

Axonal remodeling for motor recovery after traumatic brain injury requires downregulation of γ -aminobutyric acid signaling

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Remodeling of the remnant neuronal network after brain injury possibly mediates spontaneous functional recovery; however, the mechanisms inducing axonal remodeling during spontaneous recovery remain unclear. Here, we show that altered γ -aminobutyric acid (GABA) signaling is crucial for axonal remodeling of the contralesional cortex after traumatic brain injury. After injury to the sensorimotor cortex in mice, we found a significant decrease in the expression of GABA_AR- α 1 subunits in the intact sensorimotor cortex for 2 weeks. Motor functions, assessed by grid walk and cylinder tests, spontaneously improved in 4 weeks after the injury to the sensorimotor cortex. With motor recovery, corticospinal tract (CST) axons from the contralesional cortex sprouted into the denervated side of the cervical spinal cord at 2 and 4 weeks after the injury. To determine the functional implications of the changes in the expression of GABA_AR- α 1 subunits, we infused muscimol, a GABA R agonist, into the contralesional cortex for a week after the injury. Compared with the vehicle-treated mice, we noted significantly inhibited recovery in the muscimol-treated mice. Further, muscimol infusion greatly suppressed the axonal sprouting into the denervated side of the cervical spinal cord. In conclusion, recovery of motor function and axonal remodeling of the CST following cortical injury requires suppressed GABA_AR subunit expression and decreased GABAergic signaling.

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Activity in the motor cortex sometimes spontaneously recovers, to a limit, despite deleterious loss of motor function after injury. Reorganization of the neural network in the spared regions accompanies this functional recovery.¹ The motor cortex on the unaffected side may have a compensatory role to restore the lost motor function.² Subcortical efferent projections from the intact cortex including the corticospinal tract (CST) exhibit plastic changes following injury.³ CST axons newly sprout into the denervated (lesion) subcortical areas at multiple levels of the brain and spinal cord.^{4,5} Intriguingly, treatments that induce axonal growth promote functional recovery.⁴ Rewiring of the neural network may be responsible for the therapeutic effects of these treatments. However, the mechanisms that induce axonal remodeling during such spontaneous recovery remain unclear.

To obtain mechanistic insights into axonal remodeling in the contralesional cerebral cortex, we sought information on alterations in gene expression after cortical injury. Following injury, the perilesional and contralesional motor cortices exhibit downregulation of the γ -aminobutyric acid type A receptor (GABA_AR) and upregulation of the *N*-methyl-D-aspartate receptor.^{6–8} Increased neuronal excitability accompanies changes in the expressions of these proteins in the perilesional areas.⁹ Here, we hypothesize that the increased

neuronal excitability induced by decreased GABAergic signaling leads to axonal remodeling of the intact CST, thereby contributing to functional recovery.

Results

Changes in mRNA expression of GABA_AR subunits in the contralesional cortex. We employed a murine model to elucidate the role of GABAergic signaling in neuronal reorganization and functional recovery after traumatic cortical injury. We first investigated the gene expression patterns of each GABA_AR subunit in the contralesional cortex following the injury by real-time reverse transcriptase (RT)-PCR. Most GABA_ARs are heteropentamers composed of isoforms of α -, β -, γ -, and δ - subunits; the most common subtype is α 1 β 2 γ 2.^{10,11} Among the six isoforms of the α -subunit, the mRNA of the α 1 isoform in the injured mice compared with the sham mice significantly decreased at 7 days after the injury (Figure 1a, $P < 0.05$). Further, the mRNA expression of the γ 2 subunit significantly decreased in the injured group (Figure 1c, $P < 0.05$). On the other hand, the mRNA expressions of the other α -isoforms (Figure 1a), all β -isoforms (Figure 1b), other γ -isoforms, and δ -subunit

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Abbreviations: GABA, γ -aminobutyric acid; CST, corticospinal tract; GABA_AR, γ -aminobutyric acid type A receptor; RT-PCR, reverse transcriptase PCR; cDNA, complementary DNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; NCAM, neural cell adhesion molecule; PFA, paraformaldehyde; BDA, biotinylated dextran amine

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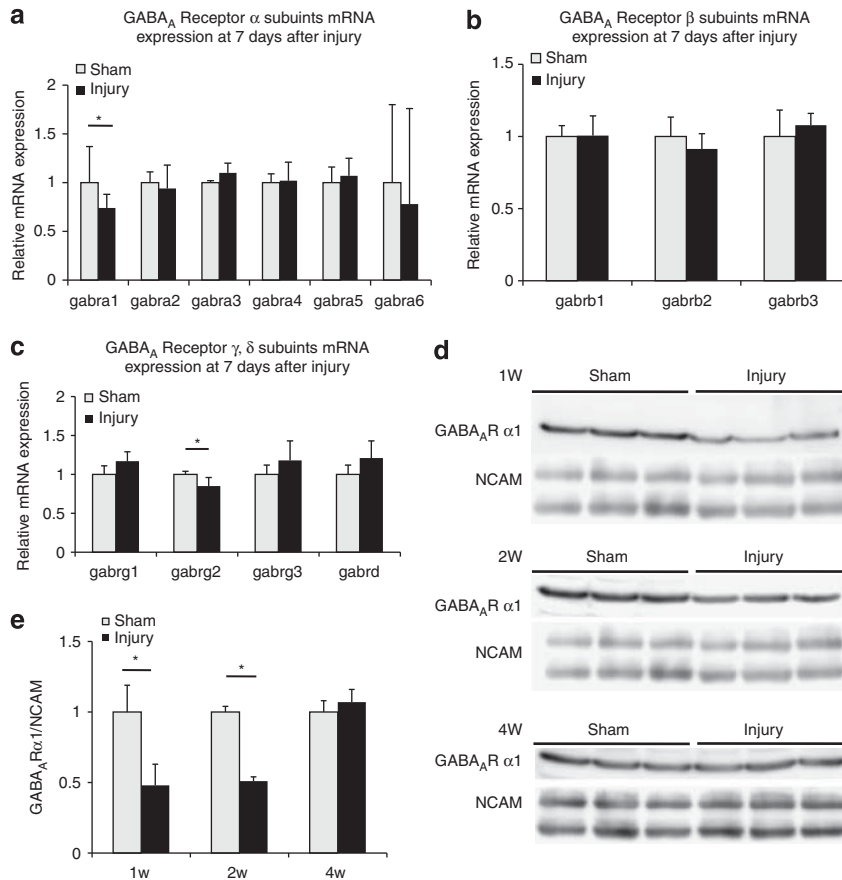


