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Variable laterality of corticospinal tract axons that regenerate after spinal cord injury as a result of PTEN deletion or knock-down

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Abstract

Corticospinal tract (CST) axons from one hemisphere normally extend and terminate predominantly in the contralateral spinal cord. We previously showed that deleting PTEN in the sensorimotor cortex enables CST axons to regenerate after spinal cord injury and that some regenerating axons extend along the “wrong” side. Here, we characterize the degree of specificity of regrowth in terms of laterality. PTEN was selectively deleted via cortical AAV-Cre injections in neonatal PTEN-floxed mice. As adults, mice received dorsal hemisection injuries at T12 or complete crush injuries at T9. CST axons from one hemisphere were traced by unilateral BDA injections in PTEN-deleted mice with spinal cord injury and in non-injured PTEN-floxed mice that had not received AAV-Cre. In non-injured mice, $97.9 \pm 0.7\%$ of BDA-labeled axons in white matter and $88.5 \pm 1.0\%$ of BDA-labeled axons in grey matter were contralateral to the cortex of origin. In contrast, laterality of CST axons that extended past a lesion due to PTEN deletion varied across animals. In some cases, regenerated axons extended predominantly on the *ipsilateral* side,

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CONFLICT OF INTEREST

O.S. has financial interest in the company “Axonis,” which holds options on patents relating to PTEN deletion and axon regeneration.

AUTHOR CONTRIBUTIONS

R.W., K.Z., K.L., Z.H. and O.S. designed research; R.W. and K.Z. performed research; R.W. analyzed data; R.W. and O.S. wrote the paper.

in other cases, axons extended predominantly contralaterally, and in others, axons were similar in numbers on both sides. Similar results were seen in analyses of cases from previous studies using shRNA-mediated PTEN knock-down. These results indicate that CST axons that extend past a lesion due to PTEN deletion or knock-down do not maintain the contralateral rule of the non-injured CST, highlighting one aspect for how resultant circuitry from regenerating axons may differ from that of the uninjured CST.

Keywords

Axon regeneration; SCI; CST

INTRODUCTION

A prominent feature of the organization of the vertebrate nervous system is lateralization of axonal tracts. Interestingly, most sensory and motor tracts are predominantly crossed, although the reason for this is unclear. With injuries to the head, Hippocrates noted that “for the most part, convulsions seize the other side of the body; for, if the wound be situated on the left side, the convulsions will seize the right side of the body...” (Adams, 1929). This observation fits with the projection of the mammalian corticospinal tract (CST), a predominantly crossed axonal tract that controls voluntary motor function (Kuypers, 1982; Lemon and Griffiths, 2005). This rule of the CST being contralateral includes CST projections in the white matter as well as terminations in the grey matter (Kuypers, 1982), and this central organizational feature has been documented in rats (Rouiller et al., 1991) and primates (Lacroix et al., 2004; Rosenzweig et al., 2009, 2010) and extends across most mammalian species (Kuypers, 2011). Rare exceptions to the contralateral rule include the bilateral CST of the goat (Haartsen and Verhaart, 1967) and elephant (Verhaart, 1963), and the uncrossed CST of the hedgehog (Palmieri et al., 1993). A few other mammals lack a CST decussation at the medullary pyramids, but their CST axons decussate at the spinal level and terminate contralateral to the cortex of origin [e.g. mole (Linowiecki, 1914), procavia (Verhaart, 1967)].

Lateralization of motor control is important for unilateral motor function, especially for bimanual tasks that require different movements by each hand (Welniarz et al., 2015). Abnormal CST lateralization has been associated with mirror movements in both mice and humans (Engle, 2010; Welniarz et al., 2015). In mice with dysfunctional ephrin B3/A4 ligand-receptor signaling, CST axons from one cortex extend bilaterally within the spinal cord, and mice have a kangaroo-like hindlimb hopping gait (Dottori, 1998; Coonan et al., 2001; Yokoyama et al., 2001). Humans with Joubert Syndrome exhibit synkinetic mirror movements and lack or have a decreased CST decussation (Friede and Boltshauser, 1978; Maria et al., 1999; Engle, 2010), reflecting aberrant ipsilateral projections. In Kallmann syndrome, 2/3 of males with mutations in *KAL1* have mirror movements and aberrant ipsilateral CSTs (Mayston et al., 1997; Krams et al., 1999; Engle, 2010). Also, mice with a mutation in *deleted in colorectal carcinoma (DCC)* lack a pyramidal CST decussation and exhibit a hopping gait (Finger et al., 2002), and a *DCC* mutation has been identified as the cause of congenital mirror movements (CMM) in humans (Srour et al., 2010).

Paralysis following spinal cord injury (SCI) is due to the interruption of long tracts of motor axons, and it is expected that regeneration of long axon tracts such as the CST will be necessary to restore motor function after severe SCI (Tuszynski and Steward, 2012). The CST is particularly refractory to regeneration (Blesch and Tuszynski, 2009; Liu et al., 2010), and it wasn't until recently that significant regeneration of CST axons was achieved (Liu et al., 2010). An approach enabling robust regeneration of the CST is deletion or knock-down of expression of the gene *phosphatase and tensin homolog (PTEN)* (Liu et al., 2010; Zukor et al., 2013). In rats, knock-down of PTEN in the cortex with AAV-shRNA coupled with injection of salmon fibrin into a spinal cord lesion enhances recovery of voluntary motor function (Lewandowski and Steward, 2014).

Notably, in our initial report of CST regeneration with PTEN deletion, we noted that in some cases, some regenerated axons extend past the lesion on the “wrong” side (Liu et al., 2010). Indeed, this was strong evidence that axons that extended past the lesion were regenerated and not spared. Our initial report documented that “wrong-sided” axon regeneration occurred, but it did not characterize the degree of laterality or the consistency of regenerated axons' lateral distribution. Because laterality is important for normal function, the present study was undertaken to determine whether there is a consistent pattern of distribution of axons that extend past a spinal cord injury due to PTEN deletion, and the degree to which axons that regenerate respect the contralateral rule of the intact CST. Using the same methods as in Liu et al. (2010) to delete PTEN as means to enhance CST regeneration, we quantitatively assess laterality of the resultant regenerative growth, and also analyze cases from a previous study documenting regenerative growth due to PTEN knock-down by shRNA (Zukor et al., 2013). Our results reveal a lack of consistent pattern of regenerated CST axon distribution, in contrast to the uninjured CST. Regenerated CST axons were ipsilateral in some cases, contralateral in some cases, and in some cases were present in similar numbers bilaterally.

MATERIALS AND METHODS

Our report consists of two studies with the experiments performed independently and in different labs. One study involved dorsal hemisection lesions performed in the Steward lab, and the second involved crush lesions performed in the He lab. Tissue and data were also reanalyzed from 2 previous studies (Liu et al., 2010; Zukor et al., 2013). All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California Irvine, or by the IACUC at Boston Children's Hospital.

Mice

For experiments involving dorsal hemisection lesions carried out at the University of California Irvine, PTEN-floxed (*PTEN^{fl/fl}*) mice [C;129S4-*Pten^{tm1Hwu}*/J, Jackson Labs] received injections of AAV-Cre (serotype 2, Vector Biolabs) into the left cortex on postnatal day one (P1), in a manner adapted from Liu et al. (2010). Briefly, pups were cryoanesthetized by placing them in crushed ice for approximately 5 minutes and then injected with 500 nl of AAV-Cre (10^{12} GC/ml) into each of three sites in the sensorimotor cortex, approximately 0.5 mm lateral from bregma and rostro-caudally spanning ~1 mm.

