Bilateral movement training promotes axonal remodeling of the corticospinal tract and recovery of motor function following traumatic brain injury in mice

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Traumatic brain injury (TBI) results in severe motor function impairment, and subsequent recovery is often incomplete. Rehabilitative training is considered to promote restoration of the injured neural network, thus facilitating functional recovery. However, no studies have assessed the effect of such trainings in the context of neural rewiring. Here, we investigated the effects of two types of rehabilitative training on corticospinal tract (CST) plasticity and motor recovery in mice. We injured the unilateral motor cortex with contusion, which induced hemiparesis on the contralesional side. After the injury, mice performed either a single pellet-reaching task (simple repetitive training) or a rotarod task (bilateral movement training). Multiple behavioral tests were then used to assess forelimb motor function recovery: staircase, ladder walk, capellini handling, single pellet, and rotarod tests. The TBI + rotarod group performed most forelimb motor tasks (staircase, ladder walk, and capellini handling tests) better than the TBI-only group did. In contrast, the TBI + reaching group did not perform better except in the single pellet test. After the injury, the contralateral CST, labeled by biotinylated dextran amine, formed sprouting fibers into the denervated side of the cervical spinal cord. The number of these fibers was significantly higher in the TBI + rotarod group, whereas it did not increase in the TBI + reaching group. These results indicate that bilateral movement training effectively promotes axonal rewiring and motor function recovery, whereas the effect of simple repetitive training is limited.

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Results

Histological assessment of cortical lesions. We employed a traumatic injury model in mice and investigated the effects of two different rehabilitative methods, reaching and rotarod training, on motor recovery after the injury. In our model, the unilateral forelimb area of the motor cortex was fully destroyed in which the CST is mostly degenerated (Figures 1a and 4a).\(^4,14\) To confirm the absence of corticospinal neurons in this model, pseudorabies virus (PRV) expressing enhanced green fluorescent protein (EGFP), a retrograde transsynaptic viral tracer,\(^15\) was injected into the forelimb muscle, and corticospinal neurons were examined for the presence of EGFP. In the sham group, most EGFP-positive neurons were found in layer V of the motor area contralateral to the injection side (Figures 1b and c). In the TBI, TBI + reaching, and TBI + rotarod groups, EGFP-positive neurons were absent in the contralateral motor area, but they were found in layer V of the ipsilateral motor area (Figures 1d–i), confirming that ipsilesional CST mostly degenerated in this model. Lesion volume was measured 34 days after TBI and was not significantly different among the TBI (28.8 ± 1.1%), TBI + reaching (27.2 ± 1.5%), and TBI + rotarod groups (29.9 ± 0.8%) (Figure 1j).

Bilateral movement enhances motor test performance after TBI. We used two different rehabilitative methods, reaching and rotarod training after TBI, and investigated the effects on motor function recovery (Figure 2). Impaired forelimb motor performance was assessed with the staircase

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**Figure 1** TBI mouse model. (a) Unilateral sensorimotor cortex contusion by TBI (34 days after injury). (b–l) Immunohistochemistry for anti-GFP and Nissl, which were injected with PRV-EGFP to the impaired forelimb, in the cortex of the sham (b and c), TBI (d and e), TBI + reaching (f and g), and TBI + rotarod groups (h and i) at day 39. (c, e, g and i) Magnified views of EGFP-positive layer V neurons in the contralateral (sham) and ipsilateral (TBI, TBI + reaching, and TBI + rotarod groups) cortex to the impaired side, respectively. Scale bars: 200 μm. (j) Percentage of lesion volume in the injured hemisphere.
We next asked whether task-specific rehabilitative training could enhance recovery of that task after TBI and found that mice subjected to single pellet-reaching training exhibited increased scores at 33 days after injury (Figure 3d; TBI + reaching versus TBI-only, two-way ANOVA followed by Tukey–Kramer test, \( P < 0.05 \)). In contrast, when mice performed the rotarod task after TBI, the differences between the TBI + rotarod and TBI-only groups were not significant (Figure 3e).

