Intraspinal rewiring of the corticospinal tract requires target-derived brain-derived neurotrophic factor and compensates lost function after brain injury

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Brain injury that results in an initial behavioural deficit is frequently followed by spontaneous recovery. The intrinsic mechanism of this functional recovery has never been fully understood. Here, we show that reorganization of the corticospinal tract induced by target-derived brain-derived neurotrophic factor is crucial for spontaneous recovery of motor function following brain injury. After destruction of unilateral sensorimotor cortex, intact-side corticospinal tract formed sprouting fibres into the specific lamina of the denervated side of the cervical spinal cord, and made new contact with two types of spinal interneurons—segmental and propriospinal neurons. Anatomical and electrophysiological analyses revealed that this rewired corticospinal tract functionally linked to motor neurons and forelimb muscles. This newly formed corticospinal circuit was necessary for motor recovery, because transection of the circuit led to impairment of recovering forelimb function. Knockdown of brain-derived neurotrophic factor in the spinal neurons or its receptor in the intact corticospinal neurons diminished fibre sprouting of the corticospinal tract. Our findings establish the anatomical, functional and molecular basis for the intrinsic capacity of neurons to form compensatory neural network following injury.

Keywords: BDNF; cortical injury; corticospinal tract; functional recovery; plasticity

Abbreviations: BDNF = brain-derived neurotrophic factor

Introduction

Cortical injury due to trauma or stroke can result in massive neuronal loss and disrupt intricate neural networks, causing deleterious functional deficits of motor, sensory, cognitive and other higher functions. However, some deficits including motor dysfunction are frequently followed by spontaneous recovery, suggesting that plasticity of the remnant neuronal network compensates for lost function (Nudo, 2006; Benowitz and Carmichael, 2009; Murphy and Corbett, 2009). One of the most critical regions for this recovery is presumptively the intact cortex contralateral to the lesion (Nudo, 2006; Benowitz and Carmichael, 2009; Murphy and...
Corbett, 2009; Stoeckel and Binkofski, 2010). Indeed, clinical and experimental evidence support the occurrence of cortical reorganization in which the contralateral intact cortex is activated (Calautti et al., 2001; Carmichael and Chesselet, 2002; Feydy et al., 2002; Johansen-Berg et al., 2002; Biernaskie et al., 2004; Cramer and Crafton, 2006). The subcortical efferent projections from the contralesional cortex newly sprout into subcortical areas on the dener- vated side such as the striatum, red nucleus and cervical spinal cord (Napieralski et al., 1996; Lenzlinger et al., 2005; Liu et al., 2007, 2009; Papadopoulos et al., 2009; Zai et al., 2009; Omoto et al., 2010; Lee et al., 2011). These observations suggest that contralesional cortex and its efferent fibres may contribute to functional recovery. However, no direct evidence to date has revealed whether the newly sprouted axons actually form a neural network that enhances functional recovery. Moreover, the underlying molecular mechanism of this reorganization is largely unknown.

In this study, we identified a critical compensatory neural circuit necessary for spontaneous motor recovery after cortical injury. The sensorimotor cortex includes layer V neurons extending the corticospinal tract axons to the contralateral side of the spinal cord. We revealed that the corticospinal tract, the main tract for fine motor function, formed sprouting fibres from the intact cortex over the midline to functionally connect with specific spinal interneurons. We further determined the molecular mechanism critical for this axonal rewiring.

Materials and methods

Animals

Adult male C57BL/6J mice (8-weeks-old, Charles River) were housed with three animals in a 12 h light–dark cycle in standard cages with food and water ad libitum. All the procedures were approved by the Guidelines for the Care and Use of Laboratory Animals of Osaka University Medical School.

Surgical procedures

The mice were stabilized in a stereotaxic frame (Narishige) after deep anaesthesia with somnopentyl (60 mg/kg, Kyoritsu Seiyaku). The scalp was retracted and using a drill, a circular craniotomy of 4 mm in diameter was made. The ventrocaudal surface of the occipital bone was revealed, and the medullary pyramid was exposed. The dura was first cut, and the pyramidal tract in the right side was then incised with a 30-gauge needle with a width of 0.5 mm and a depth of 0.25 mm. The oesophagus, trachea and muscles were repositioned, and the skin was sutured.

For lateral hemisection of the cervical spinal cord, the animals were anaesthetized and laminectomy was conducted at C4. Lateral hemisection at the left side of the spinal cord was carefully performed using a 27-gauge needle and a number 11 surgical blade to cut at the spinal level just above C4. The muscles and skin layers were then sutured.

Behavioural tests

The ladder walk test (Metz and Whishaw, 2002), the staircase test (Baird et al., 2001; Lee et al., 2004; Omoto et al., 2010), and the cylinder test (Schallert et al., 2000; Omoto et al., 2010; Lee et al., 2011) were used to assess impaired and recovered motor function of the forelimb following cortical injury, pyramidotomy or lateral hemisection. The detail protocols are described in the online Supplementary material.

Anterograde corticospinal tract labelling

The descending corticospinal tract fibres were labelled with biotinylated dextran amine (MW, 10 000; 10% biotinylated dextran amine in phosphate-buffered saline, Invitrogen), an anterograde tracer, 2 weeks before the mice were sacrificed. The mice were anaesthetized and stabilized in a stereotaxic frame. The corresponding area of skull on the right contralesional side was opened and biotinylated dextran amine (total 1.2 µl) was infused into the forelimb area of sensorimotor cortex stereotactically 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. After infusion, the skull was returned and the scalp was sutured.