Injections were made using an electronically-controlled injection system (Nanoliter 2000 injector and Micro4 pump controller, World Precision Instruments). Sesame oil was applied to the pups before returning them to their respective cages.

For experiments involving crush lesions carried out at Children's Hospital Boston, *PTEN^{fl/fl}* pups received injections of AAV2/1-Cre at P0/P1, in a manner adapted from Zukor et al. (2013). AAV2/1 was generated by inserting the AAV1 capsid gene into the AAV2 plasmid, yielding a vector with an AAV1 capsid and AAV2 inverted terminal repeat (ITR) sequences. This plasmid was selected because it was found to yield more efficient transfection of cortical motoneurons (Zukor et al., unpublished). Briefly, pups were cryoanesthetized by being placed in crushed ice and then were given 3 injections of 500 nl of AAV2/1-Cre (2×10^{12} GC/ml) into the right sensorimotor cortex using the nanoliter injection system as above. Fast green (0.5 mg/ml stock) was added to the viral vector at about 1/20 dilution to tint the solution.

PTEN^{fl/fl} mice of both sexes receiving no vector injection or spinal cord injury were used as controls to study the intact CST.

Spinal cord lesions

Eight female mice 7.5–10 weeks old that had received injections of AAV-Cre at P1 received dorsal hemisection lesions at T12, using techniques described previously (Steward et al., 2008). Briefly, mice were anesthetized with isofluorane, their eyes were protected with petroleum jelly, and the surgical area was shaved and swabbed with betadine and then 70% ethanol. Following a thoracic midline incision, overlying muscles were bluntly dissected, and a T12 laminectomy was performed. An ophthalmic scalpel (MicroScalpel Feather 15°, Electron Microscopy Sciences) was passed through the dorsal aspect of the spinal cord at a depth of approximately 0.8 mm to bilaterally sever the dorsal and dorsolateral components of the CST. Eight weeks post-injury, mice received unilateral injections of biotinylated dextran amine (BDA) to label CST axons, and were humanely killed ~10 weeks post-injury (see tissue collection, below).

Three female mice 6–7 weeks old that had received injections of AAV2/1-Cre at P0/P1 received complete crush injuries at thoracic level 8 (T8) in a manner similar to that described in Liu et al. (2010) and Zukor et al. (2013). Briefly, mice were anesthetized with ketamine/xylazine, their eyes were protected with aqua-tears, and the surgical site was shaved and cleansed with betadine and then ethanol. Following a midline incision over the thoracic vertebrae, fat and muscle were cleared from T8 and T9 and a laminectomy was performed at T8 to fully expose the spinal cord from side to side. The spinal cord was then fully crushed for 2 seconds with forceps that had been filed to a width of 0.1 mm for the last 5 mm of the tips. Care was taken to insert the tips on either side of the cord to include the full width of the cord and then gently scrape them across the ventral bone surface so as to not spare any tissue ventrally or laterally.

CST tracing

Mice received unilateral injections of BDA to trace CST axons from one side of the sensorimotor cortex. Injections of BDA (10,000 MW, 10% in dH₂O, Invitrogen) were made

in stereotaxic coordinates in a manner similar to methods previously described (Liu et al., 2010; Zukor et al., 2013). Briefly, in the dorsal hemisection study, 4 injections were made 0.6 mm deep into the left sensorimotor cortex, with coordinates being 1.0 mm lateral and 0.5 mm rostral, 0.2, 0.5, and 1.0 mm caudal to bregma (0.4 μ l per site). For the crush lesion study, BDA tinted with fast green (1/20 of 0.5 mg/ml stock) was injected 0.5 mm deep into 6 coordinates in the right sensorimotor cortex: 1.3 mm lateral, and 1.0, 0.5, 0.0, -0.5, -1.0, and -1.5 mm anterior/posterior to bregma (0.4 μ l per site). Mice were humanely killed by lethal dose of anesthesia and transcardially perfused 2 weeks post-injection.

Tissue collection

For the dorsal hemisection study, mice were euthanized with an overdose of Euthasol® (pentobarbital sodium and phenytoin sodium, Western Medical Supply, Inc.) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (4% PFA). Spinal cords and brains were dissected out and post-fixed overnight in 4% PFA and then were equilibrated in 27% sucrose for cryoprotection. The spinal cord was cut to obtain an approximately 6 mm long block with the lesion in the center, and adjacent rostral and caudal blocks approximately 2 mm long. The blocks of cryoprotected spinal cord were frozen in TissueTek O.C.T. (Sakura Finetek) for sectioning with a cryostat. Sagittal sections 30 μ m thick were collected through the lesion block, and cross sections were collected from the adjacent rostral and caudal blocks. Sagittal and rostral cross sections were collected in comparable regions of spinal cord in non-injured control mice. Coronal sections 20 μ m thick were collected from brains of mice that had regenerative CST growth. Additional rostral cross sections were collected for resin embedding from the adjacent non-frozen spinal cord of non-injured control mice using a Vibratome® set at 50 μ m.

For the crush lesion study, mice were euthanized and tissue was collected as in Zukor et al. (2013). Briefly, mice were given a lethal dose of ketamine/xylazine anesthesia and transcardially perfused with phosphate buffered saline (PBS) followed by 4% PFA. Brains and vertebral columns were post-fixed in 4% PFA overnight. Tissues were rinsed twice in PBS, and the spinal cord and right side of the brain dissected out further. Tissues were cryoprotected in 30% sucrose in PBS for 3 days before embedding in OCT. Sections 25–30 μ m thick were cut on a cryostat, directly mounted onto slides and stored at -20°C until processed. Prior to staining, slides were warmed to room temperature and dried on a 37°C slide warmer.

Primary antibodies

For PTEN immunocytochemistry, we used a monoclonal antibody from Cell Signaling (catalog #9188, RRID: AB_2174349). This antibody was raised in rabbit against amino acids 384 to 403 of the human PTEN sequence (Cell Signalling, personal communication). A rigorous control for specificity is provided by the disappearance of labeling in areas corresponding to sites of AAV-Cre injections, which delete PTEN in neurons via Cre-mediated recombination.

Some spinal cord sections stained for BDA were co-stained for glial fibrillary acidic protein (GFAP) using an antibody made in rabbit against GFAP from cow spinal cord (Dako,

catalog #Z0334, RRID: AB_10013382). This antibody detected nascent GFAP and proteolyzed products in Western blot of the mouse brain (David et al., 1997), and yields a single immunoprecipitant when incubated with cow brain extract in crossed electrophoresis (Dako, on file). Specificity is further documented by fact that immunostaining is selective for cells with astrocyte morphology.

Histology: BDA staining and immunohistochemistry

Spinal cord sections—All experiments used amplification staining for detection of BDA in the spinal cord. In the dorsal hemisection study, BDA was detected in spinal cords with fluorescence by staining via catalyzed reporter deposition (CARD) (Bobrow et al., 1989) with rhodamine, applying methods as reported by (Hopman et al., 1998). Briefly, sections were washed three times in phosphate-buffered saline (PBS) and then endogenous peroxidases were quenched with 1–3% hydrogen peroxide in PBS for 15 minutes before washing sections again three times in PBS. Sections were incubated in a 1:400 dilution of streptavidin-horseradish-peroxidase (SA-HRP, Perkin Elmer) in PBS 0.1–1% Triton X-100 (PBS-Tx) for 2 hours, and then washed three times in PBS-Tx. SA-HRP detection was performed by incubating sections in a dilution of 0.1 µg/ml tyramide-conjugated rhodamine (mg/mL) in 0.1M borate (pH 8.5) with 0.003% stabilized hydrogen peroxide (Sigma Aldrich, H-1009) for 20–30 minutes. Sections were then washed three times in PBS and mounted onto gelatin-coated slides.