Figure 1 Downregulation of GABA_AR subunit expressions in the contralesional cortex after traumatic brain injury. (a–c) Quantification of the mRNA expression of GABA_AR subunits (α (a), β (b), and γ and δ (c) subunits) in the contralesional cortex at 7 days after brain injury (by real-time PCR). The fold change of the injured group (black bars) relative to the sham group (gray bars) is shown as the mean \pm S.D. ($n = 3$, each group). (d) Western blots of the GABA_AR- α 1 subunit and NCAM (internal control) in the contralesional cortex at 1, 2, and 4 weeks after the injury. (e) Quantification of GABA_AR- α 1 protein expression in d. The expression significantly decreased at 1 and 2 weeks after the injury. ($n = 6$, each group). * $P < 0.05$ (Mann–Whitney U -test)

(Figure 1c) in the contralesional cortex did not change after the injury. Therefore, the mRNA expressions of the α 1 and γ 2 subunits in the contralesional cortex specifically decreased after the injury.

Temporal downregulation of GABA_AR- α 1 protein expression in the contralesional cortex. To assess the time course of the protein expression of the GABA_AR- α 1 subunit in the contralesional cortex, western blotting was performed with both sham and injured mice at 1, 2, and 4 weeks after the injury. Consistent with the gene expression pattern (Figure 1a), the protein level of the GABA_AR- α 1 subunit (about 51 kDa) decreased after the injury (Figure 1d). For quantification, the mean values of this protein level normalized to the expression level of neural cell adhesion molecule (NCAM) were compared between the sham and the injured mice (Figure 1e). At 1 and 2 weeks after the injury, the protein level of the GABA_AR- α 1 subunit significantly decreased in the injured mice (Figure 1e, $P < 0.05$). However, the level returned to the control level at 4 weeks after the injury (Figure 1e).

We further investigated the localization of the GABA_AR- α 1 subunit in the contralesional cortex by immunohistochemical

staining. The GABA_AR- α 1 subunit was expressed and localized in all six cortical layers (Figure 2a). At 2 weeks after the injury, the immunoreactivity for the GABA_AR- α 1 subunit significantly decreased in all the layers (Figures 2a and b). At 4 weeks after the injury, the expression in layers II, III, and V, in which pyramidal corticospinal neurons localize, was still down-regulated compared with that in the sham group, but the expression in layers I, IV, and VI returned to the control level (Figure 2c). Thus, GABA_AR- α 1 subunit expression was down-regulated in neurons of the contralesional cortex after brain injury.

Spontaneous functional recovery of impaired forelimbs diminished by muscimol infusion. The temporal downregulation of GABA_AR- α 1 subunit expression in the contralesional cortex suggests that the activity of these neurons was upregulated. Actually, we found elevated expression of one neuronal activity-dependent gene, *alvin-1*,¹² in the contralesional cortex (Supplementary Figure 1E). Thus, we next tried to explore the functional implications of this phenomenon. In accordance with previous reports,³ the injured mice showed spontaneous functional recovery after the initial motor deficits. We assessed the functional recovery with two motor tests.

