Remodeling of the Corticospinal Innervation and Spontaneous Behavioral Recovery After Ischemic Stroke in Adult Mice

Zhongwu Liu, MD, PhD; Rui Lan Zhang, MD; Yi Li, MD; Yisheng Cui, MD; Michael Chopp, PhD

Background and Purpose—To elucidate how the motor pathways rewire the denervated tissue after stroke, we investigated remodeling of the corticospinal tract (CST) in transgenic mice with yellow fluorescent protein CST labeling in conjunction with transsynaptic pseudorabies virus retrograde tracing.

Methods—Adult male CST–yellow fluorescent protein mice were subjected to permanent right middle cerebral artery occlusion (n=8/group). Foot-fault test was performed to monitor functional deficit and recovery. Pseudorabies virus tracer was injected into the left forelimb muscles at 1 or 4 weeks after middle cerebral artery occlusion (4 days before euthanasia), respectively. A third group of CST–yellow fluorescent protein mice without middle cerebral artery occlusion was used for normal control (n=6). The yellow fluorescent protein labeling of CST in the cervical cord and pseudorabies virus labeling of pyramidal neurons in the bilateral cortices were measured on vibratome sections using a confocal imaging system.

Results—Compared with normal animals, axonal density in the stroke-affected side of the cervical cord was significantly decreased at 11 days (P<0.001) and significantly increased at 32 days after stroke compared with the Day 11 values (P<0.05). Pseudorabies virus labeling was significantly decreased in the ischemic hemisphere 11 days after middle cerebral artery occlusion (P<0.001). In contrast, a significant increase was observed in pseudorabies virus labeling of bilateral cortices 32 days after stroke compared with 11 days (P<0.05). The CST axonal density in the denervated spinal cord and pyramidal neuron labeling in the bilateral cortices were significantly correlated with behavioral recovery (P<0.05).

Conclusions—Spontaneous functional recovery after stroke may, at least in part, be attributed to neuronal remodeling in the corticospinal system. (Stroke. 2009;40:2546-2551.)

Key Words: functional recovery • mice • middle cerebral artery occlusion • neuronal plasticity
nal reorganization of the cortical pyramidal neurons in bilateral hemispheres having synaptic connections with the stroke-impaired forelimb.

Materials and Methods

Animals
Adult CST-YFP mice (2 months old; body weight, 25 to 30 g) were generated by our in-house breeding colony using 1 transgenic mouse strains of B6.Cg-Tg(Thy1-EYFP)15Jrs/J and B6.129-Emx1tm1(cre)Krj/J obtained from Jackson Laboratories (Bar Harbor, Maine). In the Thy1-STOP-YFP mice, YFP expression is driven by neuron-specific regulatory elements of the Thy1 promoter after Cre-mediated excision of STOP sequences. In the Emx-Cre mice, Cre recombinase is specifically expressed in the embryonic forebrain, the area of origin of the CST. Therefore, in CST-YFP mice generated by mating Thy1-STOP-YFP with Emx-Cre strain, YFP expression is limited to the forebrain and CST. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital.

MCAO Model
For ischemic stroke, 22 mice were subjected to permanent MCAO by advancing a 6-0 surgical nylon suture with an expanded (heated) tip from the right external carotid artery into the lumen of the internal carotid artery to block the origin of the middle cerebral artery. Six mice died within the first 5 days. The remaining animals were randomly divided into 2 groups and euthanized at 11 or 32 days after MCAO, respectively (n=8 per group). A third group of naive CST-YFP mice without surgery was used for normal control (n=6).

Behavioral Tests
To evaluate the motor functional recovery, a Foot-Fault test was performed at 1 day after MCAO and weekly thereafter. This test measures the accuracy of forepaw placement on a nonequidistant grid as the percentage of foot faults of the left forepaw to total steps.

Retrograde PRV Tracing
To confirm the neuronal wiring between the motor cortex and the stroke-impaired peripheral target tissues, a transsynaptic tracer, PRV-614-mRFP (gift from Dr Lynn Enquist, Princeton University, Princeton, NJ), was used to retrogradely label the cortical pyramidal neurons from the left forelimb muscles. At 4 days before euthanasia, a 10-μL total volume of PRV-164-mRFP, divided into multiple injections of 1 to 2 μL, was injected into muscles of the left forelimb (radialor flexor) with a 10-μL Hamilton syringe through a skin incision. The animals were then transferred to a Biosafety Level 2 room to survive for an additional 94 to 96 hours for the tracer transport from axonal terminals in muscles to the cell body of pyramidal neurons in the cortex.

