

Involvement of Denervated Midbrain-Derived Factors in the Formation of Ectopic Cortico-Mesencephalic Projection after Hemispherectomy

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Neuronal remodeling after brain injury is essential for functional recovery. After unilateral cortical lesion, axons from the intact cortex ectopically project to the denervated midbrain, but the molecular mechanisms remain largely unknown. To address this issue, we examined gene expression profiles in denervated and intact mouse midbrains after hemispherectomy at early developmental stages using mice of either sex, when ectopic contralateral projection occurs robustly. The analysis showed that various axon growth-related genes were upregulated in the denervated midbrain, and most of these genes are reportedly expressed by glial cells. To identify the underlying molecules, the receptors for candidate upregulated molecules were knocked out in layer 5 projection neurons in the intact cortex, using the CRISPR/Cas9-mediated method, and axonal projection from the knocked-out cortical neurons was examined after hemispherectomy. We found that the ectopic projection was significantly reduced when integrin subunit β three or neurotrophic receptor tyrosine kinase 2 (also known as TrkB) was knocked out. Overall, the present study suggests that denervated midbrain-derived glial factors contribute to lesion-induced remodeling of the cortico-mesencephalic projection via these receptors.

Key words: axon sprouting; brain injury; circuit remodeling; corticorubral; plasticity

Significance Statement

After brain injury, compensatory neural circuits are established that contribute to functional recovery. However, little is known about the intrinsic mechanism that underlies the injury-induced remodeling. We found that after unilateral cortical ablation expression of axon-growth promoting factors is elevated in the denervated midbrain and is involved in the formation of ectopic axonal projection from the intact cortex. Evidence further demonstrated that these factors are expressed by astrocytes and microglia, which are activated in the denervated midbrain. Thus, our present study provides a new insight into the mechanism of lesion-induced axonal remodeling and further therapeutic strategies after brain injury.

Introduction

Neural circuits are reorganized after brain injury. One mechanism for the reorganization is compensation by new axonal

growth into the denervated brain region from external sources. It is well known that compensatory axonal projections are formed after cortical lesion. Fundamentally, layer 5 neurons in the motor cortical area project ipsilaterally to the brain stem and contralaterally to the spinal cord. However, after a unilateral cortical lesion, descending axons from the intact cortex sprout and project contralaterally to the midbrain and hindbrain, and ipsilaterally to the spinal cord (Tsukahara, 1981a; Takahashi et al., 2009; Benowitz and Carmichael, 2010). Lesion-induced contralateral projection to midbrain nuclei such as the red nucleus (RN) is particularly well characterized (Nah and Leong, 1976; Tsukahara, 1981b; Lee et al., 2004; Omoto et al., 2010). Functional compensation can also be achieved in the adult brain by this new projection but is insufficient for complete functional recovery. Therefore, enhancing the formation of axonal sprouting is one of the main strategies to overcome incomplete recovery after CNS injury.

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Molecular manipulations have been proposed to facilitate the ectopic contralateral projection, by various methods such as nullifying the myelin-associated axon growth inhibitor NogoA or Nogo Receptor (Lee et al., 2004; Cafferty and Strittmatter, 2006; Sato et al., 2011), degrading CSPG (Starkey et al., 2012), or overexpressing growth-promoting transcription factors such as Stat3 (Lang et al., 2013) or Klf7 (Blackmore et al., 2012). On the other hand, the formation of compensatory circuits has been shown to be robust at infant developmental stages (Tsukahara et al., 1983; Kosar et al., 1985; Murakami and Higashi, 1988; Kuang and Kalil, 1990; Omoto et al., 2010, 2011). Therefore, activation of intrinsic mechanisms may promote the growth of ectopic projections and functional recovery. So far, trophic factors and axon growth regulatory components have been shown to contribute to compensatory connections in the spinal cord (Ueno et al., 2012; Fink et al., 2017). However, the molecular mechanisms are not fully understood. Moreover, the intrinsic mechanisms underlying formation of lesion-induced cortico-mesencephalic projections are completely unknown.

In the present study, we addressed this issue by hypothesizing that some attractive or promoting factors governing the formation of the lesion-induced ectopic contralateral projection are released from the denervated region. First, we investigated the time course of the ectopic cortico-mesencephalic projections following hemispherectomy (the removal of one hemisphere) of juvenile mice. Next, lesion-induced gene expression in the midbrain was analyzed using RNA-seq. Finally, we attempted to identify the molecules that underlie the ectopic contralateral projection by means of specific gene knock-out (KO) using CRISPR/Cas9 and *in vivo* gene transfer methods.

Materials and Methods

Animals

ICR mice (Japan SLC) of either sex were used in this study. All experiments were conducted under the guidelines for laboratory animals of the Graduate School of Frontier Biosciences, Osaka University. The protocol was approved by the School's Animal Care and Use Committee.

Hemispherectomy

At postnatal day (P)6, mice were anesthetized with isoflurane (Wako). After cutting the skin, a small window ($\sim 2 \times 2$ mm) was made using micro-scissors on the right side of the skull. In most cases, the right hemisphere was completely removed using an aspirator. After the ablation, the pups recovered on a heating pad and were then returned to their mother. For sham operation, only the skin was cut. In all figures, the left side was set to the denervated side.

Anterograde axonal labeling using 1,1',3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)

Mice were killed 2, 4, or 7 d after hemispherectomy, and whole brains were fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.4). Total of 10 DiI crystals (Invitrogen) were implanted into the frontal and parietal cortical areas of hemispherectomized and control brains. After incubation for four to five weeks in 4% PFA at 37°C, 200- μ m thickness coronal sections were cut by a vibratome (DTK-1000; Dosaka). The sections were mounted in PBS under coverslips for microscopic observation (see below).

Axon labeling by *in utero* electroporation

In utero electroporation was performed on embryonic day (E)12.5 mouse embryos, according to previous studies (Fukuchi-Shimogori and Grove, 2001; Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001). In brief, a pregnant mouse was anesthetized with isoflurane (Wako) using inhalation anesthesia equipment (KN-1071-1; Natsume). Approximately 1 μ l of plasmid solution was injected into the lateral ventricle of the left hemisphere using a glass capillary and an injector (IM-30; Narishige).

Cortical axons were labeled with pCAGGS-EGFP (0.5 μ g/ μ l; Hatanaka and Murakami, 2002), and electrical pulses (37 V with five 50-ms pulses at intervals of 950 ms) were then applied with tweezer electrodes (LF650P1; BEX) connected to an electroporator (CUY21; BEX).

Hemispherectomy was performed at P6 in these electroporated mice. After surgery, they were deeply anesthetized and perfused with PBS followed by 4% PFA in 0.1 M PB. To visualize axons, the brains were postfixed with the same fixative overnight, and 200- μ m thickness coronal sections were cut by a vibratome (DTK-1000; Dosaka). The sections were permeabilized with 1% Triton X-100 for 1 h at room temperature (RT), and blocked with blocking solution (5% normal goat or donkey serum, 0.3% Triton X-100 in PBS) for 1 h at RT. They were incubated with rat monoclonal anti-GFP (1:2000, GF090R; Nacalai Tesque) in the blocking solution at 4°C overnight. After extensive washes, the sections were incubated with appropriate secondary antibodies at 4°C overnight. After washing, the slices were mounted in medium containing 2.3% DABCO, 1 μ g/ml DAPI and 50% glycerol for microscopic observation.

Sparse labeling of axons and tissue clearing

To label axons sparsely, the Supernova system was used (Mizuno et al., 2014; Luo et al., 2016). Supernova vectors pTRE-Flpe-WPRE (pK036) and pCAG-FRT-stop-FRT-tRFP-ires-tTA-WPRE (pK037) were kindly gifted from T. Iwasato, National Institute of Genetics, Japan. These vectors were co-transfected with pCAGGS-EGFP by *in utero* electroporation as described above. After surgery (see above), mice were perfused with PBS and 4% PFA in 0.1 M PB. The brain was postfixed overnight, and 1-mm coronal sections of the midbrain were cut by a vibratome (DTK-1000; Dosaka). Tissue clearing of these thick sections was performed using the SeeDB2 method as previously described (Ke et al., 2016). Briefly, sections were serially incubated in solutions with increasing concentrations of Omnipaque 350 (Daiichi-Sankyo), which contains 2% Saponin (Nacalai Tesque). Sections were imaged by confocal microscopy (TCS-SP5; Leica), and tiled images of the entire sections were acquired with a 10 $\times$ objective lens in 10- μ m steps. Approximately 100 images along z-axis were obtained, and individually labeled axons were traced and reconstructed using Simple Neurite Tracer, a plug-in for ImageJ.

Anterograde and retrograde labeling in organotypic cortical slice cultures

Organotypic cortical slice cultures were prepared as described previously (Yamamoto et al., 1989, 1992). In brief, ~ 300 - μ m-thick coronal slices were dissected from P1 mouse cortex. A block of the midbrain ($\sim 1 \times 1 \times 0.3$ mm) was dissected near the midline and ventral to the cerebral aqueduct from P8 brain with and without hemispherectomy. A cortical slice and the midbrain block were placed on collagen gel with DMEM/F-12 (Invitrogen) supplemented with 10% fetal bovine serum (FBS; HyClone). To make collagen gel, rat tail collagen was mixed with 10 $\times$ DMEM/F-12 (Invitrogen) at a ratio of 9:1 with one volume of 0.25% sodium bicarbonate. The mixed solution was put on a cell culture insert (Millicell-CM, PICMORG50; Millipore) and allowed to harden. The cultures were maintained for 24–48 h at 37°C in an environment of 5% CO₂ and humidified 95% air.

DiI crystals were implanted into the cortical slice for anterograde labeling or into the midbrain explant for retrograde labeling. DiI-labeled axons were imaged by confocal microscopy (ECLIPSE FN with EZ-C1; Nikon), and stack images were acquired with a 10 $\times$ objective lens in 5- μ m steps. Using ImageJ software, the number of axons crossing a line set to either 300 or 400 μ m from the ventricle edge was counted.