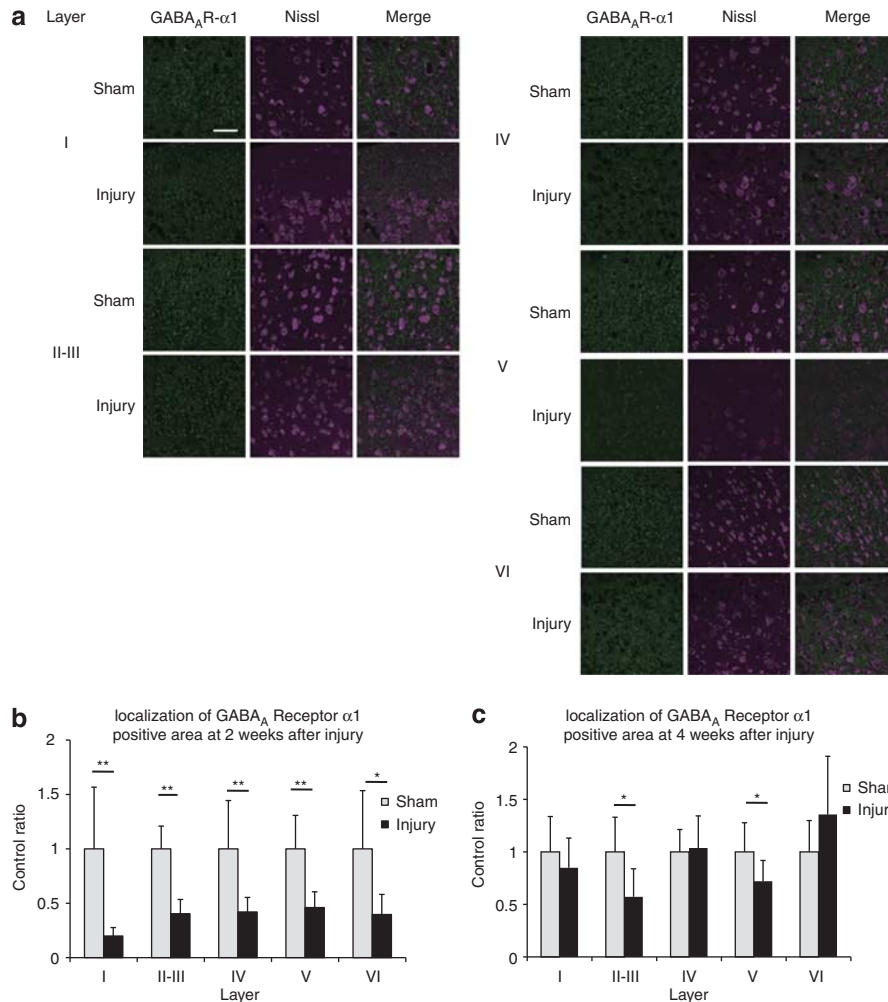


Figure 2 Downregulation of GABA_AR subunit expressions in the cortical neurons after traumatic brain injury. (a) Immunohistochemical staining of the GABA_AR-α1 subunit (green) and Nissl staining (magenta) in the contralesional cortex of the injured mice at 2 weeks after the injury. GABA_AR-α1-positive signals decreased in all the layers in injured group. Scale bar: 50 μm. (b and c) Quantification of GABA_AR-α1-positive signals by immunohistochemical staining at 2 (b) and 4 weeks (c) after the injury. The data represent the mean ± S.D. (n = 3, each group); *P < 0.05, **P < 0.01 versus the sham mice (Mann–Whitney U-test)

Although the ability to walk on a grid was severely impaired at one day after the injury, it gradually improved until 4 weeks after the injury (Figure 3a). In the cylinder test, the injured mice showed significant forelimb-use asymmetries for vertical exploration during the first week after the injury, especially reduced use of the impaired left forelimb (Figure 3b, $P < 0.01$). The independent or simultaneous use of the impaired forelimb gradually increased until 4 weeks after the injury, indicating that the function of this forelimb spontaneously recovered (Figure 3b).

We then investigated whether the downregulation of GABAergic signals in the contralesional hemisphere contributes to the functional recovery following the injury. Muscimol or saline was infused into the forelimb region of contralesional cortex for 7 days after the injury (days 3–10). Muscimol treatment induced no behavioral abnormality in the ipsilateral limbs of the mice without cortical injury, but the contralateral limbs showed transient impairment (data not shown). Further, in the grid walk test, two-way repeated measure ANOVA showed that the values in the muscimol-treated group were overall statistically different from those of

the vehicle group ($P < 0.01$), and *post hoc* analysis showed that the differences at day 7 were significant (Figure 3c). The unaffected (right) forelimb also had transient impairment during muscimol infusion (Supplementary Figure 1B), indicating that muscimol was effective in these experiments. In the cylinder test, the muscimol-treated mice used their unaffected forelimb to support their bodies more frequently than the saline-treated mice (Figure 3d). The muscimol-treated group had statistically lower function than the vehicle group ($P < 0.01$, two-way repeated measures ANOVA). At 4 weeks after the injury, the muscimol-treated group had significantly lower use ratio of the affected side than the saline-treated group ($P < 0.05$, Figure 3d). In both tests, although there were significant differences of motor functions between muscimol and vehicle-treated group by two-way repeated measure ANOVA ($P < 0.01$), the difference regarding with the time course was different. It is possible that both tests would measure different aspects of recovery in motor functions. Overall, the results indicated that the muscimol treatment inhibited the spontaneous motor recovery following the cortical injury.

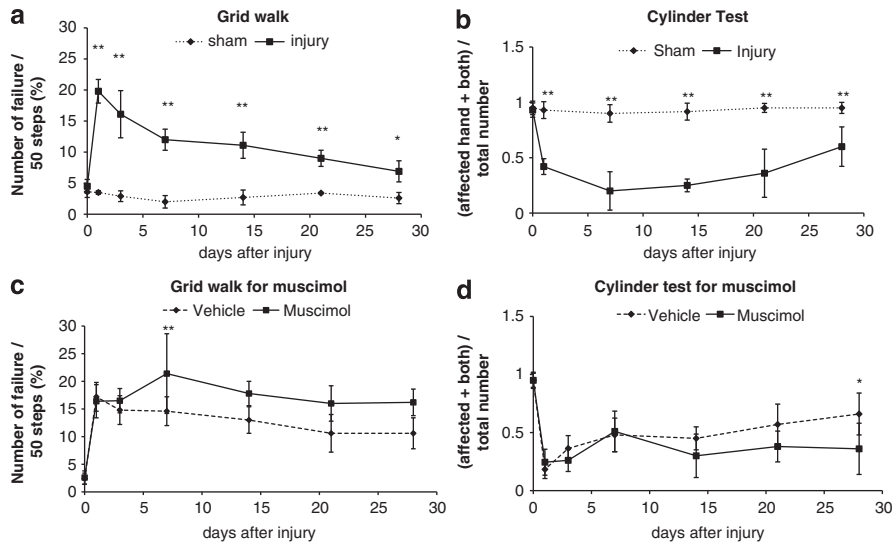


Figure 3 Spontaneous recovery of motor function after brain injury is suppressed by muscimol infusion into the contralesional cortex. (a and b) Spontaneous recovery of forelimb motor function in the grid walk (a) and cylinder (b) tests (sham group, dotted line; injured group, solid line; $n = 7$, each group). (c and d) Inhibition of spontaneous recovery of forelimb motor function by muscimol infusion in the grid walk (c) and cylinder (d) tests (saline-treated group, dotted line; muscimol-treated group, solid line). The data represent the mean \pm S.D. (vehicle $n = 7$, muscimol $n = 12$); * $P < 0.05$, ** $P < 0.01$ versus the controls (two-way repeated measure ANOVA followed by Tukey–Kramer test)