**Bilateral movement but not reaching training promotes CST rewiring after TBI.** The intact CST is capable of sprouting fibers that enter the denervated side of the spinal cord and make functional connections that are necessary for motor recovery.4 We examined anatomical changes of the CST in the cervical spinal cord (C4–C7) to determine whether rehabilitative training protocols enhanced CST plasticity at day 34 after brain injury. Biotinylated dextran amine (BDA), an anterograde tracer, was injected into the forelimb area of the uninjured sensorimotor cortex to label intact CST fibers, and we counted the number of crossing fibers into each compartment of the denervated side (Figures 4a and g). The number of BDA-labeled crossing fibers increased following TBI (Figures 4b, c, and h; TBI-only versus sham, two-way ANOVA followed by Tukey–Kramer test, \( P < 0.01 \)). The TBI + rotarod group showed a significantly higher number of crossing CST fibers compared with the TBI-only or TBI + reaching group, whereas TBI + reaching did not enhance CST rewiring compared with the TBI-only group (Figures 4c–e and h). Rotarod alone (without TBI) did not increase the number of crossing fibers compared with the sham group (sham + rotarod in Figures 4b, f, and h).

**Effect of rehabilitative training on cervical cord neuronal activity after TBI.** The finding that rehabilitative training enhances crossing CST fibers innervation suggests that neuronal activity in the cervical cord might be important for plasticity and functional recovery. c-Fos, an activity-dependent immediate-early gene, was used as a marker of neuronal activity.22 The number of c-Fos-positive cells markedly increased in the TBI + rotarod group compared with the TBI-only or TBI + reaching group at day 12 after brain injury (Figures 5a–g; two-way ANOVA followed by Tukey–Kramer test, \( P < 0.05 \)). Reach training slightly increased the number of c-Fos-positive cells, but the result was not statistically different from that in the TBI-only group (Figures 5a, b, d, e, and g). Next, we examined anatomical changes of the CST rewiring at the same day 12 to determine whether increased c-Fos-positive cells was a consequence of rewired neuronal network of CST or a prerequisite for the induction of CST rewiring. BDA-labeled crossing CST fibers at day 12 did not increase compared with those at day 33 in each group (Figures 5h–k; two-way ANOVA followed by Tukey–Kramer test, \( P < 0.05 \)). This suggests that the training increases neural activity in the spinal circuit before CST is reorganized.

**Discussion**

In this study, we assessed the effectiveness of skilled forelimb repetitive training and bilateral movement training in...
promoting functional recovery in an established TBI model and investigated whether they promoted CST axonal rewiring. The results revealed that bilateral movement training improved motor function recovery and facilitated axonal rewiring, whereas the beneficial effects of simple repetitive training were limited. Many studies have shown enhanced functional recovery by employing one rehabilitative task or a combination of tasks following CNS injuries. However, it is important to note that CST plasticity and its underlying mechanisms might differ between mice and humans.

In recent years, a form of bilateral movement training known as active–passive bilateral therapy has been described as an effective neurorehabilitation protocol following CNS-injured patients. Because rotarod training requires rhythm, balance, and coordination of bilateral motor performance, we chose it as a comparable bilateral training protocol to test in rodents. Previous studies have shown that rotarod training...

Figure 3  Motor function recovery following rehabilitative training. (a) Staircase test (sham, n = 8; TBI, n = 9; TBI + reaching, n = 9; TBI + rotarod, n = 8); (b) ladder walk test (sham, n = 8; TBI, n = 11; TBI + reaching, n = 9; TBI + rotarod, n = 8); (c) capellini handling test (sham, n = 6; TBI, n = 9; TBI + reaching, n = 9; TBI + rotarod, n = 8); (d) single pellet-reaching test (TBI, n = 8; TBI + reaching, n = 9); and (e) rotarod test (TBI, n = 8; TBI + rotarod, n = 8). Two-way ANOVA or that followed by Tukey–Kramer test, *P < 0.05.
enhances the recovery of whole-body balance and hindlimb coordination after stroke, and our results further indicate that this training is effective in recovering forelimb function following TBI (Figures 3a–c). Although the mechanism of recovery of bilateral therapy in brain-injured patients (or in rodent models) is still unknown, it is thought to restore the balance of inhibitory function within and between hemispheres that is upset by brain injury. Our data further suggest that intraspinal CST rewiring would underlie the recovery. Indeed, we recently demonstrated that intraspinal axonal regeneration of the CST is critical for recovery in a mouse model of TBI. Consistent with this, promoting rewiring by activating intrinsic regenerative capacity is strongly associated with enhanced functional recovery. Constraint-induced movement therapy after unilateral CST injury also facilitates CST fiber rewiring and functional recovery. It should be noted that although reorganization of contrale-sional CST would be indispensable for the recovery in our severe TBI model in which most of the ipsilesional CST is degenerated, in mild brain injury, ipsilesional spared CST is rewired. In any cases, although their origin differs between ipsi- and contralesional, the reorganization of CST fibers will be the key neural mechanisms for the recovery by rehabilitat-ting training.