RNA-seq analysis

Midbrain tissue was collected from hemispherectomized and sham-operated mice at P8 and P10. Total RNA was extracted from the tissue using an RNeasy Plus Mini kit (QIAGEN), and sequencing was performed on an Illumina HiSeq 2500 (BGI Japan). Sequenced reads were mapped to the mouse reference using Bowtie2 (Langmead and Salzberg, 2012), and gene expression level was calculated with RSEM software (Li and Dewey, 2011). Differentially regulated genes were defined by false discovery rate (FDR) < 0.05 ($n = 2$). To explore cell type-specific expression profiles of upregulated genes, a public database (<https://www.>

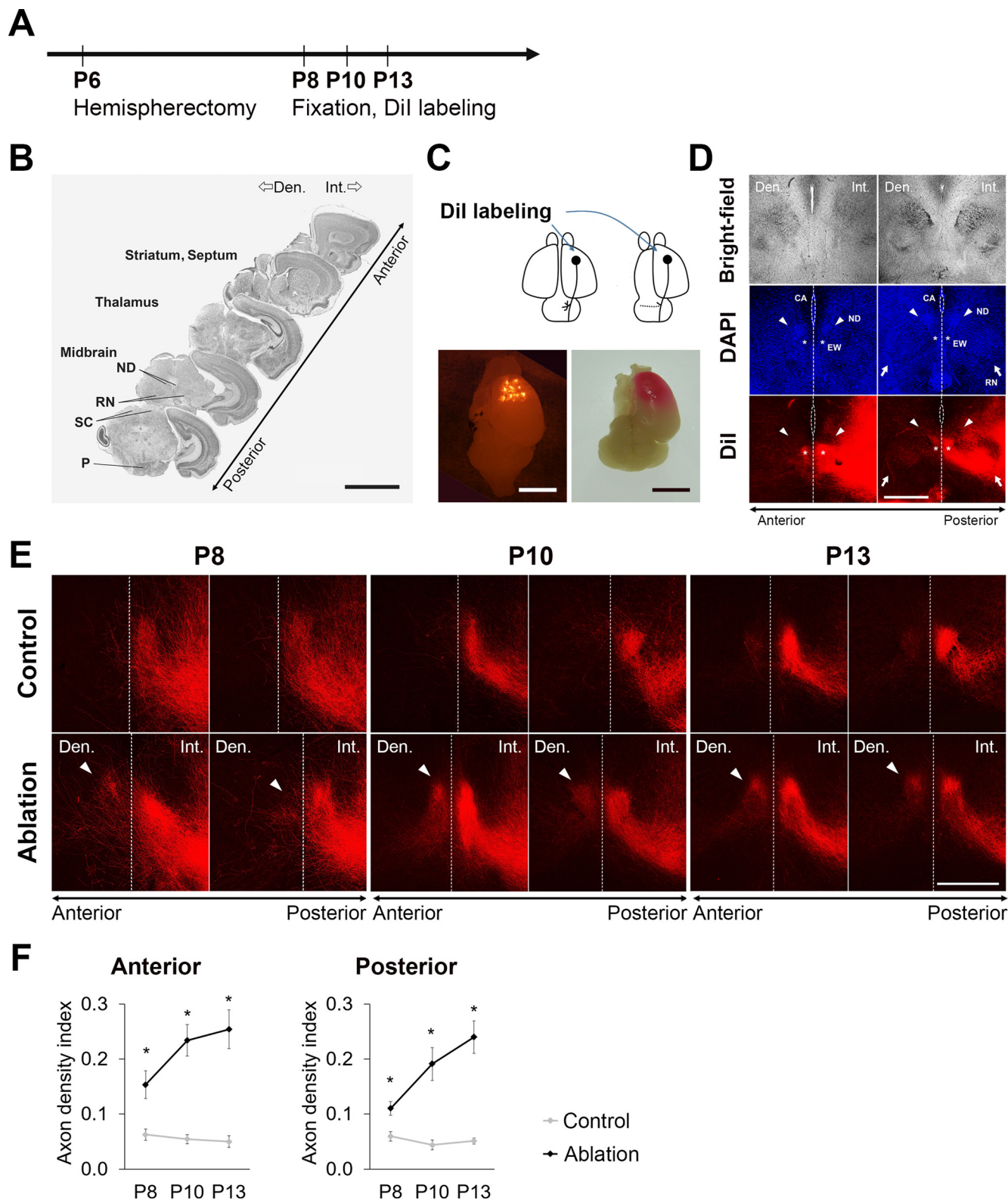


Figure 1. Midline-crossing cortico-mesencephalic axons increase after hemispherectomy. **A**, Schematic overview of the experiment. **B**, Representative coronal sections of hemispherectomized brain. SC, superior colliculus; ND, nucleus of Darkschewitsch; RN, red nucleus; P, pons; Den., denervated side; Int., intact side. Scale bar: 3 mm. **C**, Whole brain after hemispherectomy. Dil was implanted into fixed brains to label cortical axons. Scale bar: 2 mm. **D**, Dil-labeled cortical axons in the midbrain with hemispherectomy. Asterisks and arrows indicate the EW and RN, respectively, and arrowheads indicate the ND. Note that axons accumulate near the midline (dashed lines) in the Edinger–Westphal nucleus (EW) and in the region ventral to the ND. CA, cerebral aqueduct. Scale bar: 1 mm. **E**, Serial coronal sections showed that the contralateral projection to the midbrain starts to emerge only 2 d after surgery (P8) and increases at 4–7 d after surgery (P10, P13) compared with the control. Scale bar: 500 μ m. Dashed lines indicate the midline. **F**, Quantitative analysis using the axon density index (see Materials and Methods) of anterior and posterior sections ($n = 4$ for each group) confirmed a considerable increase in ectopic contralateral projection after hemispherectomy; $*p < 0.05$ against control; Mann–Whitney U test.