Retrograde labelling of corticospinal neurons

Corticospinal neurons in the sensorimotor cortex were labelled with green retrobeads (Lumafluor), a fluorescence retrograde tracer, 1 week before the mice were sacrificed. First, the mice were anaesthetized, and laminectomy was performed at vertebral level C4. They were then stabilized in a stereotaxic frame and 0.6 µl of green retrobeads was infused into the right side of the spinal grey matter (C4 at the spinal level: 0.5 mm lateral, 0.5 mm depth) using a glass capillary. After infusion, the muscles and skin layers were sutured.

Retrograde labelling of segmental interneurons and propriospinal neurons

Wheat germ agglutinin, a trans-synaptic retrograde tracer, was used for the retrograde labelling of segmental interneurons. Wheat germ agglutinin (5% in saline, total 5 µl per muscle, Sigma) was infused into the long head of the biceps brachii muscle and the long head of the triceps brachii muscle of the right forearm at five sites 1 week before sacrifice.

For retrograde labelling of propriospinal neurons, 3 days before the mice were sacrificed, green retrobeads (Lumafluor) were infused into the right side of the spinal grey matter (lateral, 0.7 mm; depth, 0.5 mm) at vertebral level C7.
Histology

The animals were transcardially perfused with 4% paraformaldehyde in 0.1M phosphate buffer. The brain and cervical spinal cord were dissected, post-fixed in the same fixatives overnight at 4°C, and immersed in 30% sucrose/phosphate-buffered saline. The tissues were embedded in OCT compound. Serial sections were cut at 20 μm on a cryostat and mounted on MA4-coated slides (Matsunami). The sections were stained with Nissl stain (Cresyl violet, Sigma) to assess the lesion volume of the cortex. Immunohistochemical procedures are described in the Supplementary material. For biotinylated dextran amine labelling, the sections were incubated in 0.3% Triton X-100/phosphate-buffered saline for 1 h, followed by incubation with Alexa-488- or Alexa-568-labelled streptavidin (1:400, Invitrogen) for 2 h at room temperature. All the sections were observed using a fluorescence microscope (Olympus BX51, DP71) or a confocal laser-scanning microscope (Olympus FluoView FV1000). Methods for histological quantification (lesion volume, midline-crossing corticospinal tract fibres, corticospinal neurons, TrkB expression in small interfering RNA experiments) are described in the Supplementary material.

Electrophysiological recording

The mice were anaesthetized with ketamine hydrochloride (100 mg/kg, i.p.) and xylazine hydrochloride (5 mg/kg, i.p.) and placed in a stereotaxic apparatus. A region of the skull, ~4 mm in diameter, was opened with a surgical drill, and the sensorimotor region of the cortex was exposed. Bipolar stimulating electrodes (tip distance, 300–400 μm) made of 50 μm-diameter Teflon-coated tungsten wires (A-M systems) were inserted into the forelimb area of the sensorimotor cortex (see detail in Supplementary material). Intracortical microstimulation was carried out with a train of 20 pulses at 333 Hz, 200 μs duration, 10–100 μA by using a stimulus generator (STG4002, Multichannel Systems). For EMG, the forelimb skin was incised, and a bipolar stainless-steel needle (tip distance, 2 mm) was inserted into the biceps or triceps muscle of both sides of the forelimbs. EMG was recorded with PowerLab (AD Instruments). Seven days after the injury or after pyramidotomy, higher currents (>100 μA) were used because the ipsilateral responses were abrogated. The lowest current that produced a response was 20 μA, and the current was increased in 20 μA steps. For EMG, the forelimb skin was incised, and a bipolar stainless-steel needle (tip distance, 2 mm) was inserted into the biceps or triceps muscle of both sides of the forelimbs. EMG was recorded with PowerLab (AD Instruments). Seven days after the injury or after pyramidotomy, higher currents (>100 μA) were used because the ipsilateral responses were abrogated. The lowest current that produced a response was 20 μA, and the current was increased in 20 μA steps.

In situ hybridization

Complementary DNA fragments used for riboprobe production are listed in the Supplementary material. Digoxigenin-labelled riboprobes were prepared by in vitro transcription. In situ hybridization was performed as described previously (Omoto et al., 2011). Signals were detected using alkaline phosphatase-coupled anti-digoxigenin antibodies (Roche Diagnostics) with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrates for colour reaction.

Reverse transcriptase real-time polymerase chain reaction

Total RNA was extracted from the right side of the spinal cord (C4–7) and reverse-transcribed for first-strand complementary DNA synthesis. Real-time polymerase chain reaction was performed with oligonucleotide primer sets corresponding to the complementary DNA sequences of brain-derived neurotrophic factor (BDNF) and GAPDH, and SYBR® Green real-time polymerase chain reaction master mix (Applied Biosystems) in 7300 real-time polymerase chain reaction system (Applied Biosystems). The relative intensity against GAPDH and the fold change relative to the control were calculated. The detailed protocols are described in the Supplementary material.