We previously demonstrated that brain-derived neurotrophic factor (BDNF) produced by intraspinal interneurons is a key factor for inducing CST axonal rewiring. This suggests that bilateral movement training has a beneficial influence on motor performance by modulating a variety of intraspinal systems. Central pattern generators (CPGs) are

Figure 4 CST rewiring in the cervical cord after rehabilitative training. (a) Diagram of the injury model. The injury to the primary motor cortex leads to the degeneration of corticofugal CST projections (dotted lines). BDA was injected into the contralesional sensorimotor cortex to label intact CST. The arrow shows the rewired crossing fibers from intact CST to the denervated side that are related to functional recovery. (b–f) Representative images of BDA-labeled CST fibers at C7 of the denervated cervical cord in sham (b), TBI (c), TBI + reaching (d), TBI + rotarod (e), and sham + rotarod groups (f) (34 days after TBI). Scale bar: 100 μm. (g) Spinal cord illustration indicating three vertical lines at 200-μm intervals (M, L1, and L2) to create compartments for counting crossing fibers. (h) The number of crossing CST fibers in each compartment (M, M–L1; L1, L1–L2; and L2, lateral to L2) of the denervated side in C4–C7. Two-way ANOVA followed by Tukey–Kramer test, *P<0.05, **P<0.01
neural networks in the spinal cord responsible for generating rhythmic movements and are important for mediating reciprocal coordination between the right and left sides of the spinal cord. In particular, commissural interneurons located in laminae VII, VIII, and X of the rodent spinal cord contain the necessary neuronal elements for generating locomotor-like activity. Interestingly, rotarod training increased neuronal activity in laminae VII, VIII, and X of the denervated side (Figures 5c, f, and g). Hence, the spinal interneurons in CPGs, including the commissural interneurons, might be involved in rotarod-induced recovery. Rotarod training increased neuronal activity in the C4–C7 regions (Figures 5c, f, and g), which corresponds to the area where CST rewiring occurred in the denervated side (Figures 4e and h). Particularly, increase of c-Fos-positive cells and crossing CST fibers in TBI + rotarod group were higher in the lower
cervical cord (C7), while increase of them was not obvious in TBI-only and TBI + reaching groups (Figures 4e, h and 5c, g). Lower cervical cord contains a number of motor neurons connecting to distal forelimb muscles, and would be important for distal forelimb movements, for example, grasping. Thus, rotarod training might strengthen the rewiring and function related to these skilled forelimb movements, especially in the lower spinal cord. Although the molecular mechanism that mediated training-induced rewiring remains unclear, neural activity and its related molecules are possible candidates. Our results showed a correlation of neural activity and CST sprouting as described above. Furthermore, c-Fos expression by rehabilitative training increased in earlier phase than CST sprouting started, suggesting that neural activity might be a prerequisite for the axonal rewiring after brain injury (Figure 5). In regard with neural activity-related molecules, BDNF might be involved in this process. Indeed, BDNF is known to be regulated by neural activity, and increased BDNF expression promotes CST rewiring and new connections onto spared descending interneurons following injury. It should be noted, however, that multiple molecules are hypothesized to be involved in postinjury rewiring.

In contrast to bilateral rotarod training, mice in the single pellet-reaching group did not show improved recovery on any tests except the single pellet test (Figures 3a–d). Although the effect of CST-dependent tasks in rehabilitative training after severe TBI has not been mostly understood, this is in line with studies using other CNS injuries that reported that single pellet-reaching task training enhanced behavioral task performance after the injury but did not facilitate improvement on the staircase test. Thus, simple repetitive training only enhances task-dependent recovery following brain injury. Consistent with this, reach training following CNS injury has been shown to induce similar movements, but other measures are not improved. Although we did not observe enhanced recovery on the rotarod test in the rotarod training group, this behavioral analysis may not be suitable for evaluating recovery in our TBI model because it has been shown that similar cortical injuries do not cause clear impairment in this test (Figure 3e, day 33). Not surprisingly, reaching training did not enhance intraspinal CST rewiring (Figures 4d and h), even though the single pellet-reaching task is considered CST-dependent. Garcia-Alias et al. demonstrated a similar result; they found that reach training alone does not increase the number of sprouting fibers from the unlesioned lateral and ventral CST following cervical cord injury. In contrast, Starkey et al. reported that reach training following unilateral pyramidotomy increased crossing CST fibers in C3–C6. The reason for these differences is unknown, but it might be due to variability in the CNS injury models and subsequent spared neural networks. In this study, neuronal activity in the cervical cord, as assessed by c-Fos expression, was not significantly increased by reach training (Figures 5b, e, and g). In the cervical cord, the lower C7–8 spinal cord segments contain motor neurons that activate muscles that control distal forelimb movements required for grasping, whereas neurons located in the upper C4 segment are associated with proximal forelimb control. In intact rats, single pellet-reaching training alters neuronal structure at C8 but not C4 neurons. Similarly, we observed that c-Fos-positive cells tended to increase in the denervated side at the lower C7 but not in the upper C4 (Figure 5g). Taken together, alteration of intraspinal neuronal activity might be insufficient to promote CST rewiring following single pellet-reach training.