In the crush lesion study, spinal cord sections were stained for BDA and glial fibrillary acidic protein (GFAP), as in Zukor et al. (2013). BDA labeling was detected with SA-HRP binding and TSA Cyanine-3 staining (Perkin Elmer). Briefly, endogenous peroxidases were quenched in slide-mounted sections with 0.3% hydrogen peroxide in PBS, then rinsed in with PBS and blocked with 10% normal serum in PBS-Tx for 1 hour. Slides were incubated with a 1:500 dilution of rabbit anti-GFAP (Dako, #Z0334, RRID: AB_10013382) overnight. Slides were then rinsed in PBS and incubated with a 1:300 dilution of SA-HRP and a 1:200 dilution of goat anti-rabbit Alexa 633 (Invitrogen) in PBS with 10% normal serum. Slides were washed and then incubated with tyramide conjugated to Cy3 (Perkin Elmer, SAT704A001, 1/200 in diluent) for 10 min to complete BDA labeling, and rinsed with PBS-Tx.

In spinal cord cross sections used for resin embedding, BDA was detected by chromogenic staining with nickel-enhanced diaminobenzidine (DAB-Ni), as previously reported (Steward et al., 2008). Briefly, sections were washed in PBS-Tx, incubated for 1–2 hrs with avidin and biotinylated horseradish peroxidase (Vectastain ABC kit, Vector Laboratories), and then washed in PBS. The DAB-Ni reaction was performed in 50 mM Tris buffer, pH 7.6, 0.024% hydrogen peroxide and 0.5% nickel chloride.

Brain sections—To visualize PTEN and BDA labeling in the brain, brain sections in both studies were fluorescently stained for BDA using direct conjugation and immunostained for PTEN using CARD. Sections were washed in tris-buffered saline (TBS), then incubated in 1% SDS in TBS for 5 minutes for antigen retrieval, and washed again in TBS. Sections were blocked in TBS with 0.3% Triton X-100 (TBS-Tx) and 5% normal

donkey serum (NDS) before incubating overnight in 1:250 dilution of rabbit anti-PTEN (Cell Signaling, #9188, RRID: AB_2174349) in the same solution. Sections were washed in TBS with 0.05% Tween-20 (TBS-Tw), before being incubated in a 1:250 dilution of donkey anti-rabbit horseradish-peroxidase (HRP) in TBS-Tx and 5% NDS. Sections were then washed in TBS-Tw before being incubated in a 4 µg/ml dilution of tyramide-conjugated fluorescein in 0.1 M borate (pH 8.5) with 0.003% stabilized hydrogen peroxide for 20–30 minutes. Sections were washed once in TBS and then 3 times in PBS-Tx before incubating in a 1:250 dilution of streptavidin-594 in PBS-Tx for 1–2 hours to label BDA. Sections were washed in PBS-Tx and mounted onto gelatin-coated slides.

In a complementary approach for visualizing PTEN alone, some brain sections were chromogenically stained for PTEN using diaminobenzidine (DAB) without nickel enhancement. Washing, blocking, and primary and secondary antibody incubations of sections were performed as above. Following incubation with donkey anti-rabbit HRP, sections were washed in TBS-Tw before performing the reaction with DAB (DAB Peroxidase Substrate kit, #SK-41000, Vector Laboratories), for 15 minutes. Sections were then washed in TBS and mounted onto gelatin-coated slides.

Resin sections

Cross sections from non-injured control mice with strong DAB-Ni staining of BDA were selected for resin embedding as previously described (Liu et al., 2010). Briefly, sections were rinsed in 0.1 M cacodylate buffer, post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer, rinsed in nanopure water, then dehydrated through a series of 70%, 85%, 90% and 100% ethanols. Sections were immersed in propylene oxide (intermediate solvent) before incubation in propylene oxide with Spurr's resin (1:1 mix) for 30 minutes and then in Spurr's resin overnight. Sections were then sandwiched between two sheets of Aclar film and polymerized at 60°C. Semi-thin sections 1 µm thick were collected from the resin-embedded sections and lightly counter-stained with toluidine blue before mounting onto slides.

Imaging

Imaging was performed on an Olympus AX-80 microscope powered by Olympus cellSens or MagnaFire software, or a Zeiss LSM with Zen software.

Reanalysis of tissue from previous studies

Mice with PTEN deletion—We also analyzed images and tissue from our previously reported experiments (Liu et al., 2010) including data from one mouse with a complete crush lesion at T8, and one mouse with a dorsal hemisection lesion at T8 (Suppl. Fig. 7 of Liu et al, 2010). Procedures and processing methods were reported in Liu et al. (2010). Briefly, newborn *PTEN^{fl/fl}* mice pups that had been injected with 2 µl of AAV-Cre into the right cortex received a dorsal hemisection or crush lesion at T8 at six weeks of age. Two weeks before termination, mice received unilateral BDA injections into the right sensorimotor cortex at the following 4 coordinates with respect to bregma: 1.5 mm lateral, and 1.0 and 0.5mm rostral and caudal to bregma. The mouse with a crush lesion was transcardially perfused with 4% PFA 12 weeks post-injury, and the mouse with a dorsal

hemisection lesion at T8 was likewise terminated 8 weeks post-injury. Sagittal spinal cord sections 25 μm thick from the mouse with a dorsal hemisection lesion at T8 were stained to detect BDA via streptavidin-horseradish peroxidase binding and TSA Cyanine 3 staining (Perkin Elmer). Laterality of labeled axons was assessed in images of the serial sagittal sections in Supp. Fig. 7 of Liu et al. (2010).

The spinal cord from the mouse with a crush lesion was sectioned in the sagittal plane at 50 μm using a Vibratome®. Floating sections were stained to detect BDA with DAB-Ni staining as described above. Eight adjacent serial sections were embedded into resin. To enhance visualization of axons through their extent in the thick resin-embedded sections, a stack of images of each section was captured under light microscopy and then made into a projection using ImageJ. Tracings were made of the BDA-labeled axons in each projection using Adobe Photoshop, rainbow color-coded according to tissue depth by section, and the tracings were aligned and superimposed onto a background image containing the central canal.

Mice with AAV-shPTEN injections—We also analyzed images from a previous study demonstrating that shRNA knock-down of PTEN enhances CST regenerative growth in mice (Zukor et al., 2013). This included 4 mice with a complete crush lesion at T8 that were terminated 8 weeks following injury. Mice had been injected with BDA at the following cortical coordinates: 1.5mm lateral and 0.5 mm anterior, 0.0 mm, 0.5 mm, and 1.0 mm caudal to bregma. BDA labeling was detected with streptavidin-horseradish peroxidase binding and TSA Cyanine 3 staining (Perkin Elmer).

Quantification

Laterality—We defined the laterality index as the number of contralateral axons divided by the number of axons on both sides. Here, the term “contralateral” refers to the cortex of origin.

$$\text{Laterality index} = \text{contralateral axons} / (\text{contralateral axons} + \text{ipsilateral axons})$$

To quantify CST laterality at the lesion level and in caudal grey matter, sagittal sections of the low thoracic spinal cord were visually overlaid with 4 dorsal-ventral lines spaced 400 μm apart, beginning at the lesion epicenter. For some mice, the line at the lesion epicenter was slightly rotated to match the dorsal-ventral axis of the lesion. In specimens with a cavity within the lesion, the center of the lesion cavity was defined as the lesion epicenter. The number of axons crossing these lines was counted at the lesion epicenter and in caudal grey matter both ipsilateral and contralateral to the injected cortex. Uninjured spinal cords were analyzed similarly. Laterality was calculated from every section from mice with a dorsal hemisection lesion, every section through the middle 400 μm in reanalysis of one mouse with a crush lesion (Liu et al., 2010), and every 4th section for mice in the present crush lesion study and reanalysis of mice with a crush lesion and PTEN knockdown (Zukor et al., 2013).