Compartmentalized microfluidic culture of cortical neurons

Cortical neurons harvested from embryonic Day (E) 18 embryo were cultured in the compartmentalized channel of a microfluidic culture devise (Axis; length of microgrooves, 150 μm; Millipore; Supplementary Fig. 5A) as described in the manufacturer’s protocol (Park et al., 2006) (details in Supplementary material). BDNF (100 ng/ml, Peprotech) or vehicle was applied into the axonal or somal compartment at 3 days in vitro when axons had elongated through the microgrooves into the axonal compartment. In some experiments, small interfering RNA for TrkB was transfected using Nucleofector® (Amaxa) before the cells were plated. The following Alexa 555-labelled small interfering RNA was used for knockdown experiments: mouse NTRK2 (TrkB; stealth small interfering RNA; Invitrogen), containing the following sequences; sense Alexa 568 (5’-GCCAUACUGAAGAGCUCAAGA-3’), antisense (5’-UGUACUGGACUCUACUGAUAG-3’). As control, control mismatch small interfering RNA was introduced. At 5 days in vitro, the neurons were fixed in 4% paraformaldehyde and stained with rabbit anti-IiIIIi tubulin (Tu1; 1:500; Covance) and Alexa 568-anti rabbit IgG (1:500; Invitrogen).

Western blot

Cultured cortical neurons at 4, 7, 10 and 14 days in vitro after TrkB small interfering RNA or control small interfering RNA transfection in 3.5 cm dishes were harvested and homogenized in lysis buffer, comprising 50 mM Tris–HCl (pH 7.8) with 150 mM NaCl, 1 mM EDTA, 2 mM Na3VO4, 1% NP-40 and protease inhibitor cocktail (Roche). After centrifugation at 12 000g for 20 min at 4°C, the protein fractions were separated on sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore). The membrane was blocked with 5% non-fat dry milk in phosphate-buffered saline containing 0.05% Tween-20 and then incubated with rabbit anti-TrkB antibody (1:200; Santa Cruz) or mouse anti-actin (1:500; Oncogene) overnight at 4°C. After washing, the membrane was incubated with horseradish peroxidase-linked anti-rabbit IgG (1:5000; Cell Signalling Technology) or anti-mouse IgM antibody (1:10,000; Santa Cruz). The detection was performed with an ECL chemiluminescence system (GE Healthcare).

ELISA in situ

To assess the effect of small interfering RNA and lentivirus (see below) on BDNF expression, ELISA in situ, a modified method from conventional ELISA, was used (Balkowiec and Katz, 2000) (Supplementary material). This method could capture relatively low amount of proteins secreted from cultured cells. N1E-115 mouse neuroblastoma cells pre-transfected with BDNF or control small interfering RNA, or pre-infected with Venus-P2A-BDNF or Venus expressing lentivirus were plated in 96-well ELISA plates coated with anti-BDNF antibody (1:100; BDNF Emax ImmunoAssay System, Promega), and grown for 3 days. The following Alexa 488-labelled BDNF small interfering RNA was used: mouse BDNF (stealth small interfering RNA; Invitrogen) containing the following sequences;
sense Alexa 488 (5′-UCGGGCCCUCUCAUGAAGGACAAA-3′), antisense (5′-UUUCGCUAUCAUGUAAGGCCCCGA-3′). As control, control mismatch small interfering RNA was used. After 3 days in vitro in ELISA plate, the cells were removed and BDNF expression was detected using BDNF Emax ImmunoAssay System (Promega). Absorbance values were read at 450 nm in a plate reader (SpectraMax M2; Molecular Devices).

Small interfering RNA transfection in vivo

Alexa 555-labelled TrkB small interfering RNA or control small interfering RNA was infused into the contralesional motor cortex at 14 days after the injury, using i-Fect™ transfection reagents (Neuromics) according to the manufacturer's instruction. The mice were anaesthetized and stabilized in a stereotaxic frame. The corresponding area of skull on the right contralesional side was opened and small interfering RNA with i-Fect™ reagent (total 0.6 μl/site) was infused into the forelimb area of sensorimotor cortex at three sites for morphological analyses of corticospinal tract (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), or 12 sites for behavioural analyses (coordinates from bregma: −0.5–2.0 mm anterior/1.0–2.0 mm lateral, all at a depth of 0.5 mm) using a glass capillary.

Alexa 488-labelled BDNF small interfering RNA or control small interfering RNA was infused into C4 and C6 level of the right cervical cord (total, 0.6 μl; C4, 0.5 mm lateral, 0.5 mm depth; C6, 0.7 mm lateral, 0.5 mm depth) at 14 days after the injury, using i-Fect™ transfection reagents (Neuromics).

Lentivirus production and its delivery in vivo

Methods for construction of lentiviral vector that co-expresses Venus and BDNF under the synapsin 1 promoter (CS-SYN-Venus-P2A-BDNF-PRE) and control vector (CS-SYN-IRES2-Venus-PRE) are described in the Supplementary material.

Lentiviruses were produced by HEK293T cells co-transfected with the expression vector and helper plasmids, pCAG-HIVgp and pCMV-VSV-G-RSV-Rev (kind gift from H. Miyoshi), using Lipofectamine® 2000 (Invitrogen). Seventy-two hours after transfection, the medium was passed through a 0.45 μm filter and centrifuged twice at 50 000 g until use. Ten-fold serial dilutions of lentivirus were added into the N1E-115 cells and determined the titres by counting the number of Venus-expressing cells. The final titre was ~10^8 IU/ml.

The viral solutions were stereotaxically injected into C4 and C6 level of the right cervical cord (total, 1.0 μl; C4, 0.5 mm lateral, 0.5 mm depth; C6, 0.7 mm lateral, 0.5 mm depth) at 7 days after the injury.