The cortical lesion volume induced by the injury did not differ between the muscimol-treated and the saline-treated groups at 4 weeks (Supplementary Figure 1C; muscimol infusion, $6.3 \pm 1.2 \text{ mm}^3$; saline infusion, $6.6 \pm 1.8 \text{ mm}^3$). Further, muscimol infusion did not lead to any apparent histological damage in the infused (contralesional) cortex (Supplementary Figure 1D). As muscimol was infused from 3 to 10 days after the injury (no effect of muscimol was observed at day 14; Supplementary Figure 1B), inactivation of GABA_AR in this period was important for motor recovery.

Enhanced GABA activation by muscimol infusion disrupted rewiring of CST fibers. The rewiring of intact corticofugal fibers, especially CST fibers, from the contralesional cortex is a main source of neuronal reorganization contributing to functional recovery after brain injury.³ As the expression of the GABA_AR- $\alpha 1$ subunit in layer V neurons including corticospinal neurons decreased (Figures 2a–c), we hypothesized that downregulation of GABAergic signaling in these neurons is necessary for enhanced axonal plasticity. To address this hypothesis, we first investigated whether CST fibers showed increased sprouting into the denervated gray matter of the cervical cord after the injury (Figure 4a). The number of biotinylated dextran amine (BDA)-labeled CST fibers recrossing the midline of the cervical cord was counted. At 2 weeks after the injury, the number of recrossing CST fibers significantly increased (Figures 4b and c). Further, quantification analysis revealed that the number of crossing CST fibers at 2 weeks increased at the C5–C7 levels (Figure 4f, $P < 0.05$). At 4 weeks after the injury, we observed a large number of crossing fibers at the C7 level in the injured mice compared with the sham mice (Figures 4d, e and g, $P < 0.05$). There were more sprouts at 2 weeks than that at 4 weeks after the injury, suggesting that progressive sprout elimination and remodeling of connections had occurred.¹³ The results

supported the notion that rewiring of CST fibers contributed to the functional recovery of motor function in the impaired forelimbs.

We then tried to investigate whether enhanced GABAergic signaling affected the sprouting of CST fibers from the contralesional cortex. We counted the number of midline-crossing CST fibers in the cervical cord at 4 weeks after the injury in the mice treated with muscimol or saline (Figures 5a and b). Fewer CST fibers crossed at the C5–C7 levels in the muscimol-treated group than in the saline-treated group (Figure 5c), although almost the same number as that in the sham group was counted (Figures 4d and g). Further, the extent of growth of the crossing fibers was assessed by measuring the BDA-positive areas in the denervated gray matter. Consistent with the decreased number of recrossing axons (Figure 5c), administration of muscimol significantly inhibited axonal outgrowth into the denervated gray matter at all the cervical levels examined (Figure 5d). These results demonstrated that suppression of GABAergic signaling was necessary for reorganization of the CST after cortical injury.

Discussion

Here, we have shown that downregulation of GABAergic signaling after traumatic brain injury in mice is necessary for rewiring of the CST and spontaneous recovery of motor function. Interestingly, when muscimol was infused from days 3 to 10 after the injury, the recovery of motor function and CST rewiring was significantly suppressed at 28 days. Thus, the period of GABAergic signal downregulation is important for neuronal plasticity and the following recovery. Indeed, this period overlaps the time window of neuronal plasticity after ischemic brain injury.¹⁴

Although functional deficits do not completely recover after central nervous system damage, partial recovery of function occurs after cortical injury in human and animals, with plastic changes in the neuronal network. For example, dendritic