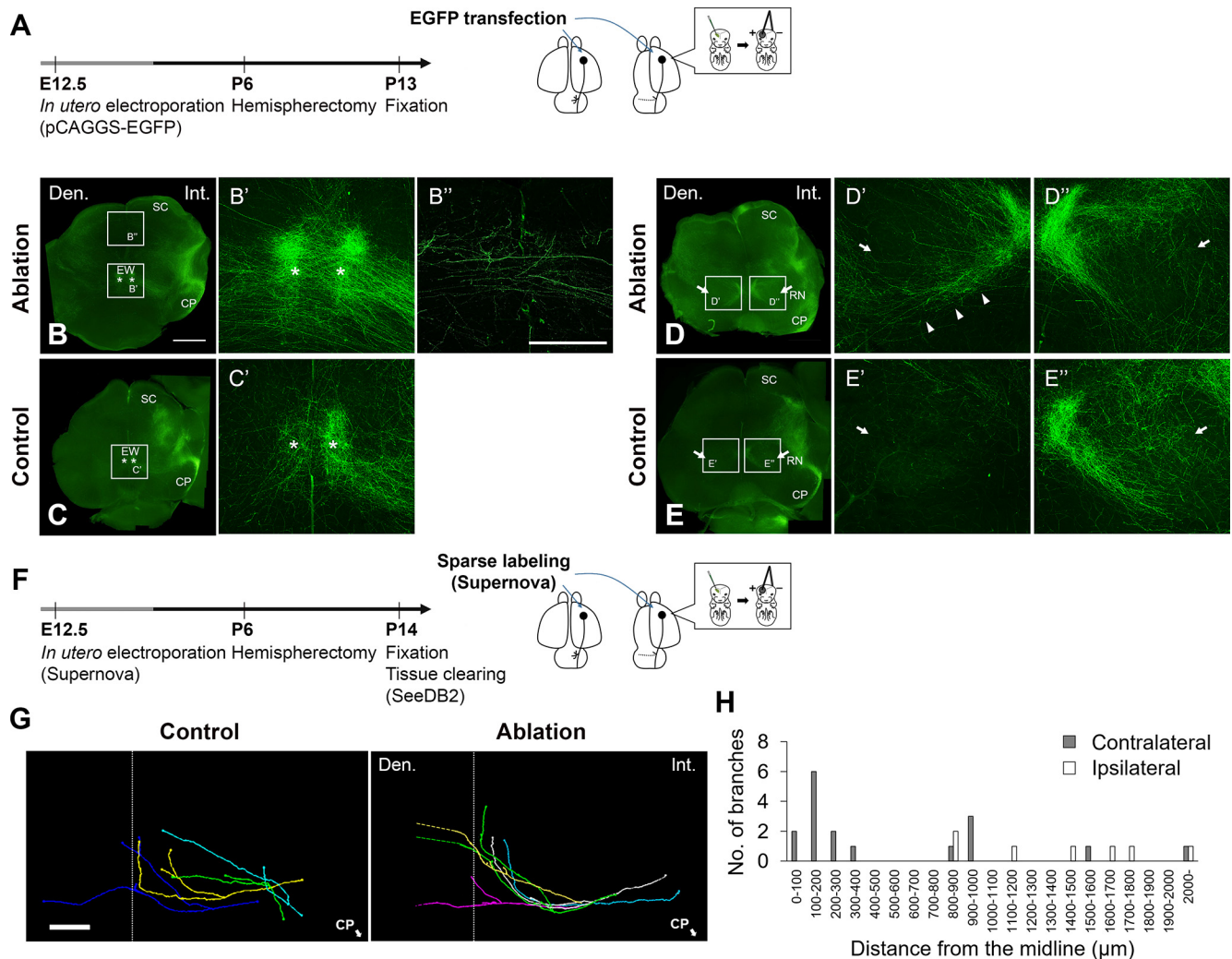


Figure 2. Contralateral cortical axons accumulate in midbrain nuclei 7 d after hemispherectomy. **A**, Schematic overview of the experiment. *In utero* electroporation-mediated transfection with EGFP was performed at E12.5 to label layer 5 cortical neurons. **B–E**, Coronal section images of hemispherectomized and control samples at P13 (7 d postsurgery). Left panels show low-magnification images of midbrain coronal sections, and right panels show magnified views of the indicated boxed areas of the left panels. After hemispherectomy, contralaterally projecting axons were found in the vicinity of the EW (asterisks), while only few midline-crossing axons were present in the control (**B**, **C**). Some of them were present more dorsally, near the SC. Bilateral projections to the RN (arrows) were formed after hemispherectomy in the posterior sections (**D**, **E**). CP, cerebral peduncle. Scale bar: 1 mm (left panels) and 500 μ m (right panels). **F**, Schematic overview of the experiment. Layer 5 neurons were sparsely labeled by the Supernova method. Tissue clearing was performed in thick (1-mm thickness) midbrain sections to trace labeled axons. **G**, Representative axon trace images of control and hemispherectomized samples. Branches of contralaterally projecting axons formed in the region adjacent to the midline (arrowheads). White lines indicate the midline, and the location of the CP is indicated by the arrows. Scale bar: 500 μ m. **H**, Histogram showing the distribution of the distance between midline and branch points. Note that contralaterally projecting axons form branches within 300 μ m of the midline, while both contralaterally and ipsilaterally projecting axons have branches in the region >800 μ m away from the midline ($n = 7$ axons for each group).

brainrnaseq.org; Zhang et al., 2014) was used, and genes with fragments per kilobase of transcript per million mapped reads (FPKM) > 10 were defined as being expressed by that cell type.

In situ hybridization

In situ hybridization was performed as previously described (Liang et al., 2000; Zhong et al., 2004). To prepare RNA probes, cDNA was synthesized from P8 mouse total RNA, and the DNA fragments of genes of interest were amplified by PCR with a pair of primers. The primer pairs were mostly designed based on the Allen Mouse Brain Atlas (Lein et al., 2007). Each cDNA fragment was then cloned into the pGEM-T vector. To produce linearized templates, the inserts were PCR-amplified with primers containing T7 and SP6 promoter sequences (TTGTAAAACGACGGCCAGT and TGACCATGATTACGCCAAGC). DIG-labeled RNA probes were then synthesized (DIG RNA Labeling Mix, Roche) following the manufacturer's instructions.

Mouse brains were fixed with 4% PFA in 0.1 M PB at 4°C and cryopreserved with 30% sucrose in PBS. The brains were sectioned into

20- μ m-thick coronal sections using a cryostat. The sections were subjected to re-fixation and acetylation. After prehybridization, they were hybridized with the DIG-labeled probe (4 μ g/ml) at 60°C. After washing, the sections were incubated with alkaline phosphatase (AP)-conjugated anti-DIG antibody (1:1000, 11093274910; Roche) at 4°C for 2 d. Finally, the hybridized probes were visualized with AP substrate (BM Purple, Roche) at RT.

CRISPR/Cas9-mediated gene KO in cortical neurons

To knock out specific genes, a plasmid containing humanized Cas9 and dual single-guide RNAs (sgRNAs) was made as previously described (px333; Maddalo et al., 2014), based on pX330-U6-Chimeric_BB-CBh-hSpCas9 (Cong et al., 2013). pX330-U6-Chimeric_BB-CBh-hSpCas9 was a gift from Feng Zhang (Addgene plasmid #42230; <http://n2t.net/addgene:42230>; RRID: Addgene_42230). The sequence of sgRNAs for each gene was chosen from the Brie sgRNA library (Itgb3: GCAGG TGGAGGATTACCCCG, AATATGGGTCTTGGCATCCG; Ntrk2: AT GACGTTGAAGCTTACGTG, AACCTGCAGATACCCAATTG; Doench

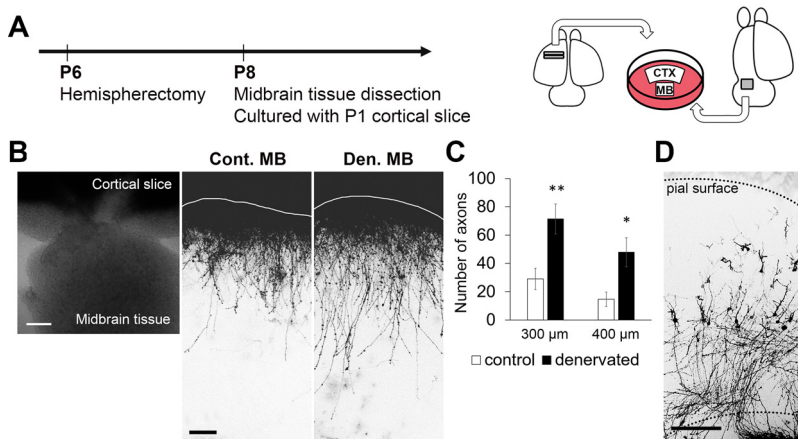


Figure 3. Coculture with denervated midbrain promotes axon growth of deep layer cortical neurons. **A**, Schematic overview of the experiment. Coculture of a cortical slice from intact P1 mice with a block of either the denervated midbrain 2 d after surgery or the same-aged midbrain without surgery. **B**, Representative images of coculture (left), and labeled cortical axons (right) when cultured with control midbrain (Cont. MB) and denervated midbrain (Den. MB). The white line indicates the edge of the ventricular side of the cortical slice. Scale bar: 200 μ m (left) and 100 μ m (right). **C**, Histogram showing the number of axons longer than 300 and 400 μ m, measured from the ventricular edge, in co-cultures with control ($n = 10$) or denervated midbrain ($n = 11$). Co-cultures with denervated midbrain showed a greater number of longer cortical axons; $*p < 0.05$, $**p < 0.01$ against control; Student's t test. **D**, Representative image of retrogradely labeled cells. Retrogradely labeled neurons were mostly found in the deep layers. Scale bar: 200 μ m.

et al., 2016). For genes that were not included in the database, sgRNAs were designed using CHOPCHOP (<http://chopchop.cbu.uib.no/>; Labun et al., 2019). The px333 plasmid designed to target a specific gene (3.5 μ g/ μ l) was electroporated together with pCAGGS-EGFP (0.5 μ g/ μ l) into the E12.5 mouse brain by *in utero* electroporation (see above). Transfection of with either the empty px333 vector or with pCAGGS-EGFP alone was performed as a control. After electroporation, hemispherectomy and visualization of cortico-mesencephalic axons were performed as described above.

Validation of KO efficiency

To validate KO efficiency, a primary cortical neuron culture was prepared as described previously (Kitagawa et al., 2017). After *in utero* electroporation at E12.5 (see above), pregnant mice were anesthetized again at E15 with pentobarbital (50 mg/kg, i.p.), and the GFP-positive area of the cerebral cortex was dissected from embryos in ice-cold HBSS. The tissue was then minced with fine scissors and incubated with 0.125% Trypsin-EDTA (Invitrogen) for 5 min and dissociated thoroughly by pipetting. After a brief centrifugation, the cells were resuspended in DMEM/F12 medium (Life Technologies) supplemented with 10% FBS and plated in four-well culture dishes (Thermo Scientific) coated with 0.1 mg/ml poly-L-ornithine (Sigma-Aldrich). The cultures were maintained at 37°C in an environment of 5% CO₂ and humidified 95% air, and were fixed after 2 d in 4% PFA in PBS at RT for 10 min. The cells were immunostained with rat anti-GFP (1:2000, GF090R; Nacalai Tesque) and goat anti-TrkB (1:250, AF1494; R&D Systems). Cells were extensively washed and incubated with appropriate secondary antibodies at 4°C overnight. Images were captured with a fluorescence microscope (IX71 with 10 $\times$ or 20 $\times$ objective lens; Olympus), and analyzed with ImageJ to measure fluorescence intensity.

Alternatively, Neuro2a cells were used for the validation. The cells were cultured in high glucose DMEM (Thermo Fisher Scientific) with 10% FBS, and transfected with plasmids (pCAGGS-EGFP and the px333 plasmids) using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. The culture was maintained until days in vitro (DIV)7, and the cell lysates were subjected to Western blot analysis (see below) to evaluate the expression level of the target gene. To estimate KO efficiency, the transfection efficiency (the ratio of the number of EGFP-positive cells to the total number of cells) was taken into account.

Immunohistochemistry and Nissl staining

For immunostaining of various molecules, the brains were perfused in 4% PFA in 0.1 M PB and postfixed with the same fixative for 4 h or

overnight. They were then equilibrated with 30% sucrose in PBS, frozen in OCT compound (Sakura Finetech), and sectioned at 20 or 50 μ m using a cryostat (CM1850; Leica Microsystems). The sections were permeabilized with 0.1% Triton X-100 for 1 h at RT and blocked for 1 h at RT in blocking solution. They were incubated with the following primary antibodies in blocking solution at 4°C overnight: mouse monoclonal anti-GFAP (1:1000, G3893; Sigma-Aldrich), rabbit polyclonal anti-GFAP (1:100, G9269; Sigma-Aldrich), rabbit polyclonal anti-Iba1 (1:500, 019–19741; Wako), goat polyclonal anti-Spp1 (1:50, AF808; R&D Systems), and rabbit anti-Plat (1:100, ASMTPA-GF-HT; Molecular Innovations). Sections were washed, incubated with appropriate secondary antibodies at 4°C overnight, and mounted. These brain sections were occasionally subjected to Nissl staining with cresyl violet.

