Proteomic analysis of trans-hemispheric motor cortex reorganization following contralateral C\textsubscript{7} nerve transfer

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Abstract

Nerve transfer is the most common treatment for total brachial plexus avulsion injury. After nerve transfer, the movement of the injured limb may be activated by certain movements of the healthy limb at the early stage of recovery, i.e., trans-hemispheric reorganization. Previous studies have focused on functional magnetic resonance imaging and changes in brain-derived neurotrophic factor and growth-associated protein 43, but there have been no proteomics studies. In this study, we designed a rat model of total brachial plexus avulsion injury involving contralateral C\textsubscript{7} nerve transfer. Isobaric tags for relative and absolute quantitation and western blot assay were then used to screen differentially expressed proteins in bilateral motor cortices. We found that most differentially expressed proteins in both cortices of upper limb were associated with nervous system development and function (including neuron differentiation and development, axonogenesis, and guidance), microtubule and cytoskeleton organization, synapse plasticity, and transmission of nerve impulses. Two key differentially expressed proteins, neurofilament light (NFL) and Thy-1, were identified. In contralateral cortex, the NFL level was upregulated 2 weeks after transfer and downregulated at 1 and 5 months. The Thy-1 level was upregulated from 1 to 5 months. In the affected cortex, the NFL level increased gradually from 1 to 5 months. Western blot results of key differentially expressed proteins were consistent with the proteomic findings. These results indicate that NFL and Thy-1 play an important role in trans-hemispheric organization following total brachial plexus root avulsion and contralateral C\textsubscript{7} nerve transfer.

Key Words: nerve regeneration; brachial plexus; brain plasticity; contralateral C\textsubscript{7} cortex reorganization; isobaric tags for relative and absolute quantitation; proteomics; nerve transfer; neurofilament light; Thy-1; neural regeneration
Introduction
Nerve transfer is the most common treatment for total brachial plexus avulsion injury (TBPI). During the past several decades, many studies have been conducted to find new donor nerves, such as the intercostal nerve (Seddon, 1963; Narakas, 1978; Krakauer and Wood, 1994), accessory nerve (Allieu et al., 1984), phrenic nerve (Brunelli and Monini, 1984; Nagano et al., 1995; Gu and Ma, 1996; Chuang et al., 2005; Liu et al., 2015b), and the contralateral C7 (CC7) nerve (Gu et al., 1998; Chuang and Hernon, 2012; Tu et al., 2014; Mathews et al., 2017).

After TBPI and CC7 nerve transfer, the movement of the injured limb will be activated by certain movements of the healthy limb at the early stage of recovery. This phenomenon is because the motor cortex for the CC7 is located on the injured side, so the movement of the healthy limb, which was originally innervated by the transferred CC7 nerve, can activate the injured limb. Some patients can regain independent motion, but in others, synchronous motion of the healthy limb is maintained (Songcharoen et al., 2001). Many studies have shown that there is a trans-hemispheric reorganization process following CC7 nerve transfer (Lou et al., 2006; Navarro et al., 2007; Jiang et al., 2010). Lundborg et al. (2006) found that after peripheral nerve injury, adjacent motor cortices encroach on the original motor cortical representation of the injured nerve and this process is referred to as phase-one reorganization, as described in previous studies (Donoghue et al., 1990; Kaas et al., 1990; Sanes et al., 1990; Merzenich and Jenkins, 1993; Qi et al., 2000). Pan et al. (2012) found that this phase-one reorganization was a harmful factor for recapture of brachial plexus motor cortices. Thus, if we can find a way to restrain or shorten this process, we may accelerate trans-hemispheric cortical functional remodeling. However, the mechanism of this process remains unclear. Previous studies have only focused on functional magnetic resonance imaging (Beaulieu et al., 2006; Sokki et al., 2012; Yu et al., 2017) and the proteins brain-derived neurotrophic factor (BDNF) and growth-associated protein 43 (GAP43) (Nicoléis et al., 1993; Lang et al., 2008; Zhao and Xu, 2008; Wei et al., 2011). There have been no studies using proteomics to analyze protein expression.

The purpose of our experiment was to screen for differential protein expression in the motor cortex after CC7 nerve transfer for TBPI by using isobaric tags for relative and absolute quantitation (iTRAQ), and western blot assays. We identified differentially expressed proteins that might play a role in the motor cortex reorganization.