Laterality of CST axons in the white matter of non-injured mice was quantified in cross sections. DAB-Ni-stained axons in the white matter outside of the dorsal column were quantified for each side in resin-embedded sections 50- μm thick. As CST axons are densely

packed in the ventral aspect of the dorsal column, semi-thin sections 1 μm thick were used to quantify DAB-Ni-stained axons in the dorsal column.

BDA labeling of PTEN-deleted neurons—Images of coronal brain sections fluorescently stained for PTEN and BDA were imported into Adobe Photoshop and a 300 μm x 300 μm square was laid over the BDA injection area. The number of PTEN-negative neurons labeled with and without BDA were counted within the square to estimate the degree of BDA labeling of PTEN-deleted neurons. Five to six sections spanning the rostrocaudal extent of the BDA injection coordinates were sampled per case.

Mapping cortical PTEN deletion

Maps of the area of PTEN deletion in the cortex were made from spans of regions with PTEN-negative cellular profiles in 20 μm coronal sections at 400 μm intervals. Based on a minimum of 2 PTEN-negative cellular profiles within 200 μm in the dorsal cortex, the spans of regions of PTEN-negative cells were plotted on a coordinate grid of the cortex with respect to bregma. The rostro-caudal distance from the section nearest bregma was calculated based on section spacing and section thickness. The section nearest bregma was estimated based on section appearance in comparison to the Paxinos mouse brain atlas (Paxinos, 2004).

RESULTS

CST Laterality in non-injured mice

Laterality of normal CST projections was established by counting BDA-labeled CST axons in the low thoracic spinal cord after BDA injections into the left sensorimotor cortex of non-injured *PTEN^{f/f}* mice. CST axons in white matter were assessed in cross section, as in previous studies in mice and other species (Rouiller et al., 1991; Brösamle and Schwab, 1997; Lacroix et al., 2004; Steward et al., 2008; Rosenzweig et al., 2009). CST axons in grey matter were assessed in sagittal sections because with sagittal sections a dorsal hemisection lesion is present in essentially every section, which aids in evaluating CST axons extending past the lesion.

The laterality of the CST in uninjured mice is illustrated in Figure 1. As shown in the thick resin-embedded section in Fig. 1A, most BDA-labeled axons in white matter are in the ventral part of the dorsal column on the right, which is contralateral to the injected cortex. In the descriptions that follow, “contralateral” and “ipsilateral” are with respect to the cortex of origin. As shown in a semi-thin section (Fig. 1B), individual BDA-labeled axons in the ventral part of the dorsal column are almost exclusively on the contralateral side, with most of the axons forming a dense bundle that represents the main CST component. A smaller number of axons were also in the dorsolateral white matter on the contralateral side, and few labeled axons were scattered in the remaining white matter of the lateral column (not shown). In the grey matter, BDA-labeled axons were present in the dorsal horn and dorsal part of the ventral horn contralateral to the injection, with fewer axons present ipsilaterally (Fig 1A, C–D). Combined data from 5 control mice (Fig. 1E) documents that $97.9 \pm 0.7\%$ of

the labeled axons in the white matter and $88.5 \pm 1.0\%$ of the labeled axons in the grey matter were contralateral to the cortex of origin (presented as means $\pm$ SEM).

Deletion of PTEN in the cortex

To delete PTEN in the sensorimotor cortex, *PTEN^{fl/fl}* mice received unilateral (left side) injections of AAV-Cre at post-natal day 1 (P1) (Liu et al., 2010). In the Steward lab, mice as adults received spinal cord injuries as described below, and then ~8 weeks later received BDA to trace the CST. Because we were interested in assessing the distribution of axons from neurons lacking PTEN, it was important to assess whether the BDA injections targeted the region of the cortex in which PTEN had been deleted. For this assessment, coronal sections through the cortex were immunostained for PTEN and stained for BDA. Consistent with previous studies, we did not observe BDA-labeled CST axons in the ventral column ipsilateral to the injected cortex in the expected position of the ventral CST if one was present.

As illustrated in Figure 2, the area of PTEN deletion in the left cortex was clearly evident in sections immunostained for PTEN due to the presence of pyramidal cell-shaped profiles in layer V that were devoid of chromogenic or fluorescent PTEN staining (Fig. 2A, C). Other smaller cells in the area, especially medium sized neurons in layers III-IV, were PTEN positive. This differential cellular and layer deletion of PTEN is consistent with AAV2 tropism (Watakabe et al., 2015). The characteristic staining pattern was distinctly different than the staining on the non-injected cortex on the right side (Fig. 2B, D). We show elsewhere that deletion or knock-down of PTEN results in strong cellular labeling for phosphorylated ribosomal protein S6, which is a downstream marker for mTOR activation (Liu et al., 2010; Zukor et al., 2013; Lewandowski and Steward, 2014). The BDA in this case clearly targeted the area containing PTEN-negative neurons, as shown with fluorescent staining (Fig. 2E–G). Using this approach, we assessed the areal overlap between BDA injections and areas of PTEN deletion in the individual mice included in this study, as described below. To estimate the degree of BDA labeling of PTEN-deleted neurons, we counted the number of PTEN-negative neurons labeled with and without BDA within a $300 \mu\text{m} \times 300 \mu\text{m}$ square of the BDA injection area, and found $37.7 \pm 4.3\%$ of these PTEN-negative neurons were labeled with BDA (mean $\pm$ SEM, N = 6).

Laterality of CST regenerative growth in *PTEN^{fl/fl}* mice with a dorsal hemisection lesion at T12

PTEN^{fl/fl} mice injected with AAV-Cre received a dorsal hemisection lesion at T12 to sever the dorsal and dorsolateral CST (Zheng et al., 2006; Steward et al., 2008). BDA was injected into the left cortex to label CST axons 8 weeks after injury, and mice were terminated 10 weeks post-injury. Two of the eight mice were not included in the analysis because of inadequate BDA labeling or having an incomplete lesion with spared labeled axons in the dorsal white matter. The remaining six cases had lesions extending down to the central canal and there were no BDA-labeled axons continuing longitudinally within either the dorsal column of white matter or the dorsal part of the lateral white matter, indicating complete lesions. Consistent with previous studies, no labeled axons were observed coursing

longitudinally in the ventral white matter through the rostro-caudal extent of the sagittal sections in the expected position of ventral CST axons if present.

In all six mice, BDA-labeled CST axons extended caudal to the lesion in a pattern comparable to what was reported in Liu et al. (2010). One case is shown in Figures 3 and 4. Serial sagittal sections in Figure 3 illustrate the laterality of BDA-labeled CST axons. Rostral to the lesion, BDA-labeled axons are mostly on the side contralateral to the injected cortex (sections #1–21), with some axons present on the ipsilateral side (sections #23–44). Large numbers of labeled axons were present just rostral to the lesion as described in Liu et al. (2010). In this case, most of the BDA-labeled axons that extend beyond the injury are on the ipsilateral side, in contrast to the contralateral rostral labeling. Higher magnification views of the midline section and 3 adjacent ipsilateral sections in Figure 4 depict axons extending from the lesion. Axons caudal to the lesion extended nearly exclusively in the grey matter, with few axons extending beyond 1.5 mm caudally. No axons were observed extending ~3mm caudal to the lesion in any of the 6 analyzed specimens with regenerative growth.