Statistics

Behavioural tests were analysed using one-way repeated-measures ANOVA with Tukey’s test. Within-group comparisons for pre- versus post-pyramidotomy or hemisection were made using the paired t-test. The features of midline-crossing corticospinal tract fibres and the ratio of corticospinal tract fibres contacted with interneurons were analysed using two-way ANOVA with the Tukey–Kramer test. The time-course of the number of midline-crossing corticospinal tract fibres, thresholds for EMG response, the number of axonal branching in compartmentalized culture, BDNF expression in ELISA in situ, and realtime polymerase chain reaction were analysed using one-way ANOVA with the Tukey–Kramer test. The number of corticospinal neurons and TrkB expression in small interfering RNA experiments were analysed using Student’s t-test. Behavioural scores between control and TrkB small interfering RNA-infused group were analysed using two-way repeated ANOVA. P < 0.05 was considered significant.

Results

Spontaneous recovery of forelimb motor function after cortical injury

We induced traumatic injury in the left sensorimotor cortex of mice with a pneumatic impact device (Omoto et al., 2010; Lee et al., 2011; Ueno and Yamashita, 2011), which produced consistent hemicortical lesions (mean lesion volume 7.30 ± 0.49 mm^3 (n = 5); Fig. 1A–C). The entire forelimb area of the sensorimotor cortex was disrupted; the retrograde tracer injected in the right cervical spinal cord labelled corticospinal neurons only in the secondary sensory cortex (Supplementary Fig. 1A–F). The corticospinal tract, the major axonal tract that signals muscles to move limbs for task execution, mostly disappeared on the right side at the level of the cervical spinal cord as assessed by immunoreactivity of protein kinase C-γ (PKCγ), a marker for the corticospinal tract (Fig. 1D and E).

We then used three behavioural assessments to evaluate impairment and subsequent recovery of forelimb motor function. In the ladder walk test, the number of faulty placements of the right paw was counted during walking on a ladder rung. One week after the injury, the number of faults was significantly higher compared to that in control mice prior to injury, although this number gradually decreased every week (Fig. 1F). In the staircase test, the total number of pellets the mice retrieved using their right forearm in a staircase was counted. One week after the injury, mice were unable to retrieve any pellets, but the score then improved from 2 to 6 weeks (Fig. 1G). In the cylinder test, the forelimb used to sustain their bodies during spontaneous vertical exploration in a cylinder was counted. After the injury, the mice tended not to use their impaired forelimb solely, but the value gradually recovered (Fig. 1H). All three tests indicated that although the function was severely impaired, it gradually recovered spontaneously but partially.

Corticospinal tract fibres cross the midline into the denervated side of the cervical spinal cord after cortical injury

We hypothesized that sprouting fibres from the intact corticospinal tract contribute to this spontaneous recovery after cortical injury. We injected biotinylated dextran amine, an anterograde tracer, into the forelimb area of the contralesional motor cortex and
determined the morphological changes to the corticospinal tract. Six weeks after the injury, many corticospinal tract axons sprouted into the denervated side of the cervical spinal cord (Fig. 2A–C). Biotinylated dextran amine-labelled corticospinal tract fibres were detected in the dorsal and dorsolateral columns but not clearly in the ipsilateral ventral column (Fig. 2B and C) consistent with the report in mice (Steward et al., 2004), suggesting that ventral corticospinal tract sprouting into denervated side is minimal compared to rats (Weidner et al., 2001). The number of fibres that crossed the midline from the intact to the denervated side (‘recrossing fibres’) increased remarkably, mainly at the C4–C5 level of the spinal cord (Fig. 2D). The number of recrossing fibres gradually increased from 2 weeks onward, concurrent with functional recovery, and peaked at 4 weeks (Fig. 2E). Thereafter, the number slightly decreased between 4 and 6 weeks, suggesting pruning of excess axons to refine the network, as observed in the recovery phase following spinal cord injury (Bareyre et al., 2004).
We injected green retrobeads, a fluorescence retrograde tracer, into the right C4 grey matter to confirm that the corticospinal tract targeted the ipsilateral side of the cervical cord. The number of corticospinal neurons retrogradely labelled in layer V of the intact motor cortex was increased (Supplementary Fig. 1A, D and G). Most labelled corticospinal neurons were located in the forelimb area, which was determined in the control cortex (Supplementary Fig. 1H). Thus, we concluded that the corticospinal tract targeted the ipsilateral side.

We further assessed the morphological features of recrossing corticospinal tract fibres in detail: axonal density, axonal length, number of branches and number of boutons, which may correspond to synaptic terminals (Fig. 2F–I). Axonal length was higher in C4–C5 at 42 days, whereas that in C6–C7 did not change compared to the control (Fig. 2G, Supplementary Fig. 2). The axonal density increased in the denervated side at all cervical levels (Fig. 2F), although the number of recrossing fibres did not increase remarkably at C6–C7 (Fig. 2D). Instead, the number of branches...
increased in the C6–C7 area (Fig. 2H, Supplementary Fig. 2C and D). These results demonstrate that the number of recrossing fibres predominantly increased at C4–C5 levels, whereas branch formation of the recrossing fibres was induced at C6–C7 levels. As the number of boutons per 100 µm of axons was constant between the control and injured groups (Fig. 2I), synaptic contacts may be formed in these axonal fibres. vGut1, a presynaptic marker (Maier et al., 2008), was localized to most boutons (Fig. 3A), demonstrating the presence of anatomical synapses.