Materials and Methods
Animals
A total of 90, 2-week-old, female Sprague-Dawley rats weighing approximately 200 g were used for surgery. The rats were supplied and housed in the Laboratory Animal Room of the Medicine College of Fudan University of China (license number: SCXK 2014-0004) with free access to food and water. All animal protocols were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals described by the U.S. National Institutes of Health and conformed to guidelines of the Society for Neuroscience and the Neurotrauma Society.

The rats were randomly and equally divided into three groups. The right forepaw was selected as the affected limb. CC7 group: The C5–T1 nerve roots for the affected limb were avulsed and the entire root of the CC7 nerve was transected and transferred subcutaneously to the median nerve, bridged by the ulnar nerve of the affected limb. TBPI group: The C5–T1 nerve roots were avulsed without any further operation on the affected limb. Control group: C5–T1 nerve roots on both limbs were exposed as for the CC7 group, but they were not avulsed and no further surgery was performed.

Model establishment of TBPI and CC7 nerve transfer
Rats were anesthetized with 40 mg/kg sodium pentobarbital and all had supraclavicular exposure of brachial plexus. The C5–T1 nerve roots on the right side were avulsed from the spinal column using forceps and 1 cm portions of the avulsed roots were excised to prevent intraplexus nerve regeneration. In the CC7 group, the CC7 nerve (left side) was transected at the level of its division; the ulnar nerve of the injured side (right side) was transected at the levels of the wrist and the axilla as the bridge between the CC7 nerve and the median nerve of the injured side. The ulnar nerve was isolated together with the superior ulnar collateral artery, which was used as its nutrient artery. The distal stump was retracted and transferred to the contralateral neck through a subcutaneous tunnel under the chest, and coapted to the CC7 root using 11-0 sutures. The proximal stump was coapted to the median nerve of the injured side. The wounds were closed with interrupted 5-0 nylon suture. In the TBPI group, the rats received C5–T1 nerve root avulsion, as in the CC7 group, without any further surgery on the affected limb. In the control group, the rats had C5–T1 nerve exposure as in the CC7 group, but no further surgery was performed.

The flexor digitorum superficiais muscles were selected as a representative muscle of the median nerve (Jiang et al., 2016). Electromyography was performed in five rats per group at 3 days, 1 week, 2 weeks, 1 month, 3 months and 5 months postoperatively using Neuromatic 2000M electrophysiological apparatus (Dantec, Les Ulis, France). The CC7 nerve was stimulated using an electrode placed in situ (single square wave shocks, 2.0 mA super pulse current, 0.2 ms pulse width, 1 Hz stimulus frequency). Compound muscle action potentials (CMAPs) were recorded from the flexor digitorum superficiais muscle using a concentric needle recording electrode inserted vertically into the muscles.

Sample collection
The motor cortex specimens from both sides of cortex were taken from five rats per group at 3 days, 1 week, 2 weeks, 1 month, 3 months and 5 months postoperatively.

Anesthetized rats were secured in a stereotactic frame, and the scalp was incised along the midline. Both sides of the skull at the area of the motor cortex were removed (size...
of craniectomy, 15 mm) using a drill and a rongeur. The coordinates of the three points from the Bregma were 4 mm rostral/1 mm lateral (coordinate A = +4, +1), 2 mm caudal/1 mm lateral (coordinate B = −2, +1), and 4 mm rostral/6 mm lateral (coordinate C = +4, +6) (Lee et al., 2009) (Figure 1). Avoiding deep structure injury, we removed the brain to a depth of 4 mm from the outer surface of the skull. We excluded rats with deep structure injury. The specimens were stored at −80°C until use.

**iTRAQ quantitative proteomic analysis**

At 3 days, 1 and 2 weeks, and 1, 3 and 5 months following surgery, five injured and normal control animals were sacrificed under sodium pentobarbital anesthesia (100 mg/kg, intraperitoneally). Motor cortex tissue from the same group (n = 5 rats) was pooled together for protein extraction. The mixed tissues were rinsed to remove blood, cut into pieces and ground into powder. The tissues were vortexed for 15 minutes after the addition of lysis buffer (7 M urea, 2 M sulfourea, 0.1% phenylmethyl sulfonylfluoride, 65 M dithiothreitol) and incubated on ice for 30 minutes. After centrifugation at 12,000 revolutions per minute for 15 minutes, supernatants were collected and stored at −70°C until further use. The high-abundance proteins were removed by a Removal System Affinity Column (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer’s instructions. After depletion, the protein concentration was assessed by the Bradford method (Xie et al., 2011).