Tissue Preparation
At 11 or 32 days after MCAO, animals were perfused transcardially with saline followed by 4% paraformaldehyde. The entire brain and the spinal cord were removed and immersed in 4% paraformaldehyde overnight. Every first 100 μm of each 500-μm thickness in the forebrain was sectioned using a vibratome to examine the mRFP-positive pyramidal neurons. The remaining 400-μm brain blocks were cut into consecutive 100-μm thick vibratome sections to detect the YFP-positive CST axons with fluorescent microscopy.

Data Analysis and Statistics
The behavior outcomes were evaluated by the numbers of the stroke-impaired left forepaw missteps between the wires with time after stroke. Lesion volume was measured by National Institutes of Health imaging software (Image J) and presented as a volume percentage of the lesion area compared with the contralerosional hemisphere as previously reported. A Bio-Rad MRC 1024 (argon and krypton) laser scanning confocal imaging system mounted onto a Zeiss microscope (Bio-Rad, Cambridge, Mass) was used to examine PRV labeling of pyramidal neurons in the bilateral cerebral cortices and the YFP labeling of CST axons in the cervical cord. The number of mRFP-positive cells was presented in 0.5 mm granularity to the bregma. The CST axonal densities in both sides of the gray matter of cervical cord were measured on 30 consecutive transverse sections with Image J. To avoid variation on fluorescent measurements, the percentage of CST density in the stroke-impaired side to the contralateral side on the same sections was calculated as an index to assess axonal remodeling in the spinal cord after stroke.

All data are presented as mean±SD. A value of P<0.05 is considered significant. One-way analysis of variance was used to evaluate functional recovery, numbers of PRV-positive pyramidal neurons, and the index of axonal remodeling. To test the correlation between behavioral outcome and neuronal reorganization, the correlation coefficients between the left forepaw motor performance and the index of axonal density in the denervated side of the cervical cord or total PRV-positive cell numbers in the bilateral cortices were calculated by Pearson’s correlation coefficients after MCAO with r>0.70 indicating a good correlation.

Results
Lesion Volume and Behavioral Recovery
The mean lesion volume in mice euthanized at 11 and 32 days after permanent suture MCAO was 29.5±4.2% (range, 21.9% to 36.2%; 30.3%±3.9% for Day 11 and 28.8%±4.6% for Day 21; Supplemental Figure I, available online at http://stroke.ahajournals.org). As assessed with Foot-Fault test (Figure 1), severe behavioral deficits were evident in all animals 1 day after stroke, which partially recovered during the first week (P<0.001). A significant progressive recovery was observed with time (P<0.001 versus 1 week).

Axonal Remodeling of the CST in the Denervated Side of the Cervical Cord
CST axons innervate the spinal motoneurons and interneurons by synaptic connections in the spinal gray matter for peripheral motor control. In CST-YFP mice, the cortical neurons in the forebrain and their axons, the CST, were specifically labeled with YFP and, therefore, were visible on vibratome sections under a fluorescent microscope (Supplemental Figure II). To examine the neuroanatomical basis of behavioral disability and recovery, we measured axonal density in the central area of the cervical gray matter in adult normal mice and mice with stroke 11 or 32 days later (Figure 1).
2). Compared with a normal animal (Figure 2A), the axonal density in the stroke-impaired side was decreased 11 days after stroke (Figure 2B), consistent with previous data. However, increased axonal density was observed at the 32-day, ie, late time point (Figure 2C), compared with Day 11 animals. To avoid subtle intersection differences induced by fluorescent imaging sensitivity, we calculated the ratio of axonal density in the denervated side to the intact side on the same sections in each animal as an index to assess the axonal remodeling. Statistical data showed that more than 60% of CST axons were eliminated in the stroke-impaired side 11 days after stroke (Figure 2D, \( P < 0.001 \)), whereas a significant recovery in CST density in the denervated side of the spinal cord was present at 32 days compared with 11 days after MCAO (\( P < 0.01 \)).

Neuronal Reorganization in the Bilateral Cortices
To confirm the establishment of neuronal connections between the bilateral hemispheres and the stroke-impaired peripheral tissue, we retrogradely labeled the neural pathways from the left forelimb muscles to the motor cortices with a transsynaptic fluorescent viral tracer of PRV-614-mRFP in CST-YFP mice (Figure 3). In the normal mouse, 4 days after tracer injection into the left forelimb muscles, most PRV-positive pyramidal neurons were found in Layer V of the forelimb motor areas in the right cerebral cortex with fluorescent labeling on the cell body and dendrites (Figure 3A), whereas only few pyramidal cells in the symmetrical areas in the left hemisphere were labeled with mRFP (Figure 3B). Eleven days after right MCAO, the number of PRV-positive pyramidal neurons was reduced in the ischemic cortex (Figure 3C) and was unchanged in the contralesional cortex (Figure 3D). Interestingly, the number of PRV-labeled cells increased within the forelimb areas in both ipsilesional and contralesional cortices 32 days compared with animals 11 days after stroke (Figure 3E–F). We counted the numbers of PRV-positive neurons on each one of 5 100-µm thick coronal sections of the rostral forebrain (Table 1). Quantitative data showed that in the right ischemic hemisphere, the number of pyramidal neurons connecting with the stroke impaired left forelimb was significantly decreased after MCAO compared with normal animals in both caudal forelimb area (−0.5 to 0 mm rostral to the bregma) and rostral forelimb area (1.5 to 2.0 mm rostral to the bregma; \( P < 0.001 \)). However, significant increases of PRV-positive neurons were observed over the caudal and rostral forelimb areas in both hemispheres at 32 days after stroke (\( P < 0.05 \) versus Day 11).

Correlation Between Functional Recovery and Neural Remodeling
To test the hypothesis that neuronal remodeling contributes to functional outcome in the subacute and chronic phases after stroke, we determined the correlation of behavioral performance with the neuronal morphological status in mice euthanized at 11 or 32 days after MCAO. Our data showed that functional scores were highly correlated with the index of axonal density in the denervated side of the spinal cord (Figure 4A) and the numbers of neurons connecting with the stroke-impaired forelimb in both ischemic (Figure 4B) and contralesional (Figure 4C) cortices (\( P < 0.05 \)). In addition, at Day 11 poststroke, a significant correlation was found between functional behavior and PRV-positive cells in the contralesional left cortex (Table 2; \( P < 0.05 \)) with no significant correlation of neurological function and PRV cells in the right ischemic cortex or axonal density in the spinal cord. In contrast, at 32 days after stroke, significant correlations were evident between functional outcome and both axonal density (\( P < 0.05 \)) and PRV labeling in the right cortex (\( P < 0.01 \)), whereas a marginal negative correlation was found between functional outcome and the PRV cells in the left cortex (\( P = 0.08 \)), suggesting that functional recovery at Day 32 is related to remodeling in the ipsilesional cortex and spinal cord, whereas at the early time point (ie, Day 11), functional...
recovery is associated with axonal changes in the contralesional intact cortex.

**Discussion**

In the present study, using transgenic and viral fluorescent labeling to the cortical pyramidal neurons in adult mice, we directly demonstrated that the neuronal rewiring in the stroke-impaired side of the cervical spinal cord originating from bilateral cortices significantly recovered during sub-acute and chronic phases after stroke. Additionally, spontaneous motor behavioral recovery after cerebral ischemic stroke is time-dependent and highly correlated with the CST axonal remodeling in the spinal cord and the pyramidal neuronal reorganization in the bilateral cortices.

Compared with traditional dye methods, the use of transgenic labeling in CST-YFP mice has salient advantages of

![Figure 3](image_url)

**Figure 3.** PRV labeling of pyramidal neurons in the bilateral cerebral cortices at the bregma level. PRV-614-mRFP (red) was injected into the left forelimb muscles. Single-layer confocal images on coronal brain sections at the bregma level show that in a normal mouse, the pyramidal neurons were labeled with PRV primarily in the corresponding area of the right cortex (A) and sparsely in the left cortex (B). PRV-positive pyramidal cells in the right cortex were reduced 11 days after MCAO (C), whereas the number PRV-labeled cells was little increased in the contralesional cortex (D). In contrast, the PRV-positive cells were increased 32 days after stroke in both ipsilesional (E) and contralesional hemispheres (F) compared with 11 days. Insets in A and B, schematic drawing of the brain coronal sections showing the position of the photomicrograph appearing in the left and right panels, respectively. Scale bar=100 μm.

**Table 1. Numbers of PRV-Positive Pyramidal Neurons in Bilateral Cortices**

<table>
<thead>
<tr>
<th>Group</th>
<th>mm to Bregma</th>
<th>0.5</th>
<th>1.0</th>
<th>1.5</th>
<th>2.0</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Left</td>
<td>2.0±1.4</td>
<td>5.7±2.5</td>
<td>0.3±0.8</td>
<td>0.3±0.8</td>
<td>3.5±2.9</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>81.2±9.2</td>
<td>134.0±11.1</td>
<td>19.0±4.3</td>
<td>2.7±2.5</td>
<td>24.2±5.8</td>
</tr>
<tr>
<td>Day 11</td>
<td>Left</td>
<td>4.4±3.6</td>
<td>6.6±3.8</td>
<td>1.9±1.8</td>
<td>0.9±1.5</td>
<td>4.8±2.7</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>34.0±13.8†</td>
<td>59.8±18.7†</td>
<td>13.8±4.4*</td>
<td>2.0±1.9</td>
<td>14.1±7.2*</td>
</tr>
<tr>
<td>Day 32</td>
<td>Left</td>
<td>12.6±5.0§</td>
<td>14.8±4.0§</td>
<td>8.9±4.0§</td>
<td>1.0±1.2</td>
<td>9.3±3.1§</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>57.9±17.6‡</td>
<td>78.3±19.7</td>
<td>15.5±5.2</td>
<td>2.8±1.8</td>
<td>23.5±6.8‡</td>
</tr>
</tbody>
</table>

Numbers are mean±SD.

*P<0.01.

†P<0.001 versus normal.

‡P<0.05.

§P<0.01 versus Day 11.
Our findings demonstrate that the corticospinal innervation is impaired early after stroke, whereas the descending motor system exhibits essentially an unilateral innervation pattern, if the motor cortex is extensively damaged, an alternative recruitment from the intact cortex is greatest in the more impaired patients.17 However, the functional contribution of the ischemic cortex may be the main source of the neuronal remodeling in the spinal cord and bilateral cortices.

An important observation from this study was that the neuronal remodeling occurred widely in extensive cerebral areas after unilateral ischemic stroke, including remote regions in the infarct hemisphere and the contralateral intact hemisphere. Although the adult mammalian corticospinal system exhibits essentially an unilateral innervation pattern, if the motor cortex is extensively damaged, an alternative network outside the damaged area, either in the ipsilateral hemisphere or contralateral hemisphere, arises to compensate for the loss of function in the damaged system.12–15 Our data demonstrate a dynamic rewiring in the denervated gray matter of the spinal cord after stroke. Acutely, within 11 days poststroke, there is a prominent reduction of CST connections between the forelimb and the ipsilesional cortex with a significant increase of CST axons in bilateral cortices originating from the forelimb at Day 32. We also found that functional recovery poststroke is highly correlated with contralateral cortical wiring, only acutely (ie, 11 days), with a negative correlation at the late time point (32 days). Similar data have also been reported in humans with increasing invariability, noninvasiveness, and sensitivity.3 Additionally, PRV is a powerful neural tracer for multisynaptic pathways without dilution in the transport process. After PRV injection into forelimb muscles, the virus replicates in the infected neurons and infectious particles are released and taken up at synapses, thus spreading along neuronal hierarchical chains.5 Moreover, transport of the attenuated PRV Bartha strain between central nervous system neurons occurs only at points of synaptic contact and proceeds in a retrograde direction.11

Our findings demonstrate that the corticospinal innervation is impaired early after stroke, whereas the descending motor signaling pathways are functionally rewired through the increased CST axons detected with YFP labeling in the denervated spinal cord 32 days after MCAO. Therefore, high correlations between functional outcome and axonal density of the CST and PRV labeling of the cortical pyramidal neurons suggest that the behavioral recovery in the late phase after stroke may, at least in part, be attributed to the neuronal remodeling in the spinal cord and bilateral cortices.