Recrossing corticospinal tract fibres form neural networks with spinal interneurons

If the recrossing corticospinal tract fibres form a precise network to exert motor functions, they should connect to specific neurons in the spinal cord. Supporting this notion, most of the recrossing fibres innervated laminae VII and VIII of the grey matter.
(Fig. 2C, 3B, Supplementary Fig. 2B and D). Therefore, we hypothesized that the interneurons that regulate forelimb motor function were counterparts of the recrossing fibres, and focused on interneurons related to motor control: segmental interneurons and propriospinal neurons (Isa et al., 2007).

As segmental interneurons connect corticospinal tract fibres and motor neurons in the same segment of spinal cord, we labelled these interneurons with wheat germ agglutinin, a trans-synaptic retrograde tracer (Yoshihara, 2002), by injecting it into the forelimb muscles of the impaired side. We selected two main muscles: the long head of the biceps brachii and triceps brachii muscles, which flex and extend the forelimb, respectively. Coinciding with a previous study in rats (McKenna et al., 2000), wheat germ agglutinin injected into the biceps labelled segmental interneurons through motor neurons mainly in C4–C5, and wheat germ agglutinin injected into the triceps labelled those in C6–C7. The labelled interneurons were mainly localized in laminae VII and VIII and were not labelled with Chat, a marker for motor neurons (Supplementary Fig. 3A–C). Many recrossing fibres made contact to these neurons with bouton structures (Fig. 3C). Quantitative analysis revealed that approximately one-third of the fibres connected with wheat germ agglutinin-labelled segmental interneurons (Fig. 3D). The total number of contacts increased with the increased number of recrossing axons after injury (Fig. 2D and F), although the ratios were not statistically different between the control and injured groups (Fig. 3C and D, Supplementary Fig. 3A and B).

Since a significant population of recrossing fibres was not connected with segmental interneurons, we assessed the possible involvement of another type of interneuron, propriospinal neurons. These interneurons are intersegmentally connected in the spinal cord including monosynaptic connections with motor neurons (Isa et al., 2007). To retrogradely label propriospinal neurons, green retrobeads were infused into the right grey matter at the C7 level. Many cell bodies of propriospinal neurons were localized to C4 and C5 (Fig. 3E, Supplementary Fig. 3D). Half the recrossing corticospinal tract fibres were in contact with propriospinal neurons with bouton structures (Fig. 3E and F). The ratio in which they connected with propriospinal neurons was increased in the injured group compared to that in controls (Fig. 3F and Supplementary Fig. 3E). These anatomical analyses suggest that recrossing corticospinal tract fibres form synapses with these two types of interneurons to regulate the forelimb muscles of the impaired side.

**Intact-side corticospinal tract is required for the activity of the ipsilateral forelimb muscles after cortical injury**

The abovementioned anatomical evidence prompted us to obtain electrophysiological evidence regarding the connection of the intact sensorimotor cortex with ipsilateral forelimb muscles after cortical injury. We conducted intracortical microstimulation in the forelimb area of the contralesional motor cortex and EMG of the biceps and triceps in both forelimbs. Intracortical microstimulation evoked a response in the ipsilateral forelimb with an average threshold of $43.8 \pm 7.0\mu\text{A}$ in the biceps and $50.0 \pm 3.5\mu\text{A}$ in the triceps in control mice (Fig. 4A–C), while the threshold was found to be lower in the contralateral forelimb. This ipsilateral response coincides with a previous study on rats (Brus-Ramer et al., 2009), which reported that the ipsilateral response required the contralateral hemisphere and the corticospinal tract originating from it. Consistent with this, the thresholds of ipsilateral response were significantly increased 7 days after injury (Fig. 4A–C), confirming that the contralateral cortex was necessary for an appropriate response. However, the thresholds significantly decreased after 42 days compared to those at 7 days (Fig. 4A–C). This ipsilateral response should not require the contralateral hemisphere, which was contused. Thus, the ipsilateral neural network from the motor cortex to the spinal cord was presumably formed after the injury. To ascertain this, we transected the corticospinal tract of the right intact side at the pyramidal level (pyramidotomy). As expected, the threshold increased approximately to the level observed at 7 days compared to that at 42 days (Fig. 4A–C). These data demonstrate that the ipsilateral projection from the motor cortex to the forelimb muscles was formed after cortical injury. The remaining ipsilateral EMG response stimulated with very high amplitude after pyramidotomy may reflect the existence of secondary neural network from the motor cortex to spinal cord, e.g. cortico-rubro-spinal or cortico-reticulo-spinal circuit, although their contributions to EMG response appears to be limited.