Proteins of each group at each time point were labeled with iTRAQ reagents (Bio-Rad, Hercules, CA, USA). Cysteine residues were then blocked for 10 minutes at room temperature by adding 1 μL iTRAQ cysteine blocking reagent and the samples were digested with trypsin (30 μg) at 37°C overnight.

The peptides were fractionated on a Waters ultra performance liquid chromatography using a C18 column (Waters BEH C18 2.1 × 50 mm, 1.7 μm). The fraction was separated by nano-high performance liquid chromatography (Eksigent, California, USA) on a secondary reverse-phase analytical column (Eksigent, C18, 3 μm, 150 mm × 75 μm). A Triple TOF 4600 mass spectrometer was operated in information-dependent data acquisition mode to switch automatically between mass spectrometry (MS) and MS/MS acquisition. MS spectra were acquired over the mass range of 350–1,250 m/z using 250 ms accumulation time per spectrum. Tandem mass spectra were scanned from 100–1,250 m/z in high sensitivity mode with rolling collision energy. The 25 most intense precursors were selected for fragmentation per cycle, with a dynamic exclusion time of 25 seconds.

Tandem mass spectra were extracted and charge state deconvoluted by MS Data Converter from AB Sciex. Mascot (version 2.3.02; Matrix Science, London, UK) which was only used to interpret samples. Mascot was set up to search the rat database (Acanthamoeba castellanii from NCBI, 30279 entries), assuming trypsin enzyme digestion. Mascot was searched with a fragment ion mass tolerance of 0.1 Da and a parent ion tolerance of 25 ppm. Methylthio of cysteine, iTRAQ8plex of lysine, and the N-terminus were specified in Mascot as fixed modifications. Oxidation of methionine and iTRAQ8plex of tyrosine were specified in Mascot as variable modifications.

**Bioinformatics analysis**

The control group was used as the baseline. Differentially expressed proteins in the CC group and the TBPI group were analyzed separately at each time point. Proteins in the CC group that showed differential expression of more than 1.5 or less than 0.67 relative to the control group at any time point were selected for further analysis. Proteins in the CC group that showed differential expression of more than 1.2 or less than 0.83 (P < 0.05) compared to those in the TBPI group at each time point were also selected for further analysis. Differentially expressed proteins selected in step 2 and step 3 were input into the DAVID network database (https://david.ncifcrf.gov/) for further analysis, including functional classification, gene ontology analysis and functional annotation clustering (Huang da et al., 2009).

**Western blot assay**

Western blot assay of the same samples from the proteomic analysis was performed to validate proteomic quantitation of selected proteins; 40 μg of protein from each motor cortex sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. The primary antibodies were mouse monoclonal antibodies against neurofilament (1:1,000; Abcam, Cambridge, UK), and CD90/Thy1 (1:1,000; Abcam). Secondary antibodies were goat anti-mouse IgG (H + L) (1:1,000; West Grove, PA, USA) and anti-rabbit IgG (H + L) (1:1,000; West Grove). Immunoreactive signals were detected by an enhanced chemiluminescence system (GE Healthcare, London, UK).

**Statistical analysis**

The results of electrophysiological study were expressed as the mean ± SD. All statistical analyses were made using SPSS version 19.0 software (IBM, Armonk, NY, USA). A two-tailed t-test was applied to compare the amplitude and latency of CMAP. Statistical significance was set at 0.05.