Because the spinal cord is an irregular cylinder, longitudinal sections at the lateral extremes of the spinal cord are often lost, and thus axons coursing in the lateral extremes can be missed. As most axons extending caudal to the lesion were in the grey matter, we limited our quantification of the laterality of axons caudal to the lesion to the grey matter to maximize consistency in quantification between mice.

In the case in Fig. 3 & 4, 34.3% of the axons in the grey matter caudal to the lesion were on the contralateral side. Laterality for the group varied, however, with the majority of axons extending caudally on the ipsilateral side in 3 mice (ratio >0.5), and on the contralateral side in 3 others (ratio <0.5, see Fig. 4E).

We wondered whether the distribution of axons caudal to the injury correlated with the point at which the axons extended through the lesion level. In the 3 mice in which the majority of regenerated axons were contralateral, axons extended through or around the lesion mostly on the contralateral side in two and on the ipsilateral side in one (Figure 4E). Similarly, in the 3 mice in which the majority of regenerated axons were ipsilateral, axons extended through the lesion level on the contralateral side in two and on the ipsilateral side in one. Thus, the point at which axons cross the lesion does not predict the side on which they extend caudally.

Regenerative growth in the white matter

Though most regenerative growth was in the grey matter, some axons caudal to the lesion extended from the grey matter into the spinal cord white matter, mostly in the ventral column and occasionally in the dorsal column. A few axons also extended into the ventral column rostral to the lesion and extended caudally past the lesion. To estimate the proportion of axons extending in the white matter, we counted the labeled axons in the ventral and dorsal columns 400 μ m caudal to the lesion and divided this number by the total number of labeled axons at that level. In the mouse with the highest proportion of axons in the caudal ventral and dorsal columns, 15 of 135 axons (11%) were in the white matter.

Figure 5 illustrates 2 examples of axons extending caudal to a lesion in the white matter. The section in Fig. 5A–B is from the side contralateral to the cortical injection and ~30 μ m from midline. An axon in this section extends caudal to the lesion in the ventral column, bypassing the lesion in the ventral column as has been described previously (Steward et al., 2008). This axon extends a short distance farther in the adjacent sections, and could not be followed beyond about 1 mm caudal to the lesion. It is noteworthy that none of the axons that extended beyond the lesion gave rise to the sort of elaborate terminal arbors that were reported by Steward et al. (2008). Fig. 5C–D are of an ipsilateral section ~210 μ m from midline, showing an axon extending caudal to the lesion that enters the ventral column.

Axons bypassed the lesion via the ventral white matter in 3 of the 6 mice; in 2 cases, axons bypassed via the ventral column, and in two cases, axons bypassed via the ventral part of the lateral column. In each case the ventral axons bypassed the lesion on the contralateral side. Axons entering the ventral white matter from the grey matter caudal to the lesion were observed on the ipsilateral side in 1 mouse (Fig. 5C–D), and on the contralateral side in 2 other mice. Thus, axons bypassing the lesion were only observed on the contralateral side, but axons entered the white matter caudal to the lesion on both sides. The fact that none of the ventral column axons that bypassed the lesion originated on the ipsilateral side indicates that these are not spared ventral CST axons because these would be ipsilateral (Steward et al., 2008).

Caudal-most extensions of regenerated axons

A recent study has reported prolonged regenerative growth of CST axons after PTEN deletion in the chronic injury period (Du et al., 2015). Thus, we wondered whether the axons that extended furthest caudally were still elongating. Although it is impossible to determine this from static images, one hint of continuing elongation would be that the ends of the axons bore a resemblance to growth cones or the ends of axons that are known to be regenerating. For example, Jin et al. (2009) used live imaging to identify axons that were actively regenerating in the lamprey spinal cord following injury, and showed that the growing tip was a simple lance tip-like structure with a larger diameter than the axon shaft. Accordingly, using the collection of serial sagittal sections from cases with dorsal hemisection injuries, we identified the termini of the CST axons that extended furthest caudally. Figure 6 illustrates a gallery of examples, with low power images illustrating the distribution of axons with the caudal-most extensions marked by arrows (Fig. 6A,C,E,G, and I). Figure 6B,D,F,H, and J illustrate confocal images of the axon tips at high magnification. In all cases, the caudal tips of the axons ended in structures with a somewhat larger diameter than the parent axon shaft, some of which had lance tip-like shapes that were similar in appearance to those described by Jin et al. It was noteworthy that in segments just caudal to the injury, CST axons exhibited extensive branching and sometimes gave rise to complex arbors whereas none of the axons that extended furthest caudally ended in complex terminal arbors.

Laterality of CST regenerative growth in PTEN-deleted mice from previous experiments

To expand the data set, we also analyzed the laterality of regenerative growth from 2 additional mice from experiments reported in Liu et al. (2010). One of the *PTEN*^{fl/fl} mice

injected with AAV-Cre had received a complete crush injury at T8 and was terminated 12 weeks later, and the other had received a dorsal hemisection lesion at T8 and was terminated after 8 weeks. Both mice had received unilateral cortical injections of BDA to label CST axons.

Laterality of labeled axons at and caudal to the lesion in the mouse with a dorsal hemisection lesion at T8 was assessed from serial sagittal images in Suppl. Fig. 7 of Liu et al. (2010). This mouse had been previously highlighted as having regenerated CST axons extending bilaterally caudal to the lesion. In this mouse, 78.5% of the labeled axons crossed the lesion on the contralateral side, and 62.9% were contralateral in the caudal grey matter (Fig. 7B).

The mouse that received a crush injury was prepared for electron microscopy in order to assess synapse formation by axons that extend past the lesion as reported in Liu et al. (2010). For this, serial Vibratome® sections were immunostained for BDA, and labeled axons were traced in each section. Figure 7A shows a reconstruction of rainbow color-coded axon tracings through 400 µm of serial sagittal sections through the lesion. The tracing colors progress to red on the ipsilateral side and to purple on the contralateral side, with the light and dark green tracings being closest to midline. One of the axons extending caudal to the lesion forms an arbor made of segments traced in progressive rainbow colors from adjacent sections, indicating continuity. This arbor extends nearly exclusively in the grey matter. The arbor is made of tracings from sections on both sides of the central canal (red through purple tracings), reflecting bilateral extension. The red tracings are of a section with confirmed synaptic structures of regenerated BDA-labeled CST axons as reported in Liu et al. (2010); this section is ipsilateral to the injected cortex. 68.0% of the BDA-labeled axons crossed the lesion on the contralateral side, but the majority (83.4%) of axons caudal to the lesion were ipsilateral (Fig. 7B). As above, the point at which axons cross the lesion did not predict the side on which they extend caudally.

Laterality of CST regenerative growth in PTEN-deleted mice following complete crush lesions

To further extend the data set, we analyzed cases from a study performed in the He lab that assessed CST regeneration following a T8 crush lesion. AAV2/1 expressing Cre was injected into the right cortex at P0/P1, and as adults the mice received a crush lesion at T8. CST axons were labeled via injections of BDA into the right sensorimotor cortex, and mice were terminated 14 weeks post-injury. Sagittal spinal cord sections from 2 of these mice are shown in Figure 8. BDA-labeled axons extended caudal to the lesion on both the ipsilateral and contralateral sides of the spinal cord with respect to the injected cortex (Fig. 8A–C). This mouse had particularly extensive regenerative growth. One mouse had a large cavity in the lesion (Fig. 8D–F), and axons coursed around the cavity to extend caudally beyond the lesion. Laterality was calculated in the same manner as above, but from every 4th sagittal section. For calculating laterality in the spinal cord with a cavity in the lesion, the center of the lesion cavity was taken as being the lesion epicenter (Fig. 8D). In each of the 3 mice, the majority of CST axons crossed the lesion on the contralateral side (75.0, 65.7, and 71.7%

contralateral) and the majority of CST axons caudal to the injury were contralateral (72.4, 63.0, and 60.0% contralateral, respectively).