**Interception of intact corticospinal tract fibres impairs recovered forelimb function**

To directly prove that the corticospinal tract from the contrale- sional cortex contributes to spontaneous functional recovery, we selectively cut the right intact corticospinal tract at the pyramid (pyramidotomy) at 42 days (Supplementary Fig. 4A and B). To evaluate the effect of corticospinal tract transection on motor recovery, we used a motor test involving only the right impaired forelimb because the left forelimb function was impaired after corticospinal tract transection. In the first task, we devised a ladder walk test by placing a board on the left half of the ladder to prevent the mice from falling off due to their left limb impairment. Similar to control mice, pyramidotomized mice without cortical injury could walk on the ladder without an increase in missed steps ($P=0.32$; Fig. 4D). However, mice with cortical injury missed foot placement more frequently 3 days after the pyramidotomy (Fig. 4D), similar to the level of injured mice before the recovery period. In the next task, we used a staircase and counted the number of pellets retrieved using their right forelimb. The pyramidotomized mice without cortical injury could retrieve pellets; the number was not significantly lower than that of control mice ($P=0.19$; Fig. 4E). However, mice with cortical injury could retrieve almost no pellets after pyramidotomy (Fig. 4E). The results of these two tasks indicated that the intact corticospinal tract is necessary for the recovery of forelimb motor function.
To further confirm that recrossing corticospinal tract fibres at the C4–C7 level contribute to functional recovery, we intercepted the corticospinal tract fibres on the intact side at the C3/C4 border just above the recrossing site by lateral hemisection at 42 days following cortical injury \( (n = 4) \). Data are represented as mean ± SEM \( **P < 0.01 \) (one-way ANOVA with Tukey–Kramer test). (C) Representative EMG responses of ipsilateral biceps at 80 \( \mu \)A in control, 7 days, 42 days and after pyramidotomy. The lowest trace indicates the stimulation. (D and E) The scores for the ladder walk test (D) and staircase test (E), obtained at 3 days after the intact corticospinal tract was transected by pyramidotomy, or at 7 days after transected at C3–C4 border \( (C3/4 \text{section}) \) 42 days after cortical injury. Control, mice with no cortical injury \( (n = 3–6 \text{ for each group}) \); injury, mice with cortical injury \( (n = 4–6 \text{ for each group}) \). Data are represented as mean ± SEM \( *P < 0.05, **P < 0.01 \) compared to the score before operation (paired t-test).

**Figure 4** Recrossing corticospinal tract fibres contribute to the recovery of forelimb movements. (A and B) Thresholds of EMG responses in ipsilateral biceps (A) and triceps (B) by intracortical microstimulation in the forelimb area of intact motor cortex. Control without cortical injury (control), at 7 and 42 days following cortical injury, and after pyramidalotomy (PTX) on the right intact side at 42 days following cortical injury \( (n = 4) \). Data are represented as mean ± SEM \( **P < 0.01 \) (one-way ANOVA with Tukey–Kramer test). (C) Representative EMG responses of ipsilateral biceps at 80 \( \mu \)A in control, 7 days, 42 days and after pyramidotomy. The lowest trace indicates the stimulation. (D and E) The scores for the ladder walk test (D) and staircase test (E), obtained at 3 days after the intact corticospinal tract was transected by pyramidotomy, or at 7 days after transected at C3–C4 border \( (C3/4 \text{section}) \) 42 days after cortical injury. Control, mice with no cortical injury \( (n = 3–6 \text{ for each group}) \); injury, mice with cortical injury \( (n = 4–6 \text{ for each group}) \). Data are represented as mean ± SEM \( *P < 0.05, **P < 0.01 \) compared to the score before operation (paired t-test).

To further confirm that recrossing corticospinal tract fibres at the C4–C7 level contribute to functional recovery, we intercepted the corticospinal tract fibres on the intact side at the C3/C4 border just above the recrossing site by lateral hemisection at 42 days (Supplementary Fig. 4C) and repeated the ladder walk and staircase test as described above. The recovered functions in both tests were impaired again after the transection (Fig. 4D and E). The results of the pyramidalotomy and C3/C4 transection indicate that the intact-side corticospinal tract and its neuronal network below the C3/C4 level were crucial for the recovery of motor function.

**Brain-derived neurotrophic factor signal is required for the rewiring of corticospinal tract fibres**

To identify factors involved in the process of corticospinal tract reorganization, we examined the gene expression of BDNF, NT-3, NT4/5, IGF-1, IGF-2, GDNF, CNTF, Netrin-1, SDF-1, Wnt7b and FGF2, factors involved in neural network formation during developmental stages, by **in situ** hybridization. Among them, BDNF, which is associated with axonal growth and
branching during development (Cohen-Cory et al., 2010), was expressed in the grey matter of cervical cord after the injury (Fig. 5A–C), while others were not clearly expressed (NT-3, NT4/5, IGF-1, IGF-2, GDNF, CNTF, FGF2) or weakly (Netrin-1, SDF-1, Wnt7b). Expression of BDNF in the denervated cervical cord was decreased at 7 days after the injury, and it increased thereafter (Fig. 5D). TrkB, a receptor for BDNF, was expressed in the motor cortex, including corticospinal neurons in layer V labelled by green retrobeads (Fig. 5E and F). Furthermore, the innervating corticospinal tract axons expressed TrkB (Fig. 5G–J). These results prompted us to hypothesize that target-released BDNF from spinal interneurons may influence the

**Figure 5** BDNF and TrkB are expressed in corticospinal circuit after injury. (A–C) *In situ* hybridization for BDNF messenger RNA (blue) in the cervical cord of control (A), and injured mice at 14 (B) and 28 days (C). Scale bar = 100 μm. (D) BDNF expression in the right denervated side cervical cord of control and injured mice at 7, 14 and 21 days after injury, detected by real-time polymerase chain reaction. Data are represented as mean ± SEM **P < 0.01, *P < 0.05 (n = 5, one-way ANOVA with Tukey–Kramer test).** (E–J) TrkB was expressed in the corticospinal neurons and sprouting corticospinal tract fibres. (E) *In situ* hybridization for TrkB messenger RNA in the motor cortex (blue). (F) Immunohistochemistry for retrograde-labelled corticospinal neurons (green) and TrkB (red, arrowheads). (G–J) Immunohistochemistry for TrkB (red) and the biotinylated dextran amine–labelled (BDA) corticospinal tract sprouting fibres (green) at 28 days after injury (arrowheads, H–J). (H–J) are magnified view of the dotted square in (G). Scale bars = 200 μm (E), 50 μm (F), 100 μm (G), 20 μm (H).
growth or arborization of corticospinal tract presynaptic axon terminals.