This study indicates that the adult CNS has limited but effective endogenous neural plasticity that can be facilitated by rehabilitative training, and the extent of plasticity is dependent on the training protocols. Further studies to elucidate the neural and molecular bases of recovery would support the development of effective rehabilitative training and clinical therapies for brain-injured patients.

Materials and Methods

Animals. Adult male C57BL/6J mice (8 weeks old, 20–25 g; SLC, Shizuoka, Japan) were group-housed under a 12-h light/dark cycle and received food and water ad libitum. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Osaka University.

Surgery for TBI. Surgical procedures for TBI were performed as described previously. The animals were deeply anesthetized with somnopentyl (55 mg/kg; Kyoritsu Seiyaku, Tokyo, Japan) and stabilized in a stereotaxic frame (Muromachi, Tokyo, Japan). The skull and dura over the sensorimotor cortex contralateral to the preferred paw (see the methods below) were removed using a drill with a 4.0-mm diameter (at the center, 0.5-mm anterior, 2.0-mm lateral to bregma), and cortical injury was induced using a pneumatic impact device (diameter 3.0 mm, depth 1.0 mm; Amacrine Instruments, Richmond, VA, USA). Thereafter, the skull was replaced, and the scalp was sutured.

Experimental design. The experimental design is summarized in Figure 2. Animals were trained on behavioral tests for the following periods before injury (single pellet test and staircase test, 2 weeks; ladder walk test, 1 week; rotarod test, 3 days; capellini handling test, training not required). The baseline values of all behavioral tests were evaluated 1 day before the injury. Thereafter, the staircase, ladder walk, and capellini handling tests were performed on days 4, 12, 19, 26, and 33 days after brain injury. The single pellet and rotarod tests were performed on days 4 and 33. Regarding rehabilitative training, reach training or rotarod training was conducted for 4 or 3 days before brain injury, respectively. Thereafter, rehabilitative training was performed for 4 weeks, starting 5 days after injury (Figure 2).

Rehabilitative procedures and behavioral tests. Single pellet-reaching training and test: The single pellet-reaching task is a simple repetitive training protocol used to test motor function. The single pellet-reaching box was made of clear Plexiglas (13 cm long × 7 cm wide × 13 cm high) with vertical slits in the center and side of the front wall (7 and 8 mm wide, respectively) and a 3-cm-wide shelf mounted 1 cm above the ground in front of the slit. A small metal bar (height 2 mm) was adhered to the base of the reaching window to create a barrier. This barrier prevented animals from simply pushing the pellet into their mouth and forced them to grasp the pellet in their paw and lift it over the bar. The pellet was placed on the shelf 1 cm from the slit. The vertical slit in the center was used for determining the preferred paw. During the initial phase, mice used both paws to reach pellets in front of the central slit. Paw preference was determined when the animal showed >70% preference over 20 reach attempts.

The animals were trained for 2 weeks before TBI to take pellets (20 mg, chocolate flavor; Bioserve, Fitchtown, NJ, USA) in the Plexiglas box (TBI, n = 8; TBI + reaching, n = 9). Food intake was restricted the night before the test. Animals were tested in the box for 20 pellets in each trial. A reach was considered successful when a mouse moved the pellet to its mouth and ate it. An attempt was counted as a miss if the animal knocked away or dropped the pellet after grasping it. The performance of each animal was scored as the number of successful retrievals. The baseline value was scored as the mean of three trials 1 day before the injury.

At 5 days after TBI, mice were subjected to single pellet training rehabilitation consisting of 60 single pellets attempts or 30 min, whichever came first, 6 times per week for 4 weeks. Food intake was restricted to 80–85% per day, and body weight was maintained at approximately 85–95% of baseline value throughout the experiment.

