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Corticospinal tract regeneration: Genetically labeled mice and wiring of regenerative growth

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Peer reviewed|Thesis/dissertation
Corticospinal tract regeneration:
Genetically labeled mice and wiring of regenerative growth

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Sciences

by

Rafer Paul Willenberg

Dissertation Committee:
Professor Oswald Steward, Chair
Associate Professor Karina Cramer
Professor Leif Havton
Professor Richard Robertson

2014
It is for this reason that, once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In adult centres the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated.

Santiago Ramón y Cajal
Degeneration and Regeneration of the Nervous System, 1928
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Willenberg R, Ertürk A, Bradke F, He Z, and Steward O.
3D Imaging of CST Axons that Regenerate After Spinal Cord Injury Due to PTEN Deletion.

Willenberg R and Steward O.
YFP-labeling of non-CST axons and dense labeling of axonal arbors in grey matter limit the utility of CST-YFP mice for studies of corticospinal tract regeneration.

CST Axons That Regenerate as a Result of PTEN Deletion Form Synapses Caudal to a Spinal Cord Lesion.

Willenberg R, Ertürk A, Bradke F, He Z, and Steward O.
3D Imaging of CST Axons that Regenerate After Spinal Cord Injury Due to PTEN Deletion.
International Symposium on Neural Regeneration; Pacific Grove CA, Dec 7-11, 2011.

CST Axons That Regenerate as a Result of PTEN Deletion Form Synapses Caudal to a Spinal Cord Lesion.
Society for Neuroscience Meeting; San Diego CA, Nov 13-17, 2010.

CST Axons That Regenerate as a Result of PTEN Deletion Form Synapses Caudal to a Spinal Cord Lesion.
Reeve-Irvine Medal Symposium; Dana Point CA, Nov 11, 2010.

CST Axons That Regenerate as a Result of PTEN Deletion Form Synapses Caudal to a Spinal Cord Lesion.

Willenberg R, Sears-Kraxberger I, and Steward O.
Regenerated Corticospinal Tract Axons in Mice Form Synaptic Contacts in Segments Caudal to the Lesion.
Roman Reed Research Meeting; Irvine CA, March 10, 2010.


Professional Training

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<td>Stanford University Biology Course 163: “Neural Systems and Behavior” – TA</td>
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2006–2008  UCSD School of Medicine: Surgery Interest Group – Treasurer
2006–2008  UCSD Cycling Team:
            Collegiate Track Cycling Nationals – 2\textsuperscript{nd} Italian Pursuit,
            2\textsuperscript{nd} Team Overall (2007)
            Collegiate Mountain Bike Nationals (2006)

Stanford University

2001–2006  Stanford Cycling Team – President, Captain
2002–2005  Stanford Bike Advocates – President, Co-founder
2002–2005  Resident Bicycle Coordinator – Founder
2001–2003  Stanford Ski Team
2002–2003  Peer Academic Advisor
For centuries regeneration was thought to be unattainable in the spinal cord, but recent tools and advancements imbue optimism. This dissertation serves to guide the field in using a genetically-labeled mouse for studying CST regeneration, and to assess wiring of CST regeneration that results from PTEN deletion.

Mice designed to have yellow-fluorescent protein (YFP) expressed by the CST (CST-YFP mice) were proposed to be useful for studying CST regeneration. We show by anterograde labeling, anterograde degeneration, and retrograde labeling that some YFP-labeled axons in the spinal cord are not of the CST. We further demonstrate that dense YFP-labeled arbors impede tracing individual YFP-labeled axons such as could be used to confirm CST origins. We also found that YFP fluorescence in these mice is faint for clearing-based 3D imaging as could be used for screening regenerative responses. These results overall limit the utility of CST-YFP mice for studying CST regeneration.
Deletion of PTEN has been demonstrated to enhance CST regeneration after spinal cord injury. For regeneration to be functionally meaningful, it is critical that the axons form synapses. We assessed whether CST axons that regenerated due to PTEN deletion form synapses at the level of electron microscopy. In PTEN-floxed mice that were injected with AAV-Cre near birth to delete PTEN and as adults given a complete thoracic crush lesion, we identified synaptic structures of regenerated CST axons caudal to the lesion. This result indicates that manipulation of the PTEN pathway can produce a regenerative response that may contribute to caudal circuitry.

Toward understanding regeneration that may contribute to circuitry of motor function, the laterality of CST regenerative growth that results from PTEN deletion was assessed. PTEN-floxed mice were injected with AAV-Cre near birth and as adults given a dorsal hemisection lesion in the low thoracic spinal cord. By anterograde tracing, CST laterality in control mice was found to be predominantly contralateral to the injected cortex. Laterality of regenerative growth caudal to the lesion was found to be variable and sometimes predominantly on the ipsilateral side. These results indicate that regenerative growth does not follow the contralateral rule of the non-injured CST.
INTRODUCTION

Overview

Regeneration has long been regarded as unattainable and non-existent in the central nervous system (CNS), dating at least as far back as the 1928 doctrine by Ramón y Cajal, *Degeneration and Regeneration of the Nervous System* (Ramón y Cajal, 1928). This view is consistent with the first known record that can be called a scientific document, the possibly 4000-year-old Edwin Smith Surgical Papyrus, in which it is noted that spinal cord injury (SCI) with paralysis is “an ailment not to be treated [cured]” (Breasted, 1930; Hughes, 1988). As illustrated in the Edwin Smith Surgical Papyrus and Ramón y Cajal’s 1928 doctrine, established dogma has been that regeneration in the CNS does not occur, and those paralyzed following SCI have lost function forever.

Five decades following Ramón y Cajal’s proclamation of the incapacity of regeneration in the CNS, