goat anti–rabbit (1:200, Alexa Fluor 488 IgG, Invitrogen) and anti–mouse (1:200, Alexa Fluor 488 IgG, Invitrogen) secondary antibodies.
Quantification

Axonal growth and sprouting in response to ischemic injury and CIMT were detected at caudal cervical enlargement (C6–C8) using a confocal microscope (Olympus FV-1000, Japan) from 6 coronal sections per rat. Three-dimensional reconstructions of the BDA-positive fibers were performed from Z-series stacks of confocal images with NIH ImageJ, by using a simple neurite tracer plugin to visualize intact CST fibers and their growth toward the denervated gray matter. The total length of the crossing CST fibers was traced and analyzed. To further analyze the number of midline-crossing fibers as well as branching of collaterals, the region between the central canal and the lateral gray matter border was divided into 3 regions named as M, D1, and D2 by 4 vertical lines, as described previously.4 Midline-crossing fibers were counted with a ×20 objective lens in the dorsal and ventral commissure at the central canal (Figure 3B, levels M), and branching of these fibers was evaluated at 2 defined regions within the gray matter (Figure 3B, levels D1 and D2).

To determine the expressions of axonal growth inhibitors, every tenth section (25 μm) between bregma levels 0.7 and 2.3 mm was selected (total of 6 sections per brain). In each section, immunofluorescence images for the axonal growth inhibitors (Nogo-A, NgR, RhoA, and ROCK) were captured at ×20 magnification in the infarcted cortex (as indicated in Figure 4A, middle) with a microscope (Olympus, Japan). The number of immunopositive cells from 6 sections was counted with NIH ImageJ, and a mean cell count was obtained.

To confirm whether the expressions of synaptic markers changed after ischemic stroke with or without CIMT, immunofluorescence images for the synaptic markers (growth associated protein-43, vGlut1, synaptophysin, and postsynaptic density-95) of every tenth section (25 μm) from C6 to C8 of the spinal cord were captured at ×20 magnification from the ventral horn. The pixel of integrated density from 6 sections per animal was measured using NIH ImageJ, and an average pixel was calculated.

Western Blots

Western blot analysis was performed at day 33 after ischemia induction. Protein concentrations were determined using a BCA protein concentration determination reagent kit (Beyotime, China). The following primary antibodies were used: anti–Nogo-A (1:1500, Abcam), anti-NgR (1:600, Abcam), anti-RhoA (1:1000, Abcam), and anti-ROCK (1:1000, Abcam). Specific proteins were visualized using an enhanced chemiluminescence reagent kit (Beyotime, China).

Behavioral Outcome Measures

The tapered/ledged beam-walking test was used to investigate the sensorimotor function of the impaired limbs.9 The performance of the rats was videotaped and later analyzed by calculating the slip ratio of the impaired (contralateral to lesion) forelimb and hindlimb (number of slips/number of total steps). Spatial learning was analyzed with a match-to-place version of the Morris water maze.10 At the end of the testing period (postoperative day 32), a probe trial of 30 s without the platform was used to assess how well the rats remembered the location of the platform (number of passes over the previous platform location).

Statistics

Statistical analyses were conducted using SPSS software (version 17). All data were analyzed using repeated-measures ANOVA or 1-way ANOVA. Statistical differences between groups were analyzed using the least significant difference post hoc test. All data were expressed as mean±SEM.

Results

Infarct Volumes Measurement

Two animals died 1 day after ET-1 injection. Four animals with no behavioral impairment (lack of limb withdrawal when hanging over the edge of a table) were excluded from the study. Gross anatomy of the brains on removal from the skull revealed visible infarcts within the sensorimotor region (Figure 4A, left). A representation of the typical damage at each level is presented in Figure 4A (right). There was no significant difference in infarct volumes between the ISC (109.3±12.6 mm³) and the ISC+CIMT groups (120.1±13.8 mm³).

Figure 3. Reconstruction of biotinylated dextran amine (BDA)-labeled corticospinal tract (CST) fibers growing toward the contralateral denervated gray matter. A, Representative pictures of reconstructed BDA-labeled CST fibers. BDA-labeled fibers are depicted in black. B, BDA-labeled fibers were quantified by counting all intersections with lines M, D1, and D2. M was placed vertically through the midline. D1 and D2 were drawn parallel to M at one third and two thirds of the distance between the central canal and the lateral gray matter border. C, Quantitative analysis was made by counting the crossing CST fibers located in regions M, D1, and D2 of the denervated spinal cord. **P<0.01; n=8 per group.
Increases in the Growth of Crossing CST Fibers by CIMT

After cerebral ischemia, axons from the undamaged CST sprout collaterals to the contralateral side, which lost its innervation.11,12 Consistent with this, the reconstructed Z-stack pictures of BDA-labeled fibers seen by confocal microscopy showed that only a few BDA-labeled CST fibers crossed the midline in the SHAM group, whereas ischemia increased the number of labeled fibers in the denervated gray matter. After 3 weeks of CIMT, there was an additional, significant increase in the growth of fibers within the denervated gray matter compared with the ISC group (Figure 2A).

We also quantified the total length of the labeled CST fibers crossing the midline. There was a significant overall group effect in the total length of the crossing CST fibers ($F_{(2,21)}$=73.539; $P<0.001$). The length of crossing CST fibers was doubled in ISC rats (1493±56 μm) and tripled in ISC+CIMT rats (2292±141 μm) compared with the length measured in SHAM rats (728±44 μm; Figure 2B).

A more detailed examination revealed that the majority of the crossing CST fibers in the SHAM and ISC groups appeared in the regions closer to the midline (M and D1), whereas the crossing CST fibers after CIMT were mainly located in the faraway regions, such as D1 and D2 (Figure 3A). In region D1, there was a significant overall group effect in the number of crossing CST fibers ($F_{(2,21)}$=10.261; $P=0.001$). The number of crossing CST fibers after CIMT was 4.3±0.5 per section, which was not different ($P=0.52$) from ISC rats (3.4±0.4 per section) and significantly increased ($P<0.01$) compared with SHAM rats (1.6±0.3 per section). In region D2, the difference among groups was significant ($F_{(2,21)}$=30.823; $P<0.001$). The number of crossing CST fibers after CIMT was 5.3±0.6 per section, which was significantly increased ($P<0.01$) compared with ISC rats (1.3±0.3 per section) and SHAM rats (1.0±0.3 per section; Figure 3C).

Decreases in Axonal Growth Inhibitors After CIMT

Next, we wanted to explore whether the expressions of various axonal growth inhibitors in the peri-infarct cortex were affected by CIMT. Indeed, there were significant overall group effects in the number of the Nogo-A, Nogo receptor (NgR), RhoA, and Rho-associated kinase (ROCK). Western blots confirmed a robust upregulation of the expressions of Nogo-A/NgR and RhoA/ROCK after cerebral ischemia, whereas CIMT significantly decreased the expressions of Nogo-A/NgR and RhoA/ROCK in the peri-infarct cortex (Figure 4C and 4D).
Restoration of Synaptic Markers by CIMT

In the ventral horn of spinal coronal sections, immunostaining for synaptic markers yielded spotted signals, which were expressed in the large cell bodies and dendritic trees of spinal motor neurons (Figure 5A). The expressions of all synaptic markers in the denervated gray matter of spinal cords were decreased after cerebral ischemia compared with SHAM animals (P<0.01). Interestingly, CIMT significantly elevated the decreased levels of these synaptic markers in response to focal stroke (P<0.05 or P<0.01; Figure 5B). Furthermore, confocal microscopy revealed that BDA was colocalized with growth associated protein-43, vGlut1, synaptophysin, and postsynaptic density-95 (Figure 5C), indicating that midline-crossing CST collaterals within the denervated spinal cords were newly grown fibers, and that functional synapses were formed.

Improved Behavioral Outcomes by CIMT

To test whether the altered structural plasticity was associated with improved behavioral outcomes in ischemic rats, sensomotor and cognitive outcomes were measured at the end of the study. There were significant overall group effects in slip ratio with the impaired forelimb (F_{2, 21}=6.747; P=0.005) and hindlimb (F_{2, 21}=7.969; P=0.003; Figure 6A and 6B) in the beam-walking test. Slip ratios with the impaired forelimb and hindlimb for the ISC rats (23.3±2.0% and 26.1±2.5%) were significantly higher than those of the SHAM rats (10.3±2.3% and 14.7±1.6%). Furthermore, there were significant differences
(P<0.05) in slip ratios with the impaired forelimb and hindlimb between the ISC and ISC+CIMT groups (15.1±3.1% and 18.2±2.0%).

When the water maze data were analyzed, there were significant group effects in escape latency (F(2, 21)=146.21; P<0.001) and path length (F(2, 21)=14.803; P<0.001; Figure 6C), but not in swimming speed (F(2, 21)=0.927; P=0.411). Compared with the ISC rats, ischemic rats treated with CIMT had shorter escape latency (P<0.001) and path length (P<0.05). In the probe trial, there was also a significant overall group effect in the number of passes over the target area (F(2, 21)=6.777; P=0.005). A significant decrease in passes was found in the ISC group compared with the SHAM group (1.88±0.23 versus 3.13±0.30; P=0.003), which was reversed in rats from the CIMT group (3.0±0.27, P=0.007). These differences in search strategies were reflected in the swim paths exhibited by the 3 groups (Figure 6D). Rats treated with CIMT showed a significant preference for the target quadrant than ischemic rats.

**Discussion**

The present study showed for the first time that CIMT enhanced the outgrowth and possible synapse formation of CST fibers from the intact side of the brain to the denervated cervical spinal cord after focal cerebral ischemia in rats. In addition, CIMT decreased the expressions of Nogo-A/NgR and RhoA/ROCK in the peri-infarct cortex. These structural and molecular changes induced by CIMT were associated with significant behavioral improvement.

A recent study showed that CIMT significantly increased the number of collateral CST fibers extending from the intact side into the denervated side of the cervical spinal cord after unilateral CST injury in rats. The growth is accompanied by the formation of new synapses and improved motor performance, suggesting that rehabilitative activity, such as CIMT, could promote axon growth and synaptogenesis. After unilateral stroke, axons of CST from the contralateral motor cortex sprout collaterals that cross over into the ipsilateral cervical spinal cord. The newly formed projections to the cortex sprout collaterals that cross over into the ipsilateral spinal cord after unilateral CST injury in rats. The growth is accompanied by the formation of new synapses and improved motor performance, suggesting that rehabilitative activity, such as CIMT, could promote axon growth and synaptogenesis. After unilateral stroke, axons of CST from the contralateral motor cortex sprout collaterals that cross over into the ipsilateral cervical spinal cord.

The present study showed that 3 weeks of CIMT after focal cerebral ischemia significantly enhances outgrowth and possible synapse formation of CST fibers crossing over the midline and sprouting into the denervated gray matter in the cervical cord in ischemic rats after 3 weeks of CIMT. In addition, CIMT led to significantly increased expression of growth-associated protein-43, which was used as a marker for axonal growth and reactive synaptogenesis. We also demonstrated that CIMT significantly increased the expressions of several synaptic markers. More important, CIMT-induced BDA-positive fibers were colocalized with the synaptic markers mentioned above, suggesting that the sprouting fibers may form functional synapses.

We also measured the expressions of the growth-inhibitory molecules, such as Nogo-A/NgR and RhoA/ROCK, at post-operative day 33 instead of early time points after stroke. We showed that stroke induced by ET-1 significantly increased the levels of Nogo-A/NgR and RhoA/ROCK in the peri-infarct cortex, which is in line with a recent study. This study showed that cerebral ischemia increased the neuronal expression of Nogo-A/NgR over time in both the ipsilesional and contralesional cortex. Remarkably, such temporal elevation of Nogo-A coincides with the failure of spontaneous sprouting after focal ischemic stroke. Although we did not measure the expression profiles of these growth-inhibitory molecules in the contralesional cortex, Nogo-A/NgR in either peri-infarct cortex or contralesional cortex seems to play a critical role in inhibiting axonal growth after stroke.

Although there are contrasting studies demonstrating that Nogo-A protein expression is decreased during the time window of 2 to 3 weeks after ischemia, the temporal expression patterns of Nogo-A and other growth-inhibitory molecules in the late ischemic period and their functional significance in poststroke axonal sprouting remain less understood. A recent study showed that anti-Nogo-A treatment, even when started as late as 9 weeks after stroke in the adult rat, remains to be effective in enhancing behavioral recovery and axonal growth originating from the intact sensorimotor cortex to the red nucleus ipsilateral to the infarct. These results indicated a potential role of Nogo-A in inhibiting the structural plasticity in the late ischemic period beyond the time window for the axonal growth. Decreasing Nogo-A/NgR expression even in the late ischemic period might improve axonal regeneration in the ischemic brain. Consistent with this assumption, we observed that enhanced axonal growth after 3 weeks of CIMT was accompanied by decreased expressions of Nogo-A/NgR and their downstream targets, RhoA/ROCK, in the peri-infarct cortex at postoperative day 33.

There is evidence suggesting that forced limb use in rodents after brain or spinal cord injury could induce other growth-promoting factors, such as neurotrophic factors. Therefore, we cannot exclude the possibility that the changed expressions of growth-promoting molecules in the peri-infarct cortex or contralesional cortex were also involved in the enhanced axonal growth induced by poststroke CIMT in our study.

How CIMT counteracts Nogo-A/NgR and its downstream targets remains unclear. However, our previous study showed that 3 weeks of CIMT after focal cerebral ischemia significantly increases the level of stromal cell–derived factor-1 (SDF-1) in the brain. SDF-1 has been shown to promote neurite outgrowth in the presence of growth-inhibitory central nervous system myelin in vitro.
infusion of SDF-1 in spinal cord injury resulted in enhanced sprouting of CST axons into both white matter and gray matter. Moreover, there was evidence suggesting that SDF-1 has the ability to inhibit the function of RhoA and its downstream effector, ROCK. All these results indicate that SDF-1 may be involved in the enhanced CST sprouting induced by CIMT by counteracting the hostile microenvironment for axonal growth.

In line with previous studies showing improved motor function after brain injury, CIMT improved behavioral performance in ischemic rats as evaluated by the beam-walking test. Moreover, there was evidence suggesting that SDF-1 has the ability to inhibit the function of RhoA and its downstream effector, ROCK. All these results indicate that SDF-1 may be involved in the enhanced CST sprouting induced by CIMT by counteracting the hostile microenvironment for axonal growth.

In our study, CIMT was initiated at poststroke day 7 and persisted for 21 days. This interval matched the critical period for stroke rehabilitation. In addition, consistent with previous studies, the initiating time point of CIMT in our study seems to be safe because no exacerbation of infarct size or behavioral impairment was observed.

In summary, our study showed that CIMT regulated structural changes at the cervical spinal cord after cerebral ischemia, leading to improved behavioral recovery. Axonal growth may occur through overcoming the intrinsic growth-inhibitory signaling.

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**Disclosures**

None.

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