**Results**

**CC7 nerve transfer improves neurological function in rats with total brachial plexus avulsion injury**

Electrophysiological testing showed that no nascent CMAPs
Figure 2  Proteomics results of NFL and Thy-1 in rat motor cortex after contralateral C7 nerve transfer (western blot assay).
(A, C) Representative western blots of NFL in the contralateral and affected cortices. (B, D) Representative western blots of Thy-1 in the contralateral and affected cortices. (E, F) Quantitative results of NFL and Thy-1 in the cerebral cortex. The data are expressed as the optical density ratio to GAPDH. (G) In contralateral cortex, the NFL level tended to be upregulated at first, and then to be downregulated. (H) Conversely, the Thy-1 level tended to be upregulated gradually. In the affected cortex, the NFL level tended to be gradually downregulated and the Thy-1 level still tended to be gradually upregulated. Data in E–H are expressed as the mean(± SD). NFL: Neurofilament light; d: days; w: week(s); m: month(s).

Figure 1 Coordinates and exposure of rat motor cortex on both sides.
(a) The coordinates of the three points from the Bregma were 4 mm rostral/1 mm lateral (coordinate A = +4, +1), 2 mm caudal/1 mm lateral (coordinate B = −2, +1), and 4 mm rostral/6 mm lateral (coordinate C = +4, +6) (Lee et al., 2009). (b) The exposure of the motor cortex on both sides.

from the flexor digitorum superficialis muscle could be recorded in the rats of the TBPI group at any time point postoperatively. In the CC7 group, nascent CMAPs could be recorded at 3 and 5 months postoperatively, but their amplitude and latency were significantly worse than those in the control group ($P < 0.05$; Table 1).

Changes in cortical protein expression and relationship to total brachial plexus avulsion injury with CC7 nerve transfer

Proteins in the CC7 group that showed differential expression of more than 1.5 or less than 0.67 relative to control group at any time point were selected for further analysis. There were 265 differentially expressed proteins in the contralateral cortex and 264 in the affected cortex of the CC7 group, as detected by iTRAQ proteome analysis. The functional classi-
Table 1 Effect of CC7 nerve transfer on the compound muscle action potential in the flexor digitorum superficialis muscle of rats after total brachial plexus avulsion injury

|                  | CC7 group | TBPI group | Control group |
|------------------|-----------|------------|---------------|
| Amplitude (mV)   | 12.48±2.79| 0          | 42.70±2.69    |
| Latency (ms)     | 15.22±2.26| 0          | 44.60±1.61    |

Data are expressed as the mean ± SD and analyzed by two-tailed t test; *P < 0.05, vs. control group; #P < 0.05, vs. TBPI group. CC7: Contralateral CC7; TBPI: total brachial plexus avulsion injury.

Table 2 Gene functional classification in contralateral and affected motor cortices of total brachial plexus avulsion injury rats treated by contralateral CC7 nerve transfer

|                  | Contralateral | Affected |
|------------------|---------------|----------|
| Nervous system development and function | - Microtubule-associated protein | - Ribosomal |
|                   | - Cysteine    | - ATP synthase |
|                   | - Ribosomal   | - Calcium binding protein |
|                   | - Molecular transport | - Cell adhesion |
|                   | - ATP synthase | - Nervous system development and function |

The differentially expressed proteins of each cortex were classified into six function clusters in the gene functional classification cluster. The clusters in bold were chosen for further analysis. ATP: Adenosine triphosphate.

Light (NFL), Thy-1, Gap43, Gdc42 and Stmn1. Among these five proteins, NFL and Thy-1 were identified for the first time by screening of peripheral nervous system injury. Compared to the TBPI group, in contralateral cortex, the NFL level was upregulated at 2 weeks after transfer then downregulated at 1 and 5 months. Conversely, the Thy-1 level was upregulated from 1 month to 5 months after transfer. In the affected cortex, the NFL level increased gradually from 1 month to 5 months after transfer.

Compared to the control group, in contralateral cortex, the NFL level tended to be upregulated at first, and then to be downregulated. Conversely, the Thy-1 level tended to be gradually upregulated. In the affected cortex, the NFL level tended to be gradually downregulated while the Thy-1 level still tended to be gradually upregulated.

Therefore, we selected these two key proteins to assess by western blot assay, the results of which were consistent with the proteomic findings (Figure 2).