Western blot analysis

Midbrain tissues from the denervated and intact sides were rapidly collected from hemispherectomized mice at P10 (4 d after the operation). The dissected tissues were homogenized in RIPA buffer containing a protease inhibitor cocktail (P8340; Sigma-Aldrich), and the supernatants were collected after centrifugation at 16,000 $\times g$ for 30 min. The protein concentrations of the supernatants were determined using a BCA Protein Assay kit (Thermo Fisher Scientific). The same amount of protein (25 μ g) of each sample was applied to SDS-PAGE using 15% polyacrylamide gels in the presence of β -mercaptoethanol. After electrophoresis, proteins were transferred to PDVF membrane (Bio-Rad) using a Mini Trans-Blot Cell (Bio-Rad). The membrane was blocked with Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and 5% skim milk (Nacalai Tesque) for 1 h at RT, and then incubated with mouse anti-BDNF antibody (1:1000, 327–100; Icosagen) in 2% skim milk in TBS-T at 4°C overnight (Kojima et al., 2020a). Primary antibody was detected by incubating the membrane with peroxidase-conjugated anti-mouse IgG antibody (1:10,000, 115-035-146; Jackson ImmunoResearch) in 2% skim milk in TBS-T for 2 h at RT. The signal was visualized by chemiluminescence with Immobilon Forte Western HRP substrate (Millipore), and imaged with an LAS-3000UV Mini (Fujifilm). The chemiluminescence intensities of the bands were analyzed with ImageJ software. As a loading control, the membrane was stained with Ponceau S (Cell Signaling Technology).

For Western blot analysis using Neuro2a cells, cells were scraped off the culture dish mechanically and homogenized in RIPA buffer. The same procedure as above was followed, with rabbit anti-itgb3 antibody (1:500, 13166; Cell Signaling Technology) and peroxidase-conjugated anti-rabbit IgG (1:10,000, 711-035-152; Jackson ImmunoResearch) as primary and secondary antibody, respectively.

Image analysis and quantification of cortico-mesencephalic axons

Images of labeled axons in the midbrain were obtained by confocal microscopy (ECLIPSE FN with EZ-C1; Nikon). For analysis, two consecutive 200- μ m thickness coronal sections were used (Fig. 1D). These sections are referred to as the anterior and posterior sections. For DiI-labeled samples, a total of 20–24 optical sections were acquired with a 10 $\times$ objective lens (1024 $\times$ 1024 pixels; 1273 $\times$ 1273 μ m) in 5- μ m steps. For *in utero*-electroporated samples, six to eight images were acquired using a 4 $\times$ objective lens with a 2 $\times$ digital zoom (1024 $\times$ 1024 pixels; 1591 $\times$ 1591 μ m) in 20- μ m steps. The projection images were thresholded using ImageJ (triangle method).

To evaluate the ectopic contralateral projection, the axon density index was defined by dividing the number of positive pixels on the contralateral (denervated) side by that of the ipsilateral (intact) side in each

region of interest (ROI). For DiI-labeled samples, the size of the ROI was 400×1024 pixels ($497 \times 1273 \mu\text{m}$), and the ROI was positioned at both sides adjacent to the midline. For EGFP-labeled samples, the size of the ROI on the ipsilateral side was 150×600 pixels ($233 \times 932 \mu\text{m}$), which includes the Edinger–Westphal nucleus (EW). The size of the ROI on the contralateral side was 600×700 pixels ($932 \times 1088 \mu\text{m}$), which contains a larger area of the denervated midbrain. A smaller ROI (450×450 pixels; $699 \times 699 \mu\text{m}$) was used to calculate the axon density index of the ventrolateral part of the denervated midbrain. To produce a pseudo-color heat-map image, the ROI of the contralateral side was divided into a grid pattern (12×14 grids, 50×50 pixels for each grid), and the axon density index was calculated for each grid. Samples in which axonal projection was rarely observed in the ipsilateral midbrain were excluded from the analysis.

Statistical analysis

All statistical values are presented as the mean $\pm$ SEM. Statistical analyses were performed using the Mann–Whitney *U* test, the Student's *t* test, and one-way ANOVA with Tukey's *post hoc* test. Differences between groups were considered to be significant at $p < 0.05$.

Results

Time course of ectopic contralateral cortico-mesencephalic projections after hemispherectomy

First, we examined the time course of ectopic contralateral cortico-mesencephalic projections after hemispherectomy (Fig. 1A). The ablation was performed at P6, when ectopic projections are known to form robustly (Takahashi et al., 2009; Omoto et al., 2010, 2011). As shown in Figure 1B, the unilateral cortical hemisphere was removed entirely with intact brain stem, although the striatum was partially lesioned in many cases. Axonal projections from the intact cortex were studied 2–7 d after hemispherectomy by implanting DiI crystals into the motor and somatosensory cortical areas (Fig. 1C). Only 2 d after the hemispherectomy, many labeled axons were found on the opposite side of the midbrain, although a small number of cortical axons also crossed the midline even without ablation (Fig. 1D,E). Sequential sections ($200\text{-}\mu\text{m}$ thickness) showed that midline crossing occurs at certain levels of the midbrain, where the EW and RN exist (Fig. 1D). This aspect was quantified in two sequential sections along the anterior–posterior axis by calculating the axon density index (see Materials and Methods), which is the ratio of the axon density in the contralateral side midbrain to that in the ipsilateral side. The axon density indices were ~ 2 -fold greater in both sections 2 d (P8) after hemispherectomy than those in the control without ablation (anterior: 0.063 ± 0.010 for control, 0.15 ± 0.025 for ablation, $p < 0.05$; posterior: 0.059 ± 0.009 , 0.11 ± 0.013 , $p < 0.05$, Mann–Whitney *U* test; Fig. 1E,F). At later times, more labeled axons were found in the contralateral side. The axon density indices were ~ 4 -fold (anterior: 0.054 ± 0.010 , 0.23 ± 0.029 , $p < 0.05$; posterior: 0.044 ± 0.009 , 0.19 ± 0.030 , $p < 0.05$, Mann–Whitney *U* test) and 5-fold (anterior: 0.050 ± 0.011 , $0.25 \pm$

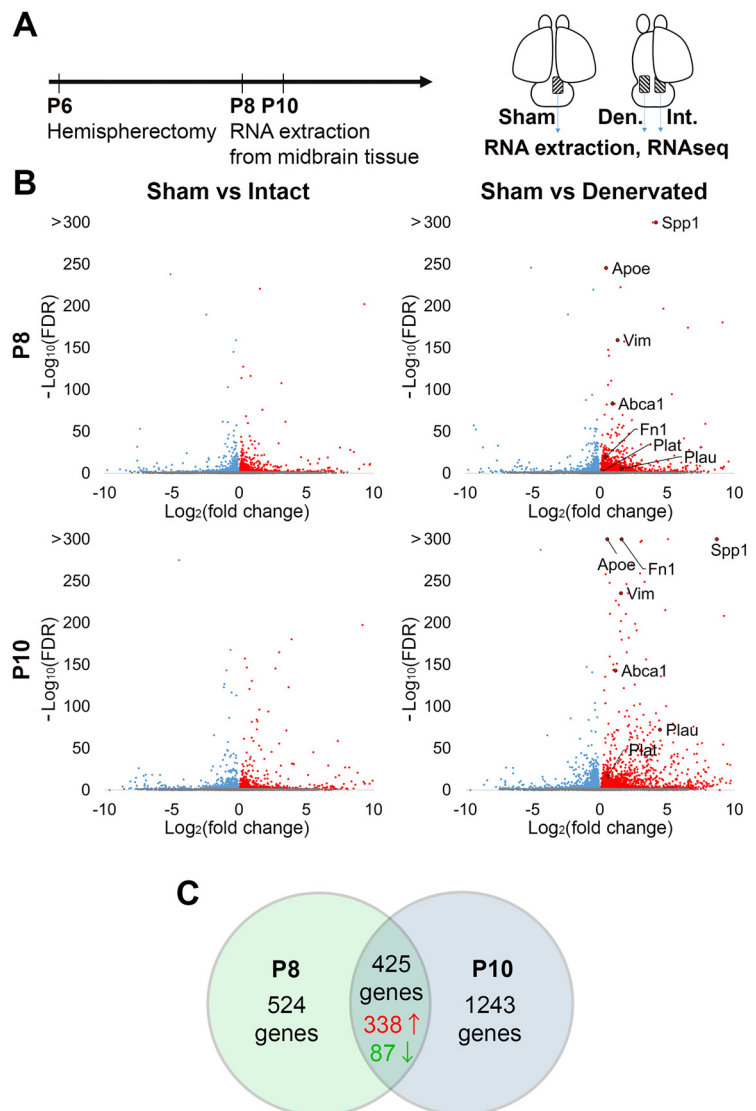


Figure 4. RNA-seq analysis shows that a subset of glial cell-derived genes is significantly upregulated in the denervated midbrain. **A**, Schematic overview of the experiment. RNA-seq was performed using P8 or P10 (2 or 4 d postsurgery) midbrain taken from sham-operated and hemispherectomized mice ($n = 2$ for each group). **B**, Volcano plots comparing sham versus intact midbrain, and sham versus denervated midbrain. Differentially expressed genes (DEGs; $FDR < 0.05$) are highlighted in blue (downregulated) or red (upregulated). **C**, Venn diagram representation of DEGs in P8 (949 genes) and P10 (1668 genes). A total of 425 genes were common to the two stages (338 upregulated and 87 downregulated). For the list of all DEGs, see Extended Data Figure 4-1.

0.035 , $p < 0.05$; posterior: 0.051 ± 0.005 , 0.24 ± 0.030 , $p < 0.05$, Mann–Whitney *U* test) greater at 4 d (P10) and 7 d (P13) after hemispherectomy, respectively (Fig. 1E,F).