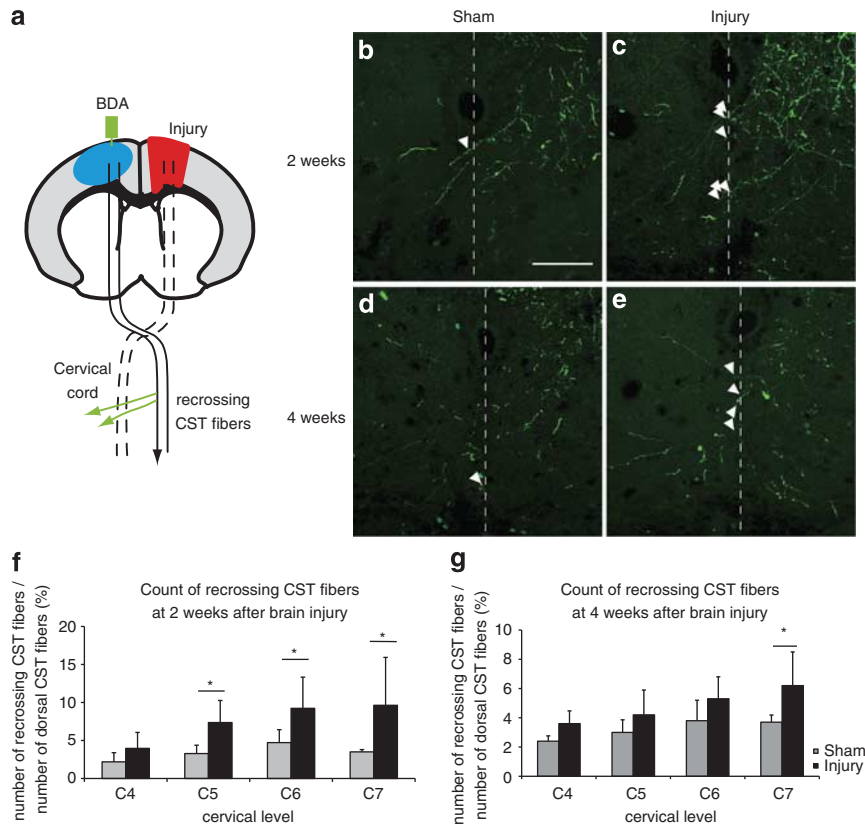


Figure 4 Increased number of midline-crossing CST fibers in the cervical spinal cord after traumatic brain injury. (a) Illustration of midline-crossing (recrossing) CST fibers after cortical injury. BDA (green) was injected into the contralesional cortex (blue) to label the CST. The BDA-labeled CST fibers sprouted and recrossed to the denervated side of the cervical spinal cord after injury (green arrows). The recrossing fibers were counted from C4 to C7. (b–e) Photographs of recrossing CST fibers labeled with BDA (green) at the C7 level in the sham (b and d) and injured (c and e) mice at 2 (b and c) and 4 (d and e) weeks after the injury (Z stack, each 0.5 μm , by confocal laser-scanning microscopy). The recrossing CST fibers were marked with arrowheads. Scale bar: 50 μm . (f and g) Quantification of the recrossing CST fibers at each cervical level 2 (f) and 4 (g) weeks after the injury. The fibers increased in the injured group (black bars) compared with the sham group (gray bars). The data represent the mean \pm S.D. (2 weeks: sham $n=6$, injury $n=4$, 4 weeks: sham $n=5$, injury $n=4$); * $P < 0.05$ (Mann–Whitney U -test)

remodeling such as dendritic arborization and spine formation occurs in periinfarct areas.^{15,16} Dendritic spine formation significantly increases, peaking at 1–2 weeks, and persists for up to 6 weeks in the periinfarct cortex.¹⁵ Further, sprouting of efferent fibers from the contralesional cortex increases on the denervated side of subcortical areas such as the red nucleus and cervical spinal cord after brain injury.^{4,5,17} Interestingly, rehabilitation and neuronal activity can contribute to promotion of these structural changes.^{18–20} Hence, neuronal activity might be the key regulator of axonal growth, branch initiation, and neuronal reorganization.^{21,22} In this case, it is conceivable that downregulation of GABA signaling might facilitate the activation of neurons and following plastic changes in our model.

Spontaneous recovery after the cortical injury was observed to the large extent during the early phase (up to a week) in the grid walk test, whereas that was observed during the late phase in the cylinder test (Figures 3a and b). Although there were significant differences in the improvement of motor functions between muscimol-treated and vehicle-treated mice, the time points, when the significantly suppressed functional recovery was observed in the muscimol-treated mice, were different in these two tests (Figures 3c and d). The effect of muscimol appears to be significant when the

spontaneous recovery rate of the function was maximal in these tests. Therefore, downregulation of GABAergic signaling in the contralateral side cortex may be required for the specific phase of the functional recovery. Reorganization of multiple tracts, including CST, corticorubro tract, corticoreticulo tract, and cortico-cortico pathways, is considered to be required for the functional recovery after the cortical injury. Thus, reorganization of other tracts, other than the CST may work for the functional recovery during the time course when we observed no significant effect of muscimol treatment in each functional test.

GABA is the major inhibitory neurotransmitter controlling the excitation and activity of neurons. The major type of GABAR in vertebrate neurons is a member of the ligand-gated ion channel family of receptors. The GABA_AR forms a heterooligomeric complex assembled from a range of homologous subunits sharing a common structure. To date, 18 GABA_AR subunits have been identified.²³ The local GABA circuits target specific subdomains on nearby pyramidal cells, such as the somaproximal dendritic region or axonal initial segment.^{24,25} The somaproximal dendritic region but not the axonal initial segment is rich in the GABA_AR- $\alpha 1$ subunit, which is located on the opposite side of synaptic boutons of parvalbumin-positive GABAergic neurons.^{24,26} In addition,