Discussion

A well-functioning upper extremity requires not only a healthy peripheral nervous system, including muscular and sensory end-organs, but also a healthy central nervous system. TBPI is devastating for patients and poses substantial challenges for surgeons; nerve transfer procedures remain the primary option for nerve reconstruction after TBPI (Tung, 2014; Liu et al., 2015a). CC7 is commonly transferred to the median nerve to restore hand function in TBPI patients. It provides 17,000 to 40,000 myelinated nerve fibers and contains both sensory and motor fibers, so the median nerve is a suitable recipient nerve for CC7 (Gu, 2007; Tu et al., 2014). Although many variables can affect the success of repair, we wanted to study the role of the central nervous system. Multiple studies have demonstrated that remodeling of neural networks, termed brain plasticity, occurs after peripheral nerve injury and repair, especially after nerve transfer. Due to the trans-hemispheric reorganization process, choosing the CC7 nerve is more challenging and requires more time for nerve regeneration (Kiper et al., 2007; Pour-
Table 3 Top 10 biological processes of differentially expressed proteins in contralateral and affected motor cortex of total brachial plexus avulsion injury rats treated by contralateral C7 nerve transfer

| Gene accession ID | Term                                      | Counts of protein involved | P         |
|-------------------|-------------------------------------------|---------------------------|-----------|
| Contralateral cortex |                                           |                           |           |
| GO:0030182        | Neuron differentiation                     | 28                        | 4.4E-21   |
| GO:0048666        | Neuron development                         | 24                        | 5.6E-19   |
| GO:0048666        | Transmission of nerve impulse              | 19                        | 1.5E-14   |
| GO:0007268        | Synaptic transmission                      | 17                        | 6.3E-14   |
| GO:0032989        | Cellular component morphogenesis           | 20                        | 1.6E-13   |
| GO:0051129        | Negative regulation of cellular component organization | 14                        | 2.1E-13   |
| GO:0030030        | Cell projection organization               | 19                        | 1.0E-12   |
| GO:0007010        | Cytoskeleton organization                  | 18                        | 1.6E-12   |
| GO:0031175        | Neuron projection development              | 17                        | 1.9E-12   |
| GO:0007267        | Cell-cell signaling                        | 18                        | 2.8E-12   |
| Affected cortex    |                                           |                           |           |
| GO:0019226        | Transmission of nerve impulse              | 20                        | 1.3E-12   |
| GO:0007267        | Cell-cell signaling                        | 21                        | 1.1E-12   |
| GO:0007268        | Synaptic transmission                      | 18                        | 2.2E-12   |
| GO:0030182        | Neuron differentiation                     | 23                        | 1.8E-12   |
| GO:0048666        | Neuron development                         | 20                        | 1.5E-11   |
| GO:0001505        | Regulation of neurotransmitter levels      | 12                        | 3.5E-10   |
| GO:0031175        | Neuron projection development              | 16                        | 6.8E-9    |
| GO:0051129        | Negative regulation of cellular component organization | 12                        | 2.4E-8    |
| GO:0044057        | Regulation of system process               | 16                        | 4.8E-8    |
| GO:0032989        | Cellular component morphogenesis           | 17                        | 4.4E-8    |

Table 4 Functional annotation cluster analysis of contralateral and affected motor cortices of total brachial plexus avulsion injury rats treated by contralateral C7 nerve transfer

| Cluster 1 | Contralateral cortex | Affected cortex |
|-----------|----------------------|-----------------|
| Neuron projection | Cell projection | Neuron differentiation | Neuron development |
| Axon development | Neuron projection | Cytoskeletal protein binding | Microtubule |
| Neuron projection | Neuron projection | Synapse | Neurological system process |
| Axon guidance | Neuron projection | Synapse | Neurotransmitter transport |
| Axonogenesis | Neuron projection | Synapse | Transmission of nerve impulse |
| Cluster 2 | Neuron differentiation | Neuron development | Cytoskeleton organization |
| Neuron development | Neuron projection | Cytoskeleton | Microtubule |
| Axonogenesis | Neuron projection | Cytoskeleton | Synapse |
| Axon guidance | Neuron projection | Cytoskeleton | Microtubule |
| Cluster 3 | Regulation of transmission of nerve impulse | Regulation of neurological system process |
| Regulation of synaptic plasticity | Regulation of synaptic transmission | Synaptic transmission |
| Regulation of synaptic plasticity | Regulation of synaptic transmission | Synaptic transmission |