Because it is difficult, in the DiI-labeled fixed brain, to observe axons clearly at late developmental stages, perhaps because of diffusion of the dye in myelinated axons, the ectopic projections were further examined about one week after hemispherectomy by labeling cortical axons with a fluorescent protein. For this, cortical neurons in the motor or somatosensory area were transfected with an EGFP-expressing plasmid by *in utero* electroporation at E12.5, when layer 5 neurons are born (Fig. 2A). In the control without ablation, EGFP-labeled axons invaded the midbrain and projected into and around the ipsilateral RN and EW, with few midline crossing axons, in accordance with the result of DiI labeling (Fig. 2C,E). In contrast, a large number of labeled axons were found to project

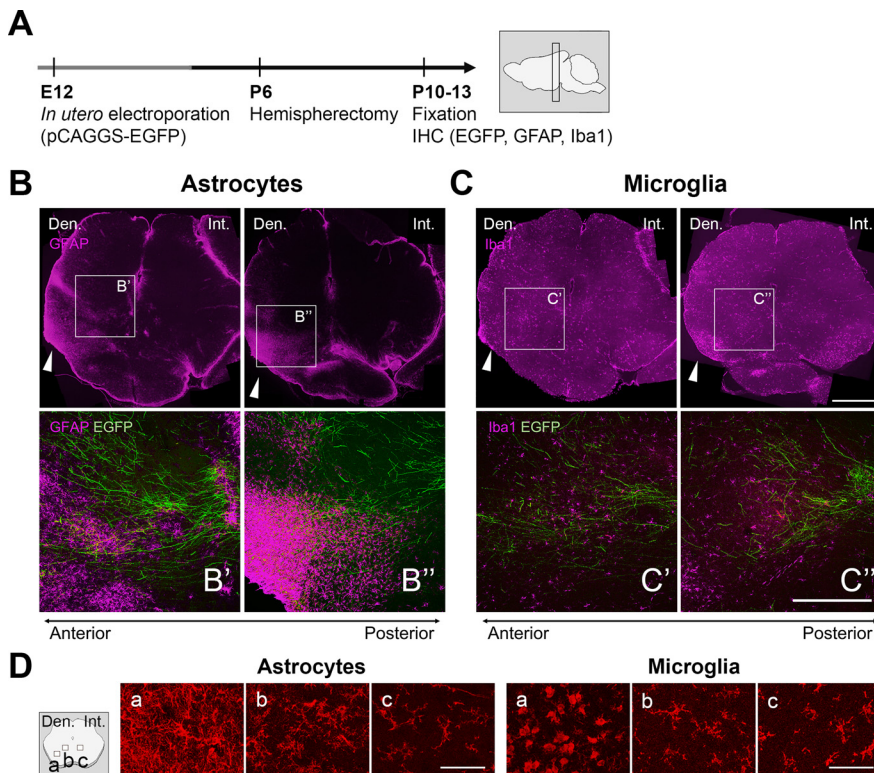


Figure 5. Distribution of glial cells in the midbrain after hemispherectomy. **A**, Schematic overview of the experiment. Immunohistochemistry with glial cell markers (GFAP and Iba1) was performed after hemispherectomy. *In utero* electroporation was conducted as described previously to label corticofugal axons. The location of sections is indicated by the illustration on the right. These sections correspond to the area of Figure 1D, which contain EW and RN. **B**, **C**, Distribution of GFAP-positive astrocytes (**B**) and Iba1-positive microglia (**C**) along the anterior–posterior axis at 7 d postsurgery. Lower panels are high-magnification views of the boxed areas above. Scale bar: 1 mm (upper panels) and 500 μ m (lower panels). Strong GFAP signals were observed near the CP on the denervated side (arrowheads), with broad distribution across the ventral part of the midbrain (**B**, **C**). Many contralaterally projecting axons run near GFAP-positive cells (**B'**, **B''**) and Iba1-positive cells (**C'**, **C''**). **D**, Magnified images of GFAP-positive astrocytes (left) and Iba1-positive microglia (right) at 4 d postsurgery. The schematic illustration on the left shows imaged regions: **a**, near the denervated CP; **b**, the medial part of the denervated midbrain; **c**, the intact side of the midbrain. GFAP-positive cells were densely distributed on the denervated side, especially near the CP. Iba1-positive microglia near the denervated CP had amoeboid morphology. Microglia distant from the CP had ramified morphology. Scale bar: 50 μ m.

contralaterally after hemispherectomy. In the anterior midbrain, labeled axons accumulated in the vicinity of the EW on not only the ipsilateral but also the contralateral side (Fig. 2B,B'). Furthermore, midline-crossing axons were present in a more dorsal region near the superior colliculus (SC; Fig. 2B''). In the more posterior midbrain, labeled axons grew further into the ventrolateral part of the denervated midbrain (Fig. 2D, arrowheads), and were distributed in the vicinity of the contralateral RN (Fig. 2D). Importantly, after hemispherectomy, axonal accumulation in the contralateral midbrain was comparable to that on the ipsilateral side, and the contralateral projections were formed in a mirror image fashion of the ipsilateral projections.

To further elucidate the morphology of the lesion-induced projection, we used a tissue clearing method and sparse labeling of the cortical neurons using the Supernova system (Mizuno et al., 2014; Luo et al., 2016) to trace individually distinguishable axons (Fig. 2F). After hemispherectomy, contralaterally projecting axons were found to form branches just before the midline (Fig. 2G). Quantitative analysis showed that axon branching of contralaterally projecting axons occurred within 400 μ m from the midline, whereas axons projecting

only ipsilaterally did not form branching close to the midline (Fig. 2H). These observations indicate that intact cortical axons form branches near the midline, project contralaterally at around 2 d after surgery, and continue to grow for several more days.

Denervated midbrain promotes axon growth of deep layer cortical neurons

Next, our hypothesis that the denervated midbrain contains axon growth-promoting activity was tested *in vitro* by co-culturing a cortical slice (P1 motor cortex) with either denervated or normal midbrain tissue (Fig. 3A). After 1 d in culture, axon elongation was examined by implanting DiI crystals into the fixed cortical slice. As shown in Figure 3B, axonal growth from the cortical explant was promoted when it was co-cultured with the denervated midbrain. The number of axons that grew to >300 or 400 μ m from the ventricular edge was counted for quantification. The result revealed that the number was significantly larger when the explant was co-cultured with the denervated midbrain than with the normal midbrain (300 μ m: 29.0 ± 7.5 for normal midbrain, 71.4 ± 10.6 for denervated midbrain, $p = 0.0083$; 400 μ m: 14.6 ± 5.2 , 47.9 ± 10.3 , $p = 0.019$, Student's *t* test; Fig. 3C). Furthermore, retrograde labeling with DiI showed that projection neurons were mostly located in the area 400–600 μ m from the pial surface, in which deep layer neurons are located (Fig. 3D). Taken together, these results indicate that the denervated midbrain contains axon growth-promoting factors for deep layer projection neurons.

RNA-seq analysis shows that glial cell-related genes are upregulated in the denervated midbrain

To investigate which genes are upregulated in the denervated midbrain, and thus candidate growth-promoting factors, we performed transcriptome sequencing and gene expression profiling. For this analysis, mRNAs were extracted from denervated midbrain 2 d (P8) and 4 d (P10) after hemispherectomy, when the ectopic projections are in the process of forming. These gene expression profiles were then compared with those of control midbrain and intact-side midbrain, and genes that were specifically upregulated or downregulated in the denervated midbrain were selected (FDR < 0.05, $n = 2$; Fig. 4A).

We found that 949 and 1668 genes were differentially regulated at P8 and P10, respectively, among which 338 upregulated genes and 87 downregulated genes were common to both stages (Fig. 4B, C). We searched a public database of cell type-specific expression in mouse brain (<https://www.brainrnaseq.org/>; Zhang et al., 2014), and found that the majority of the upregulated genes were expressed by microglia and/or astrocytes (microglia: 250/338; astrocytes: 115/338) (Toshniwal et al., 1987; Tsirka et al., 1995; Ellison et al., 1998; Dougherty et al., 2000; Dallner et al., 2002; Tom et al.,

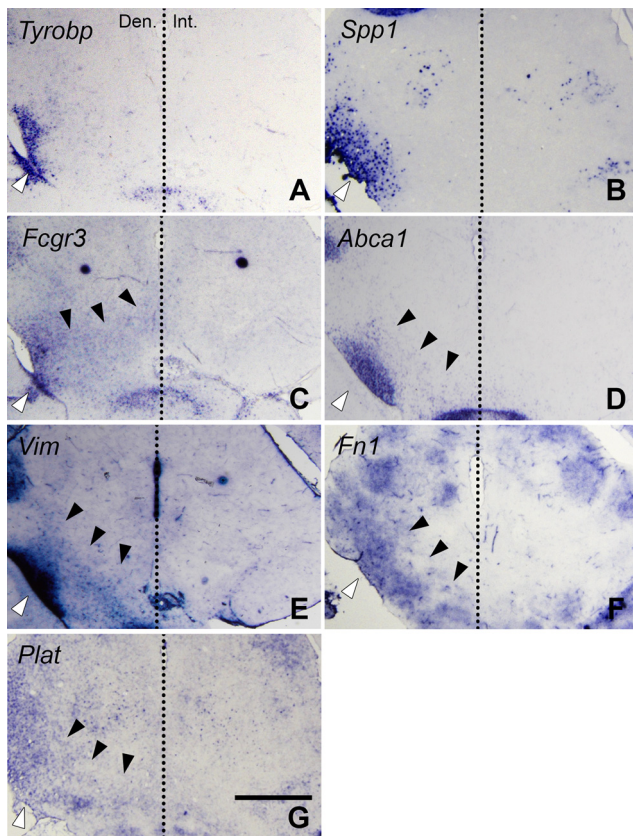


Figure 6. *In situ* hybridization confirms that lesion-induced genes are upregulated in the denervated midbrain. *In situ* hybridization was performed on P10 coronal sections of hemispherectomized mice. **A–G**, The expression of seven representative upregulated genes is shown. **A**, transmembrane immune signaling adaptor (Tyrobp); **B**, secreted phosphoprotein 1 (Spp1, osteopontin); **C**, low affinity immunoglobulin gamma Fc region receptor III (Fcgr3); **D**, ATP binding cassette subfamily A member 1 (Abca1); **E**, vimentin (Vim); **F**, fibronectin 1 (Fn1); **G**, tissue-type plasminogen activator (Plat). The midline is indicated by dashed lines. White arrowheads indicate the CP of the denervated side. Black arrowheads indicate that some genes showed widespread signals in the region adjacent to the midline. Scale bar: 1 mm.