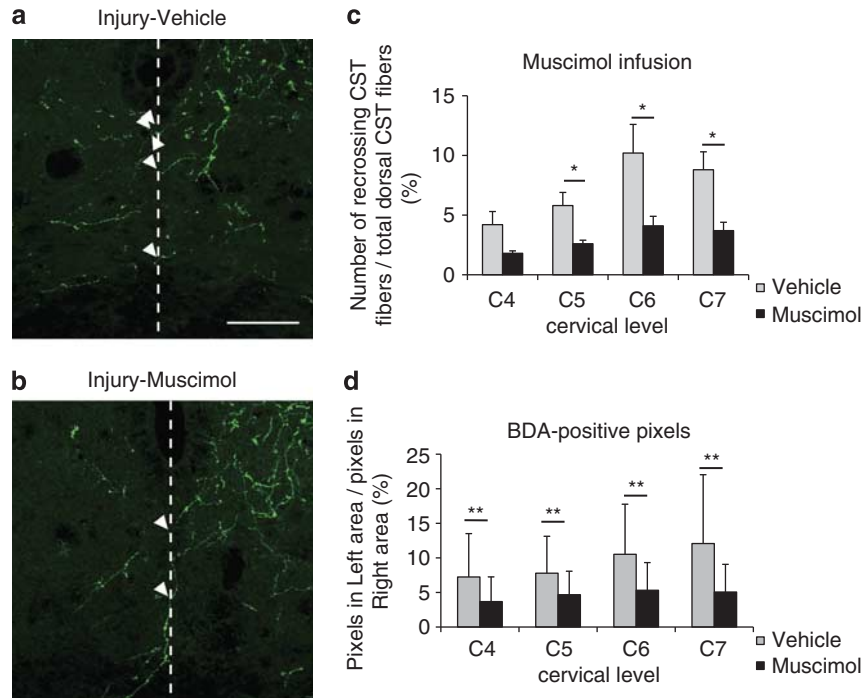


Figure 5 Sprouting of CST fibers is inhibited by muscimol infusion in the contralesional cortex after brain injury. **(a and b)** Photographs of the recrossing CST fibers labeled with BDA at the C7 level in the saline-treated **(a)** or muscimol-treated **(b)** mice (Z stack, each 0.5 μm , by confocal laser-scanning microscopy). The recrossing CST fibers were marked with arrowheads. Scale bar: 50 μm . **(c and d)** Quantification of the number of recrossing CST fibers **(c)** and their positive areas in the denervated cervical spinal cord **(d)** at each cervical level in the saline-treated (gray bars) and muscimol-treated (black bars) mice at 4 weeks after the injury. Muscimol infusion decreased the number of sprouting axons into the denervated side. The data represent the mean \pm S.D. ($n = 5$, each group); * $P < 0.05$, ** $P < 0.01$ versus the saline-treated mice (Mann-Whitney U -test)

the GABA_AR at the somaproximal dendritic region may be more effective to inhibit neuronal activation than inhibition at the dendritic shaft.²⁷ Hence, downregulation of the GABA_AR- α 1 subunit would decrease the inhibitory signal to glutamatergic neurons, resulting in enhancement of excitatory signals.

Changes in GABA_AR- α 1 gene expression have been demonstrated in other animal models of epilepsy, alcohol abuse and withdrawal, and swim stress.^{28–31} In neocortical neurons, activation of protein kinase C increases GABA_AR- α 1 subunit expression via phosphorylation of the cAMP-response element-binding protein bound to the GABA_AR- α 1 subunit promoter.³² On the other hand, decreased GABA_AR- α 1 expression depends on the inducible cAMP early repressor.³² Another report indicated that the Janus kinase/signal transducer and activator of transcription pathway regulated by the brain-derived neurotrophic factor mediate seizure-induced decrease of GABA_AR- α 1 subunit expression in epilepsy.³³ Although the mechanism of downregulation in GABA_AR- α 1 expression is still unclear in our brain injury model, these neuronal activity-related genes might be involved.

Revealing the mechanism of spontaneous compensatory motor recovery on a neural basis is important to develop new therapies for brain injury. Our study reveals that altering GABAergic signaling may be a good therapeutic target to treat patients with brain injury.

Materials and Methods

Surgical procedures for traumatic cortical injury. All experiments were conducted in accordance with the Osaka University Medical School Guide for

the Care and Use of Laboratory Animals and were approved by the institutional committee of Osaka University. Eight-week-old male C57BL/6J mice (Charles River Japan, Osaka, Japan) were anesthetized with intraperitoneal sodium pentobarbital (Somnopentyl, 50 mg/kg body weight; Kyouritu Seiyaku, Tokyo, Japan) and then individually placed in a stereotaxic frame for surgery. After a midline incision of the scalp, circular craniotomy (4 mm in diameter) was performed over the right parietal cortex with the center at 0 mm anteroposterior and 2 mm lateral to the bregma. The animal was positioned in a head holder and then subjected to traumatic cortical injury by using a pneumatic cortical impact device (AmScien Instruments, Richmond, VA, USA) with a 3-mm convex tip oriented perpendicular to the cortical surface.³⁴ The impact parameters were 4-mm/ms velocity, 1-mm depth, and 120-ms total time. The injured area covered primary forelimb and hindlimb motor area, premotor area, and sensory area of mouse cortex.³⁵ Sham-operated mice received a circular craniotomy, and the parietal cortex was exposed. The scalp was then sutured and the animal was allowed to awaken from the anesthesia. The mice were maintained according to a 12-h light/dark cycle with ad libitum access to food and water.

Quantitative real-time RT-PCR. Total ribonucleic acid (RNA) was extracted from the contralesional cortex ($n = 3$) by using TRIzol reagent (Molecular Probes, Invitrogen, Eugene, OR, USA) at 7 days after the injury and was reverse-transcribed for first-strand complementary (cDNA) synthesis with an oligo(dT) primer and SuperScript II RNase H-reverse transcriptase (Invitrogen). Real-time PCR was performed with oligonucleotide primer sets corresponding to the cDNA sequences of the GABA_AR subunits (α , β , γ , and δ), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Table 1), and alivin-1 (Supplementary Table 1). In brief, 20 μl of a reaction mixture containing 10- μl SYBR Green real-time PCR master mix (Applied Biosystems, Warrington, UK), 1–2 μl each of the sense and anti-sense primers (10 μM), and 1 μl of the cDNA sample (10 ng/ μl) was preheated at 95°C for 3 min and then treated with 40 cycles of amplification (denaturation at 95°C for 15 s, annealing and extension at 60°C for 1 min) in an ABI PRISM 7900HT sequence detection system (Applied Biosystems). The relative intensity against GAPDH and the fold change relative to the control were calculated.