Table 5 Differential protein counts of contralateral and affected motor cortices of total brachial plexus avulsion injury rats treated by contralateral C7 nerve transfer

| 3 days | 1 week | 2 weeks | 1 month | 3 months | 5 months |
|--------|--------|---------|---------|----------|----------|
| Contralateral | Up | 15 | 26 | 25 | 30 | 46 | 36 |
| Down | 15 | 31 | 51 | 18 | 49 | 97 |
| Total | 30 | 57 | 76 | 48 | 95 | 133 |
| Affected | Up | 29 | 63 | 46 | 34 | 25 | 23 |
| Down | 42 | 85 | 92 | 63 | 65 | 66 |
| Total | 61 | 147 | 138 | 97 | 90 | 89 |

The functional analysis showed that most differentially expressed proteins in both cortices of the upper limb correlated with development and function of the nervous system (including neuron differentiation and development, axonogenesis and guidance), microtubule and cytoskeleton organization, synapse plasticity, and transmission of nerve impulses after C7 root transfer. It implies that these processes were activated after C7 root transfer and that specific proteins were involved in the brain plasticity.

The peak of the differentially expressed protein counts appeared in the late stage in contralateral cortex and in the early stage in affected cortex. The function cluster showed that proteins involved in axonogenesis were upregulated in the early stage in contralateral cortex and the late stage in affect-
Table 6 The major biological processes of differentially expressed proteins in affected motor cortex of total brachial plexus avulsion injury rats treated by contralateral C7 nerve transfer

| Period          | Expression | Contralateral cortex | Affected cortex   |
|-----------------|------------|----------------------|-------------------|
| Early stage (< 1 month) | Up | Negative regulation of neuronal apoptosis | Cell-cell signaling |
|                  | Down | Axonogenesis | Synaptic transmission |
| Late stage (≥ 1 month) | Up | Protein synthesis | Material metabolism |
|                  | Down | Material metabolism | Transmission of nerve impulse |
|                  | Down | Protein synthesis | Regulation of microtubule cytoskeleton |
|                  | Down | Traumatic responses | Regulation of neurotransmitter levels |
|                  | Down | Axonogenesis | Transmission of nerve impulse |

ed cortex, and then downregulated in the late stage in contralateral cortex. Proteins involved in transmission of nerve impulses were upregulated in the late stage of contralateral cortex, while downregulated in affected cortex. There seemed to be a trend for alternative regulation. It has been reported that the postoperative dynamic changes for achieving functional independence of the injured limb after CC7 nerve transfer are substantially associated with the reorganization of neural control pathways (Lou et al., 2006) and perhaps these processes play an important role during brain plasticity.

Neurofilaments are major structural elements of neurons in the peripheral and central nervous systems and their functions are to control radial growth of axons and maintain axon caliber (Hoffman et al., 1987; Kriz et al., 2000). They are composed of NFM, neurofilament medium (NFM) and neurofilament heavy (NFH), with NFM an essential component of the neurofilament core (Yuan et al., 2012). It encodes a 62 kDa structural protein, which is a major cytoskeletal component of neurons. The radial growth of myelinated axons in animals lacking neurofilaments is severely attenuated (Kuchel et al., 1997; Jordanova et al., 2003). Zhu et al. (1997) showed that after targeted disruption of the NFL gene in mice, axonal caliber is diminished and maturation of regenerating myelinated axons is delayed. Some studies have also reported that neurofilament subunits can be detected in the cerebrospinal fluid and are associated with neuronal death and axonal degeneration in Alzheimer’s disease patients (Scherling et al., 2014; Skillbäck et al., 2014).

Some studies have suggested that the cortical plasticity process is actually an activation of existing cortical pathways that are normally suppressed under healthy conditions. This plasticity process is caused by newly formed axons. Several lines of evidence show that a unilateral lesion in the motor cortex in neonatal mice can cause recrossing of corticospinal tract axons in the white matter or the gray matter of the spinal cord (Barth and Stanfield, 1990; Kuang and Kalil, 1990; Joosten et al., 1992). Peripheral nerve injury results in functional expansion of neighboring cortical areas to occupy the corresponding cortical representation (Lundborg et al., 2006). A study of trans-hemispheric cortical reorganization in rats with CC7 nerve transfer to the median nerve showed that this cortical plasticity underwent two processes: early stage bilateral control and late stage single-sided control from the contralateral hemisphere (Lou et al., 2006).