2004; Sinclair et al., 2005; Labandeira-Garcia et al., 2017). For example, complement protein genes (e.g., C1qa, C1qb) and phagocytosis-related genes (e.g., Fcgr3), which are known to be expressed by microglia, were upregulated, along with astrocyte-derived genes such as Vim and Serpina3n (Extended Data Fig. 4–1). In support of this view, immunohistochemistry demonstrated that GFAP-positive astrocytes were densely distributed in the denervated midbrain 4–7 d after the ablation and spread out medially (Fig. 5B, D). Furthermore, Iba1-positive microglia were accumulated with amoeboid shape in the vicinity of the cerebral peduncle (CP) of the denervated midbrain, although the ramified type was found broadly in both sides of the midbrain (Fig. 5C,D). These results suggest that the upregulated genes are attributable to the glial cell response in the denervated midbrain.

The spatial distribution of upregulated genes was further investigated by performing *in situ* hybridization. Most analyzed genes were highly expressed in the vicinity of CP of the denervated side, in which a large number of axons projecting to the midbrain and spinal cord degenerated after hemispherectomy (Fig. 6). Some genes were rather restricted to the CP (e.g., Tyrobp, Spp1; Fig. 6A,B), whereas others were broadly distributed along the ventral side of the denervated midbrain spreading to the midline (e.g., Fcgr3, Abca1, Vim, Fn1, Plat; Fig. 6C–G). Thus, upregulated genes were confirmed to be expressed strongly on the denervated side.

Table 1. Upregulated genes related to axonal growth or regeneration

Gene symbol	RNA-seq (fold change)				Cell type expression		
	P8 vs control	P10 vs intact	P8 vs control	P10 vs intact	Astrocyte	Microglia	Receptor
Spp1	14.2	17.1	256.6	197.1	✓ ^(2, 3)	✓ ⁽¹⁾	Cd44, Itgb1, Itgb3
Fn1	1.3	1.6	2.9	3.0	✓ ^(1, 4)	✓ ⁽¹⁾	Itgb1, Itgb3
Grn	1.7	1.6	2.5	2.4	✓ ⁽¹⁾	✓ ⁽¹⁾	Notch1
Cntf	1.6	1.8	2.6	1.8	✓ ⁽⁵⁾	✓ ⁽¹⁾	Cntfr
Tgfb1	1.9	1.7	1.6	1.6	✓ ⁽¹⁾	✓ ⁽¹⁾	Tgfb1
Abca1	1.8	1.8	2.2	2.0	✓ ⁽¹⁾	✓ ⁽¹⁾	-
Apoe	1.3	1.3	1.4	1.4	✓ ⁽¹⁾	✓ ⁽¹⁾	Vldlr, Lrp8, Lrp1
Plau	2.4	1.9	16.2	11.4	✓ ^(1, 6)	✓ ^(1, 7)	Plaur
Plat	1.2	1.2	1.4	1.4	✓ ^(1, 6)	✓ ^(1, 7)	-
Bdnf ^(a)	1.0	0.9	1.0	1.0	✓ ⁽⁸⁾	✓ ⁽⁸⁾	Ntrk2 (TrkB)
Igf1 ^(b)	1.0	0.9	0.7	0.8	✓ ⁽⁹⁾	✓ ⁽⁹⁾	Igf1r

Their receptors are also presented in the table. Bdnf and Igf1 were not upregulated, but the signaling pathways of these genes may be affected by upregulated molecules. (a) Plau and Plat promote cleavage of proBDNF into mature BDNF (Pang et al., 2004). (b) Spp1 sensitizes corticospinal neuronal responses to IGF1, leading to an increase in the number of corticospinal sprouting neurons after cortical stroke (Liu et al., 2017). Cell type expression (astrocyte and microglia) of each gene is based on the following references: (1) Zhang et al. (2014), (2) Ellison et al. (1998), (3) Sinclair et al. (2005), (4) Tom et al. (2004), (5) Dallner et al. (2002), (6) Toshniwal et al. (1987), (7) Tsirka et al. (1995), (8) Dougherty et al. (2000), and (9) Labandeira-Garcia et al. (2017).

Formation of ectopic contralateral projections is altered after CRISPR/Cas9-mediated KO of the receptors for the upregulated molecules

From the list of upregulated genes, those related with axon growth were selected as candidate regulators of lesion-induced axonal remodeling (Table 1). To identify the underlying molecules, we knocked out receptors for the above candidate molecules in layer 5 projection neurons. Furthermore, the receptors (e.g., TrkB, Igf1r) whose signaling pathway is potentially affected by the upregulated genes were also examined (see Table 1). For KO of these receptors, *in utero* electroporation-mediated transfection with vectors expressing Cas9 and sgRNAs was conducted together with the EGFP vector (Fig. 7A,B). Among examined, obvious reduction of the contralateral projections was found in KO of integrin subunit $\beta 3$ (Itgb3) and neurotrophic receptor tyrosine kinase 2 (Ntrk2, also known as TrkB; Figs. 7C, 8), which are receptors for extracellular molecules such as Spp1 and Fn1 (Humphries et al., 2006) and Bdnf, respectively (Table 1). To quantify the reduction, axons projecting to the contralateral side for each group (only ablation without KO, Itgb3 KO, TrkB KO, and no ablation) were depicted as the axon density index (the ratio of the axon density in the contralateral side midbrain to that in the ipsilateral side), for the two sequential anterior and posterior sections (Fig. 7D; see Materials and Methods). Then the indices of Itgb3 KO and TrkB KO groups were normalized by the index of only ablation group and defined as normalized axon density index.

In Itgb3 KO samples, ectopic contralateral projections were reduced in the anterior section though not in the posterior section (Fig. 7C,D). Indeed, the axon density index in the anterior section was significantly lower in Itgb3 KO (normalized axon density index, 0.74 ± 0.043 , $p = 0.040$, one-way ANOVA with Tukey's *post hoc* test), but not in the posterior section (normalized axon density index, 0.98 ± 0.10 , $p = 0.90$, one-way ANOVA with Tukey's *post hoc* test; Fig. 7E). In particular, axons projecting further into the ventrolateral part of the denervated midbrain were greatly reduced in the anterior section (Fig. 7C,D). Quantitative analysis of the restricted area including the