Table 1 Primer sequences for real time RT-PCR

Name		Primer sequence (5'–3')
Gabra1	Forward	CAGAAAAGCCAAAGAAAGTAAAGGA
	Reverse	TGGTTGCTGTAGGAGCATATGTG
Gabra2	Forward	CCAGTCAATTGGGAAGGAACA
	Reverse	TTTCAAGTGGAAATGAGCTGTCA
Gabra3	Forward	GCTGCTCAGACTGGTAGATAATGG
	Reverse	GGGCATTGACGCTGTATTGTT
Gabra4	Forward	GGCAGACTGTATCAAGCGAGACT
	Reverse	GGTGAAGTAAACCGTCATAACAA
Gabra5	Forward	GCAGACAGTAGGCACTGAGAACA
	Reverse	GGAAAGTGAAGTCAATGATCGTAT
Gabra6	Forward	CCAGCTTTGGACCTGTGTCA
	Reverse	TGGCGGAAGAAAACATCCAT
Gabbr1	Forward	TCGATGTCGCCAGCATAGAC
	Reverse	TGCTGAAATACATGGTGAGTGT
Gabbr2	Forward	GCATCCCGATGGCACTGT
	Reverse	CATGCAGGCAGCCGTAGTT
Gabbr3	Forward	CTCCACCCTGATGGGACAGT
	Reverse	CATGCACGCTGCTGTCTGTA
Gabbrd	Forward	TGACCATATCTCAGAGGCAAACA
	Reverse	CCGCCAGCTCTGATGCA
Gabrg1	Forward	TTACTAACCCCTGCATCTGGGAAA
	Reverse	CAAGACCATGTTTTGTTTCATAGTTAA
Gabrg2	Forward	CCTGCCCCCTGGAGTTCT
	Reverse	ACTGCGCTTCCATTGATAAACA
Gabrg3	Forward	AACAGCATTGGTCTGTGTCTAT
	Reverse	TGCTCCAGCATATAATGGCATT
Gapdh	Forward	GGGCTGGCATTGCTCTCA
	Reverse	TGTAGCCGTATTTCATTGTCATACCA

Western blot analysis. At 7, 14, and 28 days after the injury, some mice ($n = 6$) were deeply anesthetized and transcardially perfused with chilled saline. Their brains were rapidly removed and the cortical regions contralateral to the injured site ($3 \times 3 \text{ mm}^2$) were sampled. The samples were frozen in liquid nitrogen and stored -80°C until use. Plasma membrane fractionation for detecting GABA_AR- $\alpha 1$ subunits was conducted with a plasma membrane protein extraction kit (BioVision, Mountain View, CA, USA). The samples were separated on 10% sodium dodecyl sulfate-polyacrylamide gels with 4.5% stacking gel. Protein was then electrotransferred onto nitrocellulose membranes. The membranes were incubated for 1 h in 0.01 M phosphate-buffered saline (PBS) containing 5% non-fat dry milk at room temperature. They were rinsed briefly in 0.05% Tween 20 in PBS and then incubated overnight at 4°C with rabbit polyclonal anti-GABA_AR- $\alpha 1$ antibody (1 : 2000; Covance, Emeryville, CA, USA) and mouse monoclonal anti-neural cell adhesion molecule (NCAM) antibody (1 : 1000; Sigma, St. Louis, MO, USA). Horseradish peroxidase-conjugated secondary antibody (1 : 5000; Cell Signaling Technology, Danvers, MA, USA) and an ECL chemiluminescence system (GE Healthcare, Buckinghamshire, UK) were used for detection.

Histological and immunohistochemical analyses. At 7, 14, and 28 days after the injury, some mice ($n = 3$) were deeply anesthetized and then transcardially perfused with 4% paraformaldehyde (PFA) in PBS. Their brains and cervical spinal cords were removed and fixed in 4% PFA at 4°C overnight and then cryoprotected in 30% sucrose for 3 days. The tissues were embedded in optimal cutting temperature compound (Tissue-Tek, SAKURA Finetek, Tokyo, Japan) and stored at -80°C . Sections of the brain and spine ($20\text{-}\mu\text{m}$ thickness) were cut coronally on a cryostat and mounted on Matsumami adhesive silane-coated slides (Matsumami Glass, Osaka, Japan). The sections were rinsed in PBS, blocked in 0.1% Triton X-100 and 5% bovine serum albumin (BSA) in PBS for 1 h, and incubated with rabbit polyclonal GABA_AR- $\alpha 1$ antibody (1 : 100; Covance) in 5% BSA in PBS overnight at 4°C . They were then washed with PBS and incubated with Alexa Fluor 488 or 568 conjugated goat anti-rabbit immunoglobulin G (1 : 100; Invitrogen) in 5% BSA in PBS for 1 h. The sections were counterstained with NeuroTrace Nissl stain (1 : 100, Invitrogen) for neuronal markers. All the sections were observed by fluorescence microscopy (Olympus BX51, DP71, Olympus, Tokyo, Japan) or confocal laser-scanning microscopy (Olympus FluoView FV1000, Olympus). For semiquantitative image analysis of GABA_AR- $\alpha 1$ subunits, we used a modified method of Redecker *et al.*⁷ In brief, measure of relative optical density of

GABA_AR- $\alpha 1$ subunit staining was performed on one section per animal. Three areas of each layer in the forelimb region of the contralesional motor cortex were photographed by using a confocal laser-scanning microscope (magnification, $\times 600$) and the images were processed with ImageJ (National Institutes of Health, Bethesda, MD, USA).