In this study, the NFL level in the contralateral cortex was upregulated 2 weeks after transfer and then downregulated at 1 and 5 months. It indicates that NFL may have promoted axonogenesis due to the occupation of adjacent motor cortices at 2 weeks. To regain single-sided control from the contralateral hemisphere, NFL levels were downregulated to restrain radial axonal growth and stop the encroachment of adjacent motor cortices at 1 and 5 months. In affected cortex, the NFL level increased gradually from 1 to 5 months after transfer. In a positron-emission tomography study of a macaque monkey model of spinal cord injury, enhanced activity (increased regional cerebral blood flow) was observed during the early recovery stage in bilateral M1 cortex, and in the contralesional primary motor cortex and ipsilateral ventral premotor cortex during the late recovery stage (Nishimura and Isa, 2009). Positron-emission tomography studies showed the cortical hand area in patients with incomplete spinal cord injury at the thoracic or lumbar level expanded towards the cortical leg area, with enhanced bilateral activation of the thalamus and cerebellum (Buonomano and Merzenich, 1998). One month is also the time that nascent CMAPs could be recorded from the flexor digitorum superficialis muscle. Thus, NFL possibly promotes axonogenesis in the affected cortex to expand the cortical C7 area to gain the control of affected limb, and then activates the contralateral motor cortex to gradually regain control of the affected limb.

Thy-1 is a major glycoprotein on mammalian neurons but its role in the peripheral and central nervous systems is poorly understood. Nevertheless, it has been inferred to be a negative regulator of neurite outgrowth. Chen et al. (2005) found that the action of Thy-1 may be involved in synaptic modification induced by learning or long-term potentiation (LTP).

Cortical plasticity following peripheral nerve injury includes short-term and long-term plasticity (Bitter et al., 2011). Mechanisms of cortical plasticity include activation of previously inactive connections (Jacobs and Donoghue, 1991), strengthening or weakening activity at existing synapses (Hess et al., 1996), and changes in neuronal membrane excitability (Halter et al., 1995), all of which are mainly functional.

In this study, we found that the Thy-1 level in contralateral cortex was upregulated from 1 to 5 months, indicating that Thy-1 may restrain radial axonal growth and stop the encroachment of adjacent motor cortices, the same as NFL. It may also strengthen the activity at existing synapses to enhance the connections between bilateral hemispheres.

In summary, NFL and Thy-1 may play an important role...
in trans-hemispheric organization following TBPI and CC nerve transfer. Further study focused on these two proteins is still needed to determine how and when they affect motor cortical reorganization. Regulating their expression may improve the prognosis of CC nerve transfer.

Author contributions: YY and XZ conceived and designed experiments. YY and XYX performed the experiments. YY and JL analyzed sequencing data. YY wrote the paper. YY, XYX, JL and XZ reviewed and edited the paper. All authors approved the final version of the paper.

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Research ethics: All animal protocols were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals described by the U.S. National Institutes of Health and conformed to guidelines of the Society for Neuroscience and the Neurotrauma Society.

Data sharing statement: Datasets analyzed during the current study are available from the corresponding author on reasonable request.

Plagiarism check: Checked twice by iThenticate.

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Open peer review report:

Reviewer: Melanie C. Urbanheck, University of Michigan, USA.

Comments to authors: The authors state that the purpose of the experiment is to screen a portion of the brain motor cortex for differential protein expression by using iTRAQ-based quantitative proteomic technique and western blot techniques after a CC nerve transfer. The CC nerve transfer is a surgical procedure to repair total brachial plexus avulsion injury. In a rat model, the experimental group had the C–T nerve roots avulsed on the affected limb and the entire root of CC nerve was transplanted and attached to the affected side median nerve using the dissected ulnar nerve of the affected side as a connecting bridge between CC and the affected medial nerve. Appropriate positive and negative control groups were also studied. Organizing software modes are used to classify the effects of differential proteins. It is assumed by the reviewer that historical data are used to infer the roles of protein differentiation during the motor cortex reorganization. These historical data are recalled in the discussion and used without statistical treatment to infer correlation relationship with the protein data found in the motor cortices of the rats studied. These described correlations need to be explained and documented more thoroughly in the results section of the manuscript. Some measurements indicate that the CC nerve transfers successfully restored nerve connections to the affected limb.

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