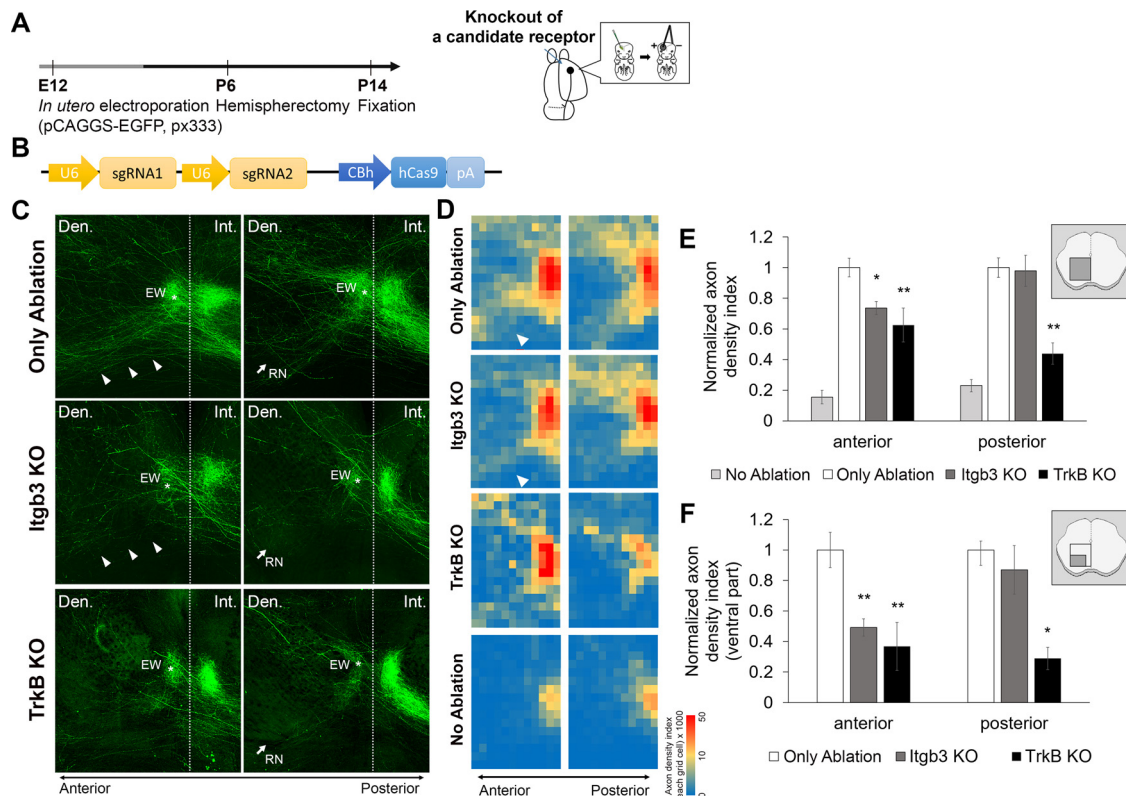


Figure 7. Formation of lesion-induced contralateral projection is impaired after KO of Itgb3 and TrkB. **A**, Schematic overview of the experiment. *In utero* electroporation was performed to knock out a specific receptor in layer 5 cortical neurons. **B**, A plasmid expressing dual sgRNAs and hCas9 (shown) was transfected into the cortex together with an EGFP expressing plasmid. **C**, After hemispherectomy, ectopic axons projecting from Itgb3 KO and TrkB KO neurons were reduced, compared with the only ablation group (without KO). Anterior and posterior sections were used for the analysis. Dashed lines indicate the midline. Note that axon projection to the ventrolateral portion is impaired in Itgb3 KO (arrowheads). Scale bar: 500 μ m. **D**, The average pattern of contralateral projection for each group is shown as pseudo-color heat-map images, based on axon density indices (see Materials and Methods). Note that the axon density indices in the ventrolateral portion of the anterior side are greatly reduced in the Itgb3 KO group (arrowheads), and overall axon density indices are reduced in the TrkB KO group (only ablation, $n = 7$; Itgb3 KO, $n = 10$; TrkB KO, $n = 6$; no ablation, $n = 6$). **E**, Normalized axon density indices (axon density index normalized by that for only ablation group) were calculated in the anterior and posterior sections for each case (no ablation, $n = 6$; only ablation, $n = 7$; Itgb3 KO, $n = 10$; TrkB KO, $n = 6$). **F**, Normalized axon density indices were similarly calculated in the ventrolateral region. The ROI for each analysis (**E**, **F**) is indicated by the illustrations on the right; * $p < 0.05$, ** $p < 0.01$ against control; one-way ANOVA with Tukey's *post hoc* test.

ventrolateral part showed a marked decrease in the axon density index (normalized axon density index, 0.49 ± 0.057 , $p = 0.0082$, one-way ANOVA with Tukey's *post hoc* test; Fig. 7F).

On the other hand, TrkB KO samples showed a substantial decrease in the axon density index after hemispherectomy in both anterior and posterior sections, with a more profound reduction in the posterior section (normalized axon density index, 0.63 ± 0.11 for anterior, $p = 0.0072$; 0.44 ± 0.070 for posterior, $p = 0.0018$, one-way ANOVA with Tukey's *post hoc* test; Fig. 7E,F). Importantly, these two receptors were expressed endogenously in layer 5 neurons (Thompson et al., 2014; Itgb3: <http://developingmouse.brain-map.org/gene/show/16189>; Ntrk2: <https://developingmouse.brain-map.org/gene/show/17979>). Thus, Itgb3 and TrkB are involved in the formation of ectopic axonal projections in a distinct fashion after hemispherectomy.

In our RNA-seq result, expression of the presumed ligands for Itgb3, Spp1, and Fn1 was substantially elevated in the denervated midbrain after the hemispherectomy (Table 1). *In situ* hybridization showed that both genes were highly expressed in the ventrolateral part of the denervated midbrain (Fig. 6B,F), where the contralateral projections were markedly reduced in Itgb3 KO samples (Fig. 7C,D). In contrast, *Bdnf* mRNA expression was not upregulated in the denervated midbrain (Table 1). Instead, the plasminogen activators (Plat, Plau), which can

promote extracellular cleavage of proBDNF into the mature type (Pang et al., 2004), were upregulated in the denervated midbrain (Table 1; Fig. 6G). In accordance with the increased expression, Western blot analysis demonstrated that mature BDNF protein was significantly more abundant in the denervated than the intact midbrain (ratio, 1.47 ± 0.11 , $p = 0.017$, Student's *t* test; Fig. 9A), suggesting that proteolytic cleavage into mature BDNF is promoted in the denervated midbrain and contributes to the contralateral projection.

As the majority of upregulated genes in the denervated midbrain were glial-related genes in our analysis (see above), we further investigated whether the receptor interacting molecules are derived from glial cells, by co-immunostaining with microglial and astrocytic markers. As shown in Figure 9B, strong puncta-like signals of Spp1 were found along the ventral part of the denervated midbrain. High magnification images showed that Spp1-positive puncta reside in and around the microglia and astrocytes (Fig. 9C,D). Plat were also strongly expressed in the denervated-side CP (Fig. 9E), and the signals were co-localized with astrocytes, but not with microglia (Fig. 9F,G). These results suggest that glial cells contribute to the production of these molecules.

Taken together, all of these results indicate that the denervated midbrain-derived factors expressed by glial cells contribute to lesion-induced remodeling of the cortico-mesencephalic projection, via receptors such as Itgb3 and TrkB.

Discussion

The present morphologic and molecular expression analyses demonstrate that glial cell-related axon growth-promoting factors are expressed in the denervated midbrain when robust remodeling of the cortico-mesencephalic projection takes place after hemispherectomy. Furthermore, CRISPR/Cas9-mediated functional analysis demonstrate that these factors are involved in the remodeling of the cortico-mesencephalic projection. In particular, KO of *Itgb3* or *TrkB* suppressed the formation of ectopic contralateral projections. Thus, the present study revealed for the first time that these specific molecular pathways contribute to remodeling of the cortico-mesencephalic projection after hemispherectomy.

The cortico-mesencephalic projection is rapidly remodeled after hemispherectomy of juvenile mice

A morphologic experiment with axon tracing showed that remodeling of the cortico-mesencephalic projection took place after hemispherectomy, in accordance with previous reports (Nah and Leong, 1976; Tsukahara, 1981a; Lee et al., 2004; Takahashi et al., 2009; Omoto et al., 2010, 2011). Robust formation of the ectopic contralateral projection is consistent with previous findings that axonal sprouting is extensive in young animals (Tsukahara, 1981b; Tsukahara et al., 1983; Kosar et al., 1985; Murakami and Higashi, 1988; Kuang and Kalil, 1990; Omoto et al., 2010, 2011; Grant et al., 2016). Moreover, our results demonstrated that only a few days after hemispherectomy, axons from the intact cortex invaded the denervated midbrain. This rapid formation of the ectopic contralateral projection raised the possibility that axonal sprouting occurs in the regions near the midline, which was supported by the observation of individually distinguishable cortical axons (Fig. 2*F–H*). Furthermore, the subsequent increase in the ectopic contralateral projection is likely because of an increase in the number of midline-crossing axons (Lee et al., 2004) rather than extensive branch formation in the small number of preexisting contralateral axons that exist even in intact animals. Such ectopic projections are thought to contribute to functional recovery by being maintained persistently (Takahashi et al., 2009; Omoto et al., 2010, 2011). Indeed, a previous study has shown that motor functions are comparable to normal in the adult after similar neonatal cortical ablation (Omoto et al., 2011).

Axon growth-promoting factors expressed in the denervated midbrain are involved in lesion-induced remodeling

Two previous studies have shown transcriptome profiles of the denervated region (Bareyre et al., 2002; Kaiser et al., 2019), but they could not lead to the identification of specific molecules that are actually involved in axonal sprouting and remodeling. Our CRISPR/Cas9-mediated KO study demonstrated that two distinct molecular mechanisms contribute to the formation of the ectopic projections.

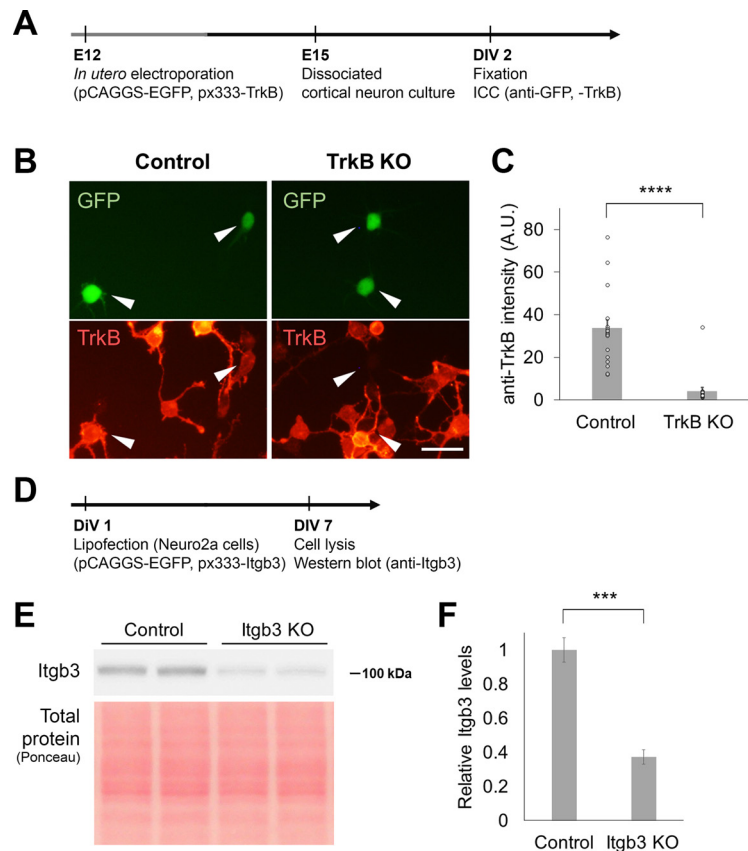


Figure 8. Validation of CRISPR/Cas9-mediated KO efficiency. **A**, Schematic overview of the experiment to validate *TrkB* KO. Dissociated neuron cultures were prepared from cortex transfected with the KO plasmid and were subjected to immunocytochemistry (ICC) with anti-*TrkB*. **B**, Most GFP-positive neurons were *TrkB*-negative (arrowheads). Scale bar: 30 μ m. **C**, Immunostaining intensity was considerably reduced by *TrkB* KO (control, 33.8 ± 3.9 , $n = 18$; *TrkB* KO, 4.2 ± 1.6 , $n = 19$); **** $p < 0.0001$, Student's t test. **D**, Schematic overview of the experiment to validate *Itgb3* KO. The plasmid for *Itgb3* KO was transfected with the GFP-expressing plasmid into Neuro2a cells by lipofection, and the cultured cells were subjected to Western blot analysis. **E**, **F**, *Itgb3* expression level was markedly decreased by *Itgb3* KO (ratio, 0.37 ± 0.042 against control, $n = 4$). As the transfection efficiency of lipofection was $66.5 \pm 4.0\%$ ($n = 4$), the KO efficiency is estimated as 94.3%; *** $p < 0.001$, Student's t test.