BDA injections overlapped areas of PTEN deletion

In these studies, our method to delete PTEN was to inject the viral vector into the cortex of mouse pups. Because this approach results in variable distribution of PTEN deletion in the adult cortex, it was important to confirm that BDA injections targeted areas in which PTEN was deleted. To assess this, we mapped the regions of PTEN deletion in sections that had been immunostained for PTEN and overlaid the respective cortical injection coordinates for BDA.

Fig. 9A–D shows representative cortical BDA labeling from a mouse with a crush injury. BDA labeling appeared as continuous along the anterior-posterior axis in the coronal sections, and individual injections could not be easily discerned due to the overlap of tracer diffusion from multiple injections. The mediolateral position of injections appeared consistent within each group.

For mapping regions of PTEN deletion, PTEN-negative cells were identified in fluorescently-stained sections as illustrated in Figure 2. BDA injection sites overlapped areas of PTEN deletion within 1 mm rostral and 0.5 mm caudal to bregma in each group of mice with regenerative growth (Figure 9E–G).

Laterality of CST regenerative growth in mice with shRNA knock-down of PTEN

Finally, we reanalyzed regenerative growth in mice from a previous report that used shRNA to knock down PTEN (Zukor et al., 2013). These C57Bl/6 mice received cortical injections of AAV-shPTEN as neonates, and then as adults received a complete crush lesion at T8 and unilateral BDA injections at 6 weeks post-injury and were terminated 8 weeks post-injury.

Regenerative growth was previously documented in these mice in Zukor et al. (2013). In the mouse illustrated in Figure 10, BDA-labeled axons that extended caudal to the lesion were more or less bilaterally symmetrical (47.1% contralateral, 52.9% ipsilateral). The same was true of one other mouse (49.2% contralateral, 50.1% ipsilateral). In the 2 other mice, the majority of axons caudal to the lesion were contralateral (91.4 and 100%). In all mice in the group, the majority of BDA-labeled axons extended through the lesion on the contralateral side (54.4, 68.0, 78.9, and 63.6% contralateral). Thus, mice with cortical injections of AAV-shPTEN had contralateral extension of CST axons at the lesion level, and varied laterality of CST regenerative growth caudal to the lesion.

DISCUSSION

A key aspect of motor function is that one side of the cortex controls the opposite side of the body. This contralateral control is almost certainly related to the fact that most corticospinal tract (CST) axons decussate at the spino-medullary junction and descend through the spinal cord contralateral to the cortex of origin. Here, we show that CST axons that regenerate after SCI as a result of PTEN deletion or knock-down do not exhibit this contralateral specificity. Our results reveal that most CST axons that extend caudal to the lesion course within the

grey matter rather than the white matter, and that the majority of axons were contralateral in 60% of the cases, ipsilateral in 27%, and approximately symmetrical in 13%. Thus, regenerating CST axons are frequently on the “wrong” side, and the degree of laterality differs across animals. These results highlight one aspect for how resultant circuitry from CST axons that regenerate may differ from that of the uninjured CST.

Regeneration vs. regenerative growth

PTEN deletion or knock-down has been shown to enable regenerative growth and bona fide regeneration of CST axons after SCI (Liu et al., 2010; Zukor et al., 2013; Du et al., 2015). We use the term “regeneration” as has been previously proposed, to refer to regrowth of an injured or transected axon beyond a lesion (Tuszynski and Steward, 2012). We use the more general term “regenerative growth” to describe growth extending caudal to a lesion that cannot be definitively identified as originating from an injured axon.

We confirm here that PTEN deletion enables CST axons to regenerate into and through a complete crush injury (Liu et al., 2010; Du et al., 2015). With dorsal hemisections, some axons course around the lesion, mostly via the ventral grey matter, and we cannot exclude that some growth caudal to the lesion originates from collaterals in the grey matter. Accordingly, for the dorsal hemisection model, extension caudal to the lesion is termed regenerative growth.

CST laterality

Our quantitative analysis revealed that ~98% of the BDA-labeled CST axons in the white matter of the mouse thoracic spinal cord are contralateral to the cortex of origin. This is consistent with previous studies in rats, where ~98% of CST axons in the white matter at cervical levels are contralateral (Rouiller et al., 1991). Based on the pattern of BDA labeling in uninjured mice with cortical deletion of PTEN, there are no apparent differences in the lateral specificity of the CST due to PTEN deletion alone (Liu et al., 2010).

In our analysis, ~89% of the BDA-labeled CST axons in the grey matter of the mouse thoracic spinal cord were contralateral. Rouiller et al. (1991) report that based on axon density in the cervical grey matter and retrograde labeling in the cortex following unilateral injections of retrograde tracer into the spinal cord, ~96–98% of the CST contribution in rat comes from the contralateral cortex. In mice, reports based on unilateral injections of retrograde tracers into the lumbar spinal cord indicate ~80% (Yokoyama et al., 2001) and ~98% (Iwasato et al., 2007) of the CST contribution comes from the contralateral cortex. Our results from CST axon density in the grey matter are intermediate between these values.

It is possible that our quantification underrepresents the extent to which CST axons in grey matter are normally contralateral. When the density of labeled axons is high, axons overlap and may be missed in the most densely labeled regions of contralateral grey matter. Potential underrepresentation is unlikely in assessments of the laterality of regenerative growth as the axons extending caudal to a lesion were sparse and readily distinguished.

Laterality of regenerative growth is inconsistent across cases

We noted previously that CST axons induced to regenerate following PTEN manipulation extend bilaterally in caudal segments (Liu et al., 2010; Du et al., 2015), although the extent of contralateral vs. ipsilateral extension was not quantified. In the 7 mice in which laterality was assessed after dorsal hemisection, the majority of BDA labeled axons caudal to the lesion were ipsilateral in 3 and contralateral in 4.

The assessment of laterality in 3 mice that received crush injuries in the He lab revealed that the majority of the axons caudal to the injury were contralateral in all mice. This experiment performed in the He lab used AAV2/1 in contrast to AAV2/2 for all the other mice analyzed, and we cannot exclude that serotype may have an effect on laterality, though we have no speculation on how this could occur. Assessments of laterality of regenerated axons in a mouse with a crush lesion that was described in Liu et al. (2010) revealed that most axons were ipsilateral. The same was true of 2 of 4 mice with shRNA knock-down of PTEN (Zukor et al., 2013). Taken together, our data indicate that there is no lateral specificity in CST axon growth caudal to either a dorsal hemisection or contusion injury. Laterality of regeneration can range from being mostly contralateral to mostly ipsilateral in individual cases. One limitation in our study is that the groups were small. However, there were cases with balanced bilateral or mostly ipsilateral axon extension in most experiments, with 40% of all cases having mostly ipsilateral or roughly symmetrical extension. A lack of lateralized specificity of regenerating CST axons thus seems to be a generalized phenomenon and not a chance result.