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Rotarod training and test: The rotarod is used to assess motor coordination in rodents\(^8\) and served as an effective bilateral motor training protocol in this study. Animals were placed on a rotating rod (diameter 30 mm) that accelerated from 4 to 40 r.p.m. within a 5-min period.\(^9\) The animals were trained two times a day for 3 days before injury (TBI, n = 8; TBI + rotarod, n = 8). Total time was counted until the mouse fell off the rod or gripped and spun around two times. The baseline value was scored as the mean of three trials 1 day before TBI. The data were expressed as the percentage of duration relative to baseline.

For rotarod rehabilitation, animals trained for 30 min. 6 days per week, starting 5 days after TBI. Because motor function was impaired, animals were initially run at a constant 20 r.p.m. speed on the first day, and speed was gradually increased to 25 r.p.m. at 2–4 days, 30 r.p.m. at 5–6 days, and 35 r.p.m. from 4 weeks on.

Staircase test: The staircase test was used to assess skilled forelimb reaching.\(^10\) At 2 weeks before the injury, mice were trained to reach pellets on the staircase (Melquest, Toyama, Japan) for 30 min ( sham, n = 8; TBI, n = 9; TBI + reaching, n = 9; TBI + rotarod, n = 8). Each staircase consisted of seven steps with two food pellets placed on each step, and the number of pellets retrieved in 30 min was counted for each side. The pellets on the first and second stairs were not counted because animals could retrieve them using only their forepaw. Food intake was restricted the night before the test, and the baseline value was assessed 1 day before TBI.

Ladder walk test: The ladder walk test was used to assess skilled walking and measure forelimb placing, stepping, and interlimb coordination.\(^17\) The animals were placed on a 1-m runway comprised of randomly spaced metal rungs (6–18 mm intervals) 15 cm above the ground ( sham, n = 8; TBI, n = 11; TBI + reaching, n = 9; TBI + rotarod, n = 8). The mice were video recorded in slow motion as they walked across the 1-m runway (Sony Handycam: Sony, Tokyo, Japan), and foot faults for each forepaw were counted during continuous steps.\(^17\) The animals were trained for 1 week (each for 10 min) before injury and were tested three times per session after training. The baseline value was assessed 1 day before the injury.

Capellini handling test: The capellini handling test was used to characterize forepaw dexterity while eating a piece of pasta.\(^18\) The animals were placed on a mirror in a clear box ( sham, n = 6; TBI, n = 9; TBI + reaching, n = 9; TBI + rotarod, n = 8). Uncooked pasta pieces (diameter 0.9 mm and length 26 mm) were eaten in three sequential trials after training. The number of forepaw adjustments made while eating the pasta was counted using slow-motion video (×10 × zoom). An adjustment was defined as any distinct removal and replacement of the paw or digits on the pasta (re-contacts the previous piece, regrips the piece, or extension–flexion or abduction–adduction of the digits). Cases when the paw slid across the pasta piece with no digit movement or repositioning were not counted, nor were paw movements that did not contact the pasta. Because the test was performed the same day as the staircase test, food intake was restricted. The baseline value was scored as the mean of three trials 1 day before injury. The data were expressed as the percentages of duration relative to baseline.

Immunohistochemistry. The animals were deeply anesthetized and transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). Following perfusion, the brain and spinal cord were postfixed in the same fixative solution at 4°C overnight and subsequently transferred to 30% sucrose in phosphate-buffered saline (PBS) at 4°C overnight. The brain and spinal cord were cut into 20-μm-thick coronal sections using a cryostat and placed on MAS-coated glass slides (Matsunami, Osaka, Japan). For immunohistochemistry, the sections were immersed for 5 min × 3 in PBS and blocked with 5% bovine serum albumin(0.1–0.3%) Triton X-100/PBS for 1 h. Subsequently, the sections were incubated with primary antibodies overnight at room temperature (RT) or at 4°C. Mouse anti-neuronal nuclei (NeuN) (1:100; Millipore, Temecula, CA, USA), rabbit anti-c-Fos (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit anti-GFAP (1:1000; Invitrogen, Carlsbad, CA, USA) were used as primary antibodies. The sections were then washed three times with PBS-Tween-20, followed by incubation with secondary antibodies for 1 h at RT in the dark. Goat-anti-rabbit or mouse IgG Alexa 488 or 568 (1:500; Invitrogen) were used as secondary antibodies. The sections were counterstained with Neurotracer Nissl (1:1000; Invitrogen). For BDA labeling, the sections were immersed three times in PBS and incubated in 0.3% Triton X-100/PBS for 1 h, followed by incubation with Alexa Fluor 568-conjugated streptavidin (1:400; Invitrogen) for 2 h at RT. All images were acquired with a fluorescence microscope (Keyence, Biorevo BZ-9000, Osaka, Japan) or a confocal laser-scanning microscope (Olympus Fluoview FV1000, Tokyo, Japan).