First, *Itgb3* KO showed a significant decrease of the ectopic projections in the ventrolateral portions of the midbrain where *Spp1* and *Fn1* are upregulated (Figs. 6*B,F*, 9*B–D*). It is plausible that developmental mechanisms guide axons to their proper targets in both intact and denervated midbrain, as the lesion-induced contralateral projection resembles the intact ipsilateral projection (Fig. 2*D*). The axonal growth effect of *Spp1* and *Fn1* may enhance this process, helping axons to reach their targets. In support of this view, these molecules have been reported to facilitate axonal growth and regeneration of CNS and PNS neurons (Tom et al., 2004; Myers et al., 2011; Wright et al., 2014; Duan et al., 2015; Liu et al., 2017). Furthermore, *Spp1* and *Fn1* have shown to be upregulated in the hippocampus and cortex after lesion (Tate et al., 2007; Park et al., 2012). Thus, these extracellular molecules may be involved in axonal extension commonly after injury.

Second, *TrkB* KO showed a striking decrease of the ectopic projections, which may be because of non-utilization of mature BDNF, whose concentration should be elevated by proteolytic cleavage in the denervated side (see below; Fig. 9*A*). BDNF–*TrkB* signaling may also contribute to the final connections with target cells, as it is known to promote axonal branch formation (Cohen-Cory et al., 2010; Granseth et al., 2013). Previous work

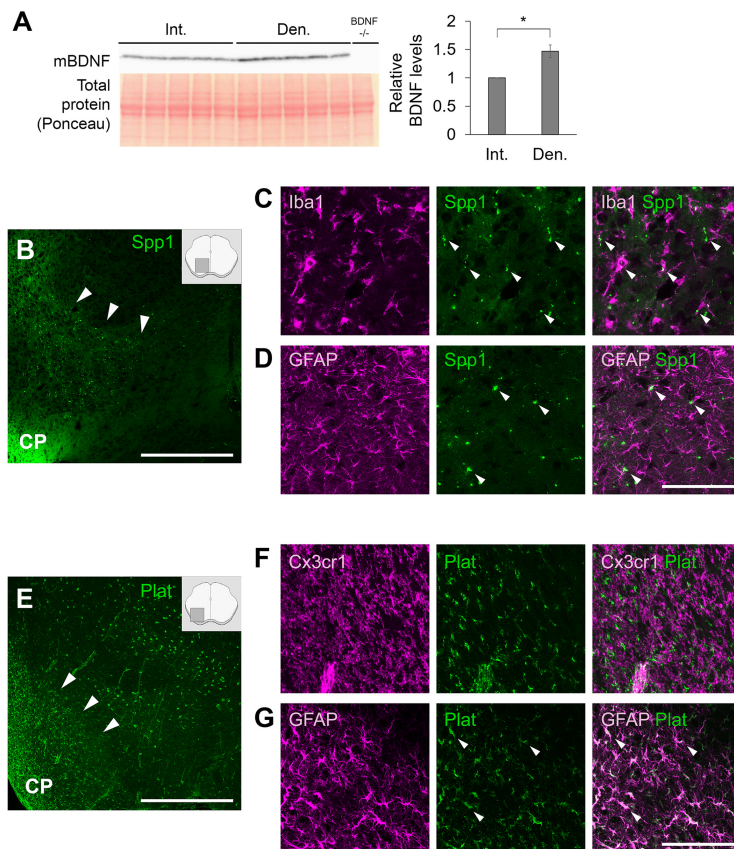


Figure 9. The expression of upregulated molecules in relation to glial cells. **A**, Western blot analysis shows a significant increase of mature BDNF amount in the denervated-side midbrain ($n = 5$) compared with the intact side ($n = 5$) taken from P10 mice (4 d postsurgery); $*p < 0.05$; Student's t test. **B**, Punctate signals of Spp1 were observed broadly on the ventral side of the denervated midbrain (arrowheads), with strong signals on the ventrolateral side near the CP. Illustration (insert) indicates the location of the imaged area. Scale bar: 500 μm . **C**, Immunostaining of Iba1 and Spp1 shows that Spp1 was detectable within the Iba1-positive microglia (arrowheads). **D**, Immunostaining of GFAP and Spp1 showed that Spp1-positive puncta reside in and around GFAP-positive astrocytes (arrowheads). Scale bar: 100 μm . **E**, Immunopositive signals for Plat were observed on the area near the CP (arrowheads). Scale bar: 500 μm . **F**, Immunostaining of Cx3cr1 and Plat shows that Plat signals do not co-localize with Cx3cr1-positive microglia. **G**, Immunostaining of GFAP and Plat showed that Plat-positive signals co-localize with GFAP-positive astrocytes (arrowheads). Scale bar: 100 μm .

has demonstrated that blocking BDNF–TrkB signaling hinders the lesion-induced sprouting of the corticospinal projection (Ueno et al., 2012), in which Bdnf expression was not upregulated in the denervated spinal cord after the cortical lesion. The present results also show that the Bdnf mRNA level was unchanged in the denervated midbrain, but that the level of mature BDNF protein was upregulated (Fig. 9A). It is known that proBDNF is released extracellularly (Yang et al., 2009), and posttranscriptional modification of proBDNF mediated by plasminogen activators has been implicated in various aspects of neural plasticity (Lee et al., 2001; Mataga et al., 2004; Pang et al., 2004; Kojima et al., 2020b). Thus, it is likely that plasminogen activators upregulated in the denervated midbrain promote the extracellular cleavage of proBDNF and production of mature BDNF.

Glial cells as the source of the axon growth-promoting factors

Which cell types produce these axon growth-promoting factors? Our RNA-seq analysis showed that many glial cell-related genes were upregulated in the denervated midbrain after hemispherectomy. Importantly, previous and the present results demonstrated that the upregulated genes Spp1 (Ellison et al., 1998;

Sinclair et al., 2005), Fn1 (Tom et al., 2004), and plasminogen activators (Toshniwal et al., 1987; Tsirka et al., 1995) are expressed by glial cells (Fig. 9B–G). We also observed that contralateral cortical axons ran in and around GFAP-positive astrocytes which were broadly distributed in the denervated midbrain (Fig. 5). A plausible scenario is that reactive glial cells (Streit et al., 1999; Sofroniew and Vinters, 2010) expressing these factors spread in the denervated region and provide a growth-permissive or promoting environment for cortical axons. In accordance with this view, glial cells have been demonstrated to respond to axon degeneration with characteristic gene expression in the denervated CNS region, although the upregulated genes are distinct from those in the present study (Kaiser et al., 2019; Tsujioka and Yamashita, 2019). Recently, it has also been demonstrated that microglial cells in the cortex express BDNF in responding to PNS injury (Huang et al., 2021). Such glial cell responses could be efficient to make a cellular environment for spreading the molecules that induce ectopic axonal growth and formation of compensative neuronal circuits after brain lesion.

On the other hand, astrocytes and microglia have been shown to be the source of a glial scar at the lesion site which suppresses axon regeneration by expressing inhibitory molecules such as Nogo and CSPG (Silver and Miller, 2004; Yiu and He, 2006; Schwab and Strittmatter, 2014). It is likely that reactive glial cells in the denervated midbrain have different properties from the scar-forming glial cells, as the denervated midbrain is distant from the lesion site. Glial cells may respond differently to distinct stimuli, as in the case of alternative activation (Hu et al., 2015; Liddelow and Barres, 2017). That is to say, glial cell activation may be context-dependent (Mosser et al., 2017).

Other possible mechanisms for lesion-induced remodeling

In the present study, impairing Itgb3-mediated and TrkB-mediated signaling did not block sprouting completely, which implies that other mechanisms also operate in this process. The expression of axon growth-inhibitory molecules may decrease after hemispherectomy, as manipulations that remove inhibitory molecules such as Nogo or CSPG can facilitate axonal sprouting (Cafferty and Strittmatter, 2006; Starkey et al., 2012). However, we could not find any decline of these inhibitory molecules from our RNA-seq result. Another possibility is that a midline barrier exists which prevents axon growth, similar to the Ephrin-B3/EphA4 signaling pathway in the spinal cord (Kullander et al., 2001; Yokoyama et al., 2001; Katori et al., 2017). However, this is also unlikely, as the cortico-mesencephalic projection has been shown to be normal in these KO mice (Yokoyama et al., 2001; Serradaj et al., 2014).

The involvement of neuronal activity has to be considered, because it has been reported that neuronal activity promotes axonal branching (Uesaka et al., 2005). Indeed, lesion-induced sprouting in the corticospinal tract is enhanced by electrical stimulation of the motor cortex (Carmel et al., 2010; Carmel and

Martin, 2014) and rehabilitation (den Brand et al., 2012; Wahl et al., 2014), which led to functional recovery. The expression of sprouting-inducing factors and/or receptor molecules may also be regulated in an activity-dependent fashion (Yap and Greenberg, 2018). Furthermore, neuronal activity including synaptic activity may play a role in synapse formation and maintenance in their target cells (Hoerder-Suabedissen et al., 2019).

In summary, our study elucidates a novel intrinsic mechanism of lesion-induced axonal remodeling, mediated by Itgb3 and TrkB signaling pathways. To date, a model in which axonal regeneration and/or sprouting is enhanced by removal of growth-inhibitory factors has been emphasized. In addition to this approach, molecular manipulations that enhance the present endogenous mechanism may also be effective for axonal remodeling, and may provide a novel insight into new therapeutic strategies for functional recovery after CNS injury.

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