A consideration regarding the laterality of regenerating axons is whether the variability is attributable to our separate AAV and BDA injections. Our BDA injections roughly overlapped regions with PTEN-negative cells (Fig. 8), but not all (~38%) of PTEN-negative cells were labeled with BDA in the BDA-injection area and some PTEN-positive neurons also labeled with BDA (Fig 2). This highlights a general limitation of tract tracing studies in that it is not possible to determine with certainty which neurons in or near an injection site actually take up sufficient quantities of the tracer to yield detectable axonal labeling. Despite this caveat, it is not obvious how differences in the degree of labeling of PTEN-negative neurons could account for the individual differences in laterality of regenerated axons. In the absence of an alternative explanation, our results suggest that the individual differences are not related to some tract tracing artifact.

Breaking the rules of laterality

Other examples of CST axon growth following injury also involve loss of lateral specificity. Following a lateral hemisection in the mouse cervical spinal cord, there is an increase in the number of non-injured CST axons extending across midline (Lee et al., 2010). Unilateral pyramidotomy in rats also leads to increases in the number of non-injured CST axons extending across midline (Maier et al., 2008; Ghosh et al., 2009). Also, in mice with PTEN deletion before or after unilateral pyramidotomy, transmidline sprouting of CST axons was enhanced at segmental levels, shifting the ratio of CST axon density in the grey matter toward the ipsilateral side (Liu et al., 2010; Du et al., 2015).

Why do regenerating axons fail to follow the normal rules of laterality? One possibility is that during development, the laterality of the CST and other major pathways is determined at critical way stations, and cues determining laterality are only present at these sites at particular times during development. For example, CST axons cross in the pyramidal decussation, and repulsion of CST axons by midline expression of ephrin-B3 (Coonan et al., 2001; Kullander, 2001; Yokoyama et al., 2001) diminishes by adulthood (Omoto et al., 2011). CST axons that regenerate from a spinal cord injury site would be growing at the wrong time and place to be subject to the cues that determine laterality during development.

Alternatively, deletion or knock-down of PTEN may render axons insensitive to axon guidance cues. For example, downregulation of PTEN or expression of a dominant negative form diminishes chemorepulsive growth cone turning and collapse in vitro (Chadborn et al., 2006; Henle et al., 2013). Also, deletion or knock-down of PTEN enables axons to grow through a normally repulsive lesion (Pasterkamp and Giger, 2009; Henle et al., 2013). Against this interpretation is that mice without a spinal cord injury that had cortical PTEN deletion at postnatal day 1 (P1) did not have significantly altered CST laterality in the grey matter relative to control mice (Supp. Fig. 3 in Liu et al., 2010). CST axons are still growing at P1 in rodents (Stanfield, 1992; Gianino et al., 1999), so if deletion of PTEN disrupted growth specificity, one would expect disruption of normal laterality.

In consideration of whether the timing of PTEN deletion affects the laterality of regenerating axons, Du et al. also observed bilateral extension of regenerating CST axons in mice with chronic SCI that had PTEN deletion in adulthood (2015). Thus, the lack of contralateral specificity is not tied to the timing of post-birth PTEN deletion.

Implications for function

If normal voluntary motor function depends on lateral specificity of CST projections, it follows that regenerative growth on the “wrong” side might not be optimal for restoring function. In particular, one prediction is a loss of ability to voluntarily control one limb independently. In this regard, previous studies have correlated bilateral CST projections with a hopping gait in EphA4-null mice (Dottori, 1998; Coonan et al., 2001), ephrin-B3-null mice (Kullander, 2001; Yokoyama et al., 2001), and DCC-null mice (Finger et al., 2002). An alternative interpretation, however, is that the hopping gait is attributable to misformation of the spinal cord’s central pattern generator (CPG) (Kullander, 2003; Iwasato et al., 2007; Rabe Bernhardt et al., 2012).

In rats, knock-down of PTEN with AAV-shRNA combined with injection of salmon fibrin into a cervical dorsal hemisection enhances regenerative growth and recovery of forelimb motor function (Lewandowski and Steward, 2014). Notably, enhanced recovery was of both forelimbs, despite PTEN knock-down being limited to one cortex. In another study involving *PTEN^{fl/fl}* mice, however, conditional genetic deletion of PTEN in one cortex led to enhanced motor recovery of the contralateral forepaw (Danilov and Steward, 2015). Critically, both of these studies involved group comparisons. Because laterality of CST axon growth varies across animals, it will be important in future studies to relate recovery of individual limbs to the laterality CST axon growth on a case-by-case basis.

In terms of overall recovery of function, inability to voluntarily move each limb independently is almost certainly better than inability to move at all. Also, it may be possible to learn to use circuitry that is somewhat abnormal. For instance, training improves motor recovery after stroke (Nudo et al., 1995, 1996; Kleim et al., 1998; Biernaskie and Corbett, 2001), as well as motor performance after SCI (Edgerton et al., 2001; Fong et al., 2005). Rats that recovered forelimb function with PTEN deletion and salmon fibrin (Lewandowski and Steward, 2014) were tested extensively over the postoperative period, which is also a training experience. Thus, learning may enable use of regenerated connections regardless of their laterality. It remains to be seen whether lack of lateral specificity of regenerative growth limits recovery in individual cases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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From experiments using PTEN deletion or knock-down to enhance CST regeneration, we show that laterality of CST extension beyond a lesion is sometimes mostly ipsilateral, mostly contralateral, or similar between sides. This variability highlights how resultant circuitry from regenerating axons may differ from that of the uninjured CST.

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Figure 1.

CST laterality in the non-injured mouse. A, Resin-embedded cross section from the low-thoracic spinal cord showing the distribution of nickel-enhanced DAB-Ni staining of BDA-labeled CST axons. Note that the vast majority of BDA-labeled CST axons are on the right side, contralateral to the injected cortex. B, Semi-thin section counter-stained with toluidine blue showing that the main component of CST axons are almost exclusively on the contralateral side at the base of the dorsal column. C–D, Sagittal sections showing fluorescent staining of BDA-labeled CST axons in the ipsilateral (C) and contralateral (D) grey matter. E, Laterality of the CST in the white matter and grey matter for 5 non-injured *PTEN^{fl/f}* mice. The laterality index is defined as the ratio: (contralateral axons) / (ipsilateral axons + contralateral axons). Ipsi, ipsilateral; Contra, contralateral; GM, grey matter; WM, white matter. Error bars indicate SEM. Scale bars, 200 μ m (A, C–D), 50 μ m (B).

Figure 2.

Deletion of PTEN in the cortex. Chromogenic (A–B) and fluorescent (C–D) PTEN staining from an adult *PTEN^{fl/f}* mouse reveals areas with cellular shapes devoid of PTEN in the cortex injected with AAV-Cre at P1 (inset, A; arrows, C) in contrast to the right cortex that had no injection (inset, B; D). Staining for BDA which was also injected into the left cortex (E, enlarged in G) revealed BDA-labeling of some cells devoid of PTEN (white arrows, F–G), and BDA-labeling of other cells with PTEN staining (black arrows, F–G). Note the hole

in the tissue that is consistent with the cross section of a blood vessel (arrowheads) and not a PTEN-deleted cell. Roman numerals indicate cortical layers. Scale bars, 200 μ m.

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Figure 3a

Figure 3b

Figure 3c**Figure 3.**

Greyscale serial sagittal sections from one mouse showing regenerative growth of CST axons extending caudal to a spinal cord lesion mostly on the ipsilateral side. The lesion intersects midline in #22; the side of the spinal cord ipsilateral to the injected cortex is in #23–44. The laterality index measured for the regenerative growth in this specimen was 0.34. Higher magnification images from sections #22–25 are presented in Figure 4. cc, central canal. Scale bar, 500 μ m.