Lesion volume measurement. At 34 days after TBI, the animals were deeply anesthetized and perfused with 4% PFA (TBI, n = 5; TBI + reaching, n = 5; TBI + rotarod, n = 5). The brains were sectioned at 40 μm per 200 μm in the coronal plane at the level of the forelimb area (bregma +1.2 to −1.0 mm) and stained with Nissl. The lesion volume of each animal was calculated as a percentage by dividing the volume of the injured hemisphere by that of the intact contralateral hemisphere using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).\(^31\)

Anterograde tracing of CST fibers. The descending CST fibers were labeled with the anterograde tracer BDA (MW, 10,000; 10% BDA in PBS; Invitrogen) 2 weeks before the animals were killed. BDA injection procedures were performed as described previously.\(^4\) The animals were anesthetized and stabilized in a stereotaxic frame. The corresponding skull area on the contralateral side was opened, and 2.7 μl BDA was stereotactically infused into the forelimb area of the sensorimotor cortex at three sites (coordinates from bregma: 0 mm anterior/1.0 mm lateral, 0.5 mm anterior/1.0 mm lateral, and 0.5 mm anterior/1.5 mm lateral, all at a depth of 0.5 mm) using a glass capillary attached to a microsyringe.

CST fibers’ quantification. The numbers of midline-crossing BDA-labeled CST fibers were counted at ×400 magnification in each section (20 sections per segment, C4–C7; sham, n = 5; TBI, n = 5; TBI + reaching, n = 5; TBI + rotarod, n = 5; sham + rotarod, n = 4; for Figure 4h: TBI, n = 5; TBI + reaching, n = 6; TBI + rotarod, n = 5; for Figure 5k). The numbers of CST fibers in the denervated side of the cervical cord were counted in each of three compartments, which were defined by three vertical lines: M, L1, and L2. The first vertical line M was drawn through the central canal, and L1 and L2 were parallel to M at distances of 200 and 400 μm, respectively (Figure 4f). To correct for tracing differences among individual animals, the results were normalized to the mean number of BDA-labeled CST fibers in the dorsal funiculus at C1 (three serial transverse sections).\(^15\)

Quantification of c-Fos-positive neurons. Animals at 12 days after injury were used to quantify c-Fos-expressing neurons in the cervical cord after training by same protocols (TBI, n = 4; TBI + reaching, n = 4; TBI + rotarod, n = 4). To ensure adequate time for c-Fos expression, animals were returned to their cage after training and allowed to rest for 1 h. Then, the mice were perfused, and the tissues were processed as described above. c-Fos-positive neurons were counted in 10 sections per cervical level (C4–C7), and the mean number was calculated in each animal. Neurons were identified by double staining with NeuN. They were quantified within lamina VII, VIII, and X of the denervated side in the cervical cord. The localization of c-Fos-positive cells was plotted with Neurolucida (MBF Bioscience, Williston, VT, USA).

Retrograde transsynaptic tracing with PRV. The PRV was used as a transsynaptic retrograde tracer to verify neuronal connections between the motor cortex and the impaired forelimb.\(^18\) The day after the final behavioral test, animals were anesthetized and injected with PRV expressing EGFP under a cytomegalovirus promoter (PRV-152, gift from Dr. JP Card and LW Enquist) at five sites (2 μl per site, titer: 4.2 × 10^7 PFU/μl) in the extensor muscle of the impaired forelimb by using a glass capillary attached to a microsyringe. After 120 h, the animals were perfused, and the tissues were processed as described above. The brains were sectioned at 20 μm in the coronal plane at the level of the forelimb area of the motor cortex (bregma +1.2 to −1.0 mm), and the sections were stained with an anti-GFP antibody and Neurotracer Nissl.

Statistics. Lesion volume was statistically analyzed by one-way ANOVA. Behavioral tests were analyzed by two-way ANOVA, and Tukey-Kramer post hoc test was applied when appropriate. CST fibers and c-Fos-positive cells results were analyzed using two-way ANOVA followed by Tukey-Kramer test. All data are represented as mean ± S.E.M., and statistical significance was accepted at P < 0.05.

Conflict of Interest
The authors declare no conflict of interest.
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