We next investigated whether local application of BDNF to axons could induce axonal arborization, as indicated in the sensory and retinotectal circuits (Cohen-Cory et al., 2010). To investigate the effect of BDNF on axons in vitro, cortical neurons were cultured in a compartmentalized microfluidic culture platform (Taylor et al., 2005) (Supplementary Fig. 5A). BDNF treatment to the axonal compartment increased the number of branches (Supplementary Fig. 5B, C and E). In contrast, BDNF application to the somal compartment did not induce such branching (Supplementary Fig. 5E). To determine the involvement of TrkB receptor in BDNF-induced branching, TrkB small interfering RNA was introduced into neurons. TrkB small interfering RNA efficiently reduced TrkB expression, and the effect continued for at least 14 days in vitro (Supplementary Fig. 5F and G). The BDNF-induced increase in branching was significantly reduced by TrkB small interfering RNA transfection (Supplementary Fig. 5D and E). These results indicate that TrkB-BDNF signalling in axons induced axonal arborization in mouse cortical neurons.

Finally, we assessed whether BDNF-TrkB signalling was involved in axonal rewiring of the corticospinal tract after brain injury. To test this possibility, the signal was blocked by TrkB small interfering RNA infusion into the forelimb area of the contralateral motor cortex. TrkB small interfering RNA infusion effectively decreased TrkB expression in layer V for at least 10 days (Supplementary Fig. 6A–E). Since sprouting fibres increased in the cervical cord later than 14 days after the injury (Fig. 2E) and the effect of small interfering RNA continued for 10 days, TrkB small interfering RNA was infused at 2 weeks after the injury. To label the corticospinal tract, biotinylated dextran amine was infused simultaneously. Two weeks after small interfering RNA infusion, the sprouting of biotinylated dextran amine-labelled corticospinal tract fibres was analysed. The axonal density and branching of corticospinal tract fibres in the denervated side significantly decreased by TrkB small interfering RNA infusion, but not control small interfering RNA infusion, following cortical injury (Fig. 6A–D, Supplementary Fig. 6F and G). To examine whether the decrease in axonal density affected behavioural outcome, three motor tests, ladder walk, staircase and cylinder tests were conducted on TrkB small interfering RNA- and control small interfering RNA-infused mice. All three tests showed that the recovery was impaired in TrkB small interfering RNA-infused mice (Fig. 6E–G).

Next, BDNF small interfering RNA was infused into the denervated side of the grey matter of C4 and C6 level at 2 weeks after injury. The BDNF small interfering RNA effectively reduced the expression of BDNF in N1E-115 neuroblastoma cells in vitro, as assessed by ELISA in situ (Supplementary Fig. 7A). Since Alexa 488-labelled BDNF small interfering RNA spread over ~250 µm anterior or posterior from the injection site, axonal density and branching of the corticospinal tract were estimated within this area. Four weeks after the injury, the axonal density and branching of biotinylated dextran amine-labelled corticospinal tract fibres significantly decreased (Fig. 6H–K, Supplementary Fig. 7B and C). These results indicate that TrkB-BDNF signalling is required for the corticospinal tract rewiring that contributes to behavioural recovery.

Finally, to assess whether BDNF could enhance corticospinal tract axonal innervation, BDNF was overexpressed in spinal neurons using lentivirus encoding BDNF and Venus, a GFP variant, under synapsin 1 promoter (Hokii et al., 2009; Tamura et al., 2009). The lentivirus encoding BDNF increased BDNF expression (Supplementary Fig. 7A). This virus was infused into the denervated side of the grey matter of C4 and C6 level at 1 week after injury. Venus was specifically expressed in spinal neurons over ~250 µm anterior or posterior from the injection site (Supplementary Fig. 7D). Four weeks after the injury, the axonal density and branching of biotinylated dextran amine-labelled corticospinal tract fibres significantly increased (Fig. 6L–O, Supplementary Fig. 7E and F).

Discussion

Although axonal and dendritic remodelling occur and brain maps change after cortical injury (Murphy and Corbett, 2009), whether these changes actually contribute to functional recovery has remained unknown. The results of the present study reveal that the remodelled corticospinal tract axons connect to specific types of spinal interneurons and contribute to recovery following cortical injury. Further, a part of the molecular basis for this plasticity is revealed.

These findings may contribute to the development of therapeutic strategies for brain injuries. In particular, they raise the possibility that increasing the sprouting of corticospinal tract fibres and their connections can aid in the recovery process. In that respect, sprouting after cortical or unilateral corticospinal tract injury has been successfully promoted by enhancing neural activity (electrical stimulation and rehabilitation) (Brus-Ramer et al., 2007; Maier et al., 2008; Carmel et al., 2010), activating intracellular neuronal signals (inosine treatment, P3K/Akt, and mTOR pathway) (Chen et al., 2002; Zai et al., 2009; Liu et al., 2010; Yip et al., 2010), blocking axonal inhibitory factors (Nogo signalling) (Thalmair et al., 1998; Lee et al., 2004), and trophic support (transplantation of bone marrow stromal cells) (Liu et al., 2007). Some of these experiments further observed correlations between increased sprouting and greater motor function recovery. Although other neuronal reorganization such as intracortical (Nudo, 2006; Murphy and Corbett, 2009; Li et al., 2010), cortico-rubro-spinal (Lee et al., 2004) and cortico-reticulo-spinal circuit (Umeda et al., 2010), might also be involved in the recovery process, our present data strongly support the notion that the effect of the candidate therapies can be appropriately assessed by focusing on the formation of the restorative neural network, i.e. corticospinal tract, identified in the present study.