Figure 4.

Regenerative growth of CST axons ipsilateral to the injected cortex. A–D, BDA-labeled axons extend caudally from the lesion in the dorsal spinal cord at midline (A) and on the ipsilateral side (B–D). Note that axons extend caudally nearly exclusively in the grey matter (marked by arrowheads). E, Laterality of axons in the grey matter in control mice and at the lesion level or in caudal grey matter in mice with regenerative growth. The mean and SEM are indicated for the control mice. Data in grey correspond to the specimen depicted in A–D. Mice were injected with AAV2-Cre and survived 10 weeks post-injury. Panels A–D were each adjusted for contrast to enhance the grey matter. D, dorsal; V, ventral; R, rostral, C, caudal; Regen, regenerative growth. Scale bars, 200 μ m.

Figure 5.

CST axons sometimes extend into white matter caudal to the lesion on either side of the spinal cord. A–B, Greyscale images from a sagittal section in the spinal cord contralateral to the injected cortex showing an axon bypassing the lesion by extending caudally in the ventral column (arrowheads). C–D, Overlaid images from a sagittal section in the ipsilateral spinal cord showing an axon extending caudal to the lesion that enters the ventral white matter. D, dorsal; V, ventral; R, rostral, C, caudal. Scale bars, 500 μm (A), 200 μm (B–D).

Figure 6.

Gallery of the caudal-most tips of regenerated axons. Termini of the CST axons that extended furthest caudally were identified in the collection of serial sagittal sections from cases with dorsal hemisection injuries. A,C,E,G, and I illustrate examples from 3 different cases; arrows point to the caudal-most tips in each section. B,D,F,H, and J illustrate confocal images of the tips indicated by arrows. Scale bar represents 1 mm for A,C,E,G, and I, and 130 μ m for B,D,F,H, and J.

Figure 7.

Laterality of CST regenerative growth caudal to a T8 dorsal hemisection lesion and a T8 crush. A, CST regeneration through a T8 crush lesion. Tracings of BDA-labeled axons are rainbow color-coded by tissue depth in 50 µm increments and overlaid onto a micrograph of one section containing the central canal. The light green tracings are from axons in the background image; the light green and dark green tracings are closest to midline. The yellow, orange, and red axons are on the ipsilateral side with respect to the injected cortex; the cyan, blue, and purple tracings are on the contralateral side. The section with axon tracings made in red was used to confirm the presence of synapses of regenerated axons by electron microscopy in a previous study (Liu et al., 2010). Laterality was calculated by the crossings of the tracings and the superimposed white lines at the lesion and caudal to the lesion. B, Laterality of axons at the lesion level and of caudal regenerative growth in the grey matter of the mouse with a T8 crush lesion from panel A and the mouse with a T8 dorsal hemisection from Suppl. Fig. 7 in Liu et al (2010). cc, central canal; D, dorsal; V, ventral; R, rostral, C, caudal; ipsi, ipsilateral; mid, midline; contra, contralateral; Regen, regenerative growth. Scale bars, 300 µm.

Figure 8.

Laterality of CST regenerative growth in mice with a T8 crush lesion and cortical PTEN deletion. BDA is shown in magenta (A–C, E–F) or white (D), and GFAP is shown in green (A–C, E–F). A–C, Sagittal sections at 240 μm intervals from a mouse with extensive regenerative growth of BDA-labeled axons caudal to the crush lesion. Axons extend on the ipsilateral side (A), at midline (B), and on the contralateral side (C). D–F, Regenerative growth on the contralateral side in one mouse with a large cavity in the lesion. Sections are 120 μm from midline (D, E) and 360 μm from midline (F). Superimposed vertical lines (D) show the locations used for calculating laterality at the lesion epicenter based on the cavity center (orange line) and of caudal regenerative growth (white lines). Note that D and E are the same section. G, Laterality of axons at the lesion level or in caudal spinal grey matter through every 4th sagittal section (120 μm intervals). Data in grey correspond to the case depicted in A–C; § indicates the case in D–F. These mice were injected with AAV1/2-Cre and were killed 14 weeks post-injury. D, dorsal; V, ventral; R, rostral, C, caudal; cc, central canal; Regen, regenerative growth. Scale bar, 500 μm.

Figure 9.

Overlap of BDA injection coordinates and areas of PTEN deletion.

A-D, Coronal sections showing cortical BDA labeling +0.8 mm (A), 0.0 mm (B), -0.8 mm (C), and -1.6 mm (D) from bregma from BDA injections targeted at 1.5 mm lateral to midline. This mouse had a spinal crush lesion and 6 BDA injection coordinates from 1.0 mm anterior to 1.5 mm posterior to bregma. E-G, Maps of BDA injection coordinates and cortical PTEN deletion show the spans of regions with PTEN-negative cells as represented by colored bars for 3 mice with dorsal hemisection and mostly ipsilateral regenerative growth (E) or mostly contralateral regenerative growth (F), and 3 mice with a crush lesion and mostly contralateral regenerative growth (G). Locations of BDA injection coordinates are overlaid in transparent red circles. Cyan lines indicate the rostro-caudal extent of data presented, and each bar color within each panel represents data from a different mouse. Data in E and F are from cases presented in Figure 4 and AAV-Cre and BDA injected into the left cortex; data in G are from cases presented in Figure 8 and AAV-Cre and BDA injected into the right cortex. Dorsal Hx: dorsal hemisection; Ipsi, ipsilateral; Contra, contralateral; Regen, regenerative growth. Scale bar, 1mm.

Figure 10.

Laterality of CST regenerative growth caudal to a T8 crush lesion in mice with cortical AAV-shPTEN injections. A–F, Sagittal sections at 120 μm intervals showing BDA-labeled axons (red) extending caudally from the crush lesion on the ipsilateral side (A–B), at midline (C), and on the contralateral side (D–F). GFAP is shown in green, and nuclei in blue. G, Laterality of axons at the lesion level or in caudal spinal grey matter through every 4th section (120 μm intervals). CST regeneration was previously documented from these cases in Zukor et al (2013). Data in grey correspond to the case depicted in A–F. D, dorsal; V, ventral; R, rostral, C, caudal; cc, central canal; Regen, regenerative growth. Scale bar, 500 μm.

Table 1

Experiments.

Manipulation and Lesion	Mouse Strain	AAV injection	Lab
Non-injured controls	<i>PTEN^{fl/f}</i>	none	Steward
PTEN Deletion + T12 Dorsal Hemisection	<i>PTEN^{fl/f}</i>	AAV2-Cre	Steward
PTEN Deletion + T8 Crush	<i>PTEN^{fl/f}</i>	AAV2/1-Cre	He

Table 2

Reanalyzed Tissue and Data from Prior Studies.

Manipulation and Lesion	Mouse Strain	AAV injection	Lab	Publication
PTEN Deletion + T8 Dorsal Hemisection	<i>PTEN^{fl/f}</i>	AAV2-Cre	He	Liu et al., 2010
PTEN Deletion + T8 Crush	<i>PTEN^{fl/f}</i>	AAV2-Cre	He	Liu et al., 2010
PTEN Knock-down + T8 Crush	<i>C57Bl6</i>	AAV2-shPTEN	He	Zukor et al., 2013

Table 3

Primary Antibodies.

Antigen	Antibody Type	Company, Catalog #	RRID	Dilution
GFAP	rabbit, polyclonal	Dako, Z0334	AB_10013382	1:500
PTEN	rabbit, monoclonal	Cell Signaling, 9188S	AB_2253290	1:250