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Table 2. Within-Group Comparisons Between Functional Outcome and Neuronal Status

<table>
<thead>
<tr>
<th>Group</th>
<th>Percent of CST Density</th>
<th>Right PRV Labeling</th>
<th>Left PRV Labeling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 11</td>
<td>r=0.20</td>
<td>r=0.27</td>
<td>r=0.74</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.05</td>
<td>p=0.64</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>Day 32</td>
<td>r=0.71</td>
<td>r=0.91</td>
<td>r=0.66</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.05</td>
<td>p&lt;0.01</td>
<td>p=0.08</td>
</tr>
</tbody>
</table>

The present finding of CST axonal remodeling provides neuroanatomical evidence for brain functional reorganization after stroke. Namely, the cortical pyramidal neurons residing outside the infarct region extend their arborization through axonal outgrowth in the spinal cord to take over the lost innervation from neurons in the ischemic infarct area or neurons with CST axonal disruption on spinal motoneurons to re-establish the corticospinal motor control. Moreover, with time poststroke, the increase of PRV labeling was mostly found in the ischemic hemisphere, suggesting that the ischemic cortex may be the main source of the neuronal recovery. Interestingly, in the Day 32 group, animals having higher contralesional PRV labeling showed worse behavioral performances, supporting a previous report that functional recruitment from the intact cortex is greatest in the more impaired patients.17 However, the functional contribution of the contralesional cortex requires further validation. Additional studies on the relationship of specific deficits/recovery with anatomic sets of neuronal remodeling and demonstrating causality by selective inhibition of CST remodeling as well as studies using different stroke models and therapeutic interventions are also warranted.

In the adult mammal, the injured central nervous system is a highly inhibitory environment for axonal regeneration. However, after ischemic lesions, the adult central nervous system can induce cellular responses needed for neurite growth and synaptic formation.18,19 Such molecules might support CST axonal sprouting in a time window of 2 to 3 weeks poststroke.20 Indeed, CST plasticity in the spinal cord associated with enhanced functional outcome has recently been found in animals after experimental stroke.21–23 Furthermore, our previous study demonstrated that the increase of CST axons in the spinal cord is attributed to increased arborization of neighboring uninjured fibers through short-range axonal sprouting within the spinal gray matter, a region without any direct ischemic damage.24 Such local short-range sprouting has important benefit to overcome glial inhibition in the central nervous system.25 Therefore, the CST axonal remodeling in the spinal cord may be considered as a potential target for cerebral stroke treatment by enhancing activation in the sensorimotor cortex from early contralesional activity to late ipsilesional activity during recovery from hemiparesis.16 Thus, the cortical rewiring formed in the mouse and its correlation with neurological function parallels observations in the patient with stroke. Our data, for the first time, demonstrate a robust anatomic correlation between the CST and functional recovery. Thus, spontaneous recovery after stroke may depend on a time-dependent rewiring of the CST.

Figure 4. Sketch graphs of correlations between neural remodeling and behavioral recovery. The functional recovery assessed by Foot-Fault test was highly correlated with the CST axon density in the denervated side of the spinal cord (A) and numbers of PRV-positive pyramidal neurons in the bilateral cortices (B–C, P<0.05).
axon growth stimulative factors in the brain and/or reducing axonal growth-inhibitory factors in the spinal cord.

Summary
Using transgenic CST-YFP labeling and retrograde transsynaptic PRV tracing in adult mice, we demonstrated that CST axonal remodeling in the spinal cord and neuronal reorganization in the bilateral cortices are time-dependent and highly correlated with spontaneous behavioral recovery after ischemic stroke.

Acknowledgments
We gratefully acknowledge the gifts of pseudorabies viral tracers from Dr Lynn W. Enquist, Princeton University, Princeton, NJ.

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References
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http://stroke.ahajournals.org/content/40/7/2546

An erratum has been published regarding this article. Please see the attached page for:
/content/40/12/e719.full.pdf
In the article “Remodeling of the Corticospinal Innervation and Spontaneous Behavioral Recovery After Ischemic Stroke in Adult Mice”, by Liu et al., there are several errors in Table 2. First, in the title, “States” should read “Status”. Second, the values for row “Day 32”, column “Right PRV Labeling” should read $r=0.91$ and $P<0.01$, and for column “Left PRV Labeling” should read $r=-0.66$ and $P=0.08$. The values were inadvertently transposed for publication. The authors regret these errors.

The corrected version can be viewed online at http://stroke.ahajournals.org.