For analysis of the cortical lesion volume, tissue sections were obtained at every $100 \mu\text{m}$ and stained with Nissl stain (cresyl violet; Sigma) or hematoxylin and eosin. Each section was digitalized by using a charge-coupled device camera and the cavity area in each section was measured by using ImageJ. On each image, the cavity was outlined with the polygon selection tool by visual inspection, with the dorsal aspect of the lesion estimated from mirror image of the contour of the uninjured hemisphere. The total volume of the cortical cavity was calculated by integrating the measured area from each section.³⁴ The average volume was $6.28 \pm 2.0 \text{ mm}^3$.

Anterograde labeling of the CST. At 0 or 2 weeks after the injury, BDA (10% in PBS, molecular weight 10 000; Invitrogen) was injected into the left motor cortex ($1.2 \mu\text{l}$ per cortex; 0–0.5 mm anterior and 1–1.5 mm lateral to the bregma, 0.5-mm depth) under anesthesia to label CST fibers. During each injection, $0.2 \mu\text{l}$ of BDA was delivered for 30 s via a glass capillary (inner diameter, 15–20 μm) attached to a microliter syringe (ITO, Shizuoka, Japan). Fourteen days after BDA injection, the animals were sacrificed by perfusion with 4% PFA. The cervical spinal cords were sampled, post-fixed overnight in the same fixatives, and cryopreserved in 30% sucrose in PBS. They were embedded in TissueTek OCT compound (SAKURA Finetek). The blocks were sectioned coronally (20 μm) from vertebrae C4 to C7. The sections were incubated in 0.3% Triton X-100 in PBS for 4 h and then incubated for 2 h with Alexa Fluor 488-conjugated streptavidin (1 : 400; Invitrogen) in PBS at room temperature.³⁶

Muscimol treatment. Supplementary Figure 1 illustrates the experimental procedures for the muscimol treatment. A stainless steel cannula (Brain infusion kit 3; Alzet, Cupertino, CA, USA) connected to a microsmotic pump (model 1004; Alzet) was implanted in the forelimb area of the left motor cortex (1.0 mm anterior and 1.0 mm lateral to the bregma, 0.65-mm depth from the cortical surface) at 3 days after the injury. For reliable infusion, the cannula was implanted slightly deeper than BDA infusion, because the cannula is thicker than the glass capillary for BDA infusion. Muscimol (10 mM in saline; Sigma), a GABA R agonist, was infused continuously for 7 days (days 3–10; $0.11 \mu\text{l/h}$).^{37,38} Saline (Otsuka, Tokyo, Japan) was administered as a control.³⁹ To assess the histological changes caused by muscimol infusion, cryosections (20 μm) of the infused cortex (1 mm anterior and 1 mm lateral to the bregma) were stained with hematoxylin and eosin (Supplementary Figure 1D).

Grid walk test. The grid walk test assesses the ability to place the forepaws accurately on the rungs of a grid during spontaneous exploration. The mice were placed on a grid of $0.24 \times 0.2 \text{ m}$ (length \times width) with 10-mm^2 openings to explore freely for 4 min. Step errors were videotaped with a camera placed underneath the grid. We counted a step failure when the limb did not bear the body weight and the foot slipped through the grid hole.⁴⁰ The percentage step failure was calculated by the formula (step failure/50 steps) $\times 100$. The test was performed once a day from a day to 4 weeks after the injury.

Cylinder test. The cylinder test evaluates forelimb use during spontaneous vertical exploration within a cylinder. The mice were placed in a cylinder (115-mm height and 80-mm diameter), and the number of times each forelimb or both forelimbs were used to support the body on the wall of the cylinder was counted. Two mirrors were set behind the cylinder to view all directions. The behaviors were videotaped and the total behaviors of 20 tests were scored.¹⁶

Quantification of midline-crossing CST fibers. The number of BDA-labeled midline-crossing CST fibers in the cervical spinal cord was compared between the sham and the injured mice with saline and muscimol treatment, respectively. For quantification, the number of fibers crossing the midline (see Figures 4a–e; arrowheads) was counted by using a fluorescence microscope (final magnification, $\times 400$) in 20 cross-sections (20 μm) per cervical (C4–C7) level. To correct variations in BDA uptake of CST neurons in the motor cortex among the animals, the number of midline-crossing axons was normalized by the number of BDA-labeled dorsal CST fibers, the main pyramidal tract, in the first section at the C4 level, photographed by confocal laser-scanning microscopy (magnification, $\times 600$). The results were calculated as the percentage of the total number of dorsal

CST fibers. To quantify the density of crossing CST fibers, 10 cross-sections at each cervical (C4–C7) level were photographed (magnification, $\times 100$) and the BDA-positive areas in the affected and contralesional gray matter were measured by using ImageJ. These results were calculated as the ratio of positive areas in the affected (denervated) gray matter to those in the contralesional gray matter.

Statistical analysis. All data represent the mean \pm S.D. For mRNA and protein expressions, and data of BDA-labeled crossing CST fibers, non-parametric Mann–Whitney *U*-test was used for comparisons of groups. All behavioral scores were evaluated using a two-way repeated measure ANOVA to detect significant differences between and among treatment groups; Tukey–Kramer *post hoc* analysis was used to determine where those differences occurred. $P < 0.05$ was considered significant.

Conflict of interest

The authors declare no conflict of interest.

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