It should be noted that several reports have indicated that activation of contralosional cortex in the chronic phase was negatively correlated with functional recovery, although it may be necessary for recovery in the early phase (Calautti et al., 2001; Johansen-Berg et al., 2002; Liu et al., 2009). These controversial results may partly reflect the severity of the lesion (Fedy et al., 2002; Johansen-Berg et al., 2002; Nudo, 2006; Murphy and
Figure 6  BDNF-TrkB signalling is required for corticospinal tract axonal branching after brain injury. (A and B) Biotinylated dextran amine-labelled corticospinal tract axonal arborization (green) in the denervated side of the cervical cord (C6) after infusion of control (A) or TrkB small interfering RNA (siRNA) (B) at 28 days after injury. (C and D) The extent of axonal density (C) and branch formation (D) of corticospinal tract after infusion of control or TrkB small interfering RNA (*p = 4). (E-G) Behavioural scores of ladder walk (E; control small
Corbett, 2009; Stoeckel and Binkofski, 2010). Indeed, the contribution of contralesional intact cortex was shown to increase in larger lesions (Biernaskie et al., 2005). Along these lines, recent studies have indicated that ipsilesional remnant corticospinal tract fibres can reorganize after smaller injuries than ours (Liu et al., 2009; McNeal et al., 2010). Collectively, it is conceivable that although their origin differs between ipsi- and contralesional, the reorganization of corticospinal tract fibres is indispensable to compensate for lost functions.

We provide evidence that midline-crossing corticospinal tract fibres connect with two types of interneurons—segmental and propriospinal neurons—after cortical injury. In cats, segmental interneurons mediate the command for taking food (grasping with digits), and propriospinal neurons mediate forelimb target reaching (Isa et al., 2007). We observed recovery of behavioural score in the ladder walk test, in which target reaching to the forward rung is an important part of the task, more so than the staircase test in which grasping is critical. This difference may be reflected by the involvement of propriospinal neurons rather than segmental interneurons in recovery. Propriospinal neurons can partially compensate for lost grasping function in monkeys (Isa et al., 2007) and locomotor function in rodents after spinal cord injury (Bareyre et al., 2004; Courtine et al., 2008). Their importance is highlighted further by our finding. Thus, propriospinal neurons may be critical for compensation of lost motor functions after injuries to the spinal network.

Despite the wealth of knowledge on manipulations that enhance axonal sprouting and the resultant motor function recovery as mentioned above, the intrinsic mechanism underlying spontaneous recovery had not yet been elucidated until the present study. Several studies have attempted to identify the intrinsic factors that induced corticospinal tract rewiring after cortical stroke or pyramidotomy using DNA microarray analysis, but the critical molecules have not been determined (Bareyre et al., 2002; Maier et al., 2008; Zai et al., 2009). The present study identified BDNF-TrkB signalling as required for corticospinal tract reorganization. We revealed that postsynaptic BDNF triggered morphological changes in TrkB-expressing presynaptic axons to reorganize the network. BDNF is considered to work locally in the target zone for collateral axonal branching but not for axonal pathfinding during development (Cohen-Cory et al., 2010). Our data indicate a similar function in injured adult CNS, because local branching and growth were effectively blocked by TrkB or BDNF knockdown, but the effects on recrossing and pathfinding into the specific area were limited. This observation raises the possibility that other molecules are also involved in the corticospinal tract rewiring and pathfinding. Further investigation is needed to reveal the broader mechanism of these plastic changes, particularly how the axons are attracted to the denervated side and how they recognize specific partners to form synapses. Understanding the underlying molecular mechanism could be helpful in establishing a new therapeutic method for the recovery of impaired cortical function.

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Supplementary material

Supplementary material is available at Brain online.

References


Bareyre FM, Kerschensteiner M, Raineteau O, Mettenleiter TC, Weinmann O, Schwab ME. The injured spinal cord spontaneously

Figure 6 Continued

interfering RNA, n = 14; TrkB small interfering RNA, n = 15), staircase (F; control small interfering RNA, n = 7; TrkB small interfering RNA, n = 15), and cylinder test (G; control small interfering RNA, n = 14; TrkB small interfering RNA, n = 15) in control and TrkB small interfering RNA infused mice after injury. **P < 0.01 compared to control small interfering RNA (two-way repeated ANOVA). (H and I) Corticospinal tract arborization (biotinylated dextran amine, red) in the denervated side (C6) after infusion of control (H) or BDNF small interfering RNA (I) at 28 days. (J and K) The extent of axonal density (J) and branch formation (K) of corticospinal tract after infusion of control or BDNF small interfering RNA (control small interfering RNA, n = 3; BDNF small interfering RNA, n = 6). (L and M) Corticospinal tract arborization (biotinylated dextran amine, red) in the denervated side (C6) after infection of Venus (L) or Venus-BDNF expressing lentivirus (M) at 28 days. Insets are Venus expression detected by GFP antibody in the same section. (N and O) The extent of axonal density (N) and branch formation (O) of corticospinal tract after infection of Venus or Venus-BDNF expressing lentivirus (n = 3). Scale bars = 100 μm. Data are represented as mean ± SEM. **P < 0.01, *P < 0.05 compared to control small interfering RNA or Venus (two-way ANOVA followed by Tukey-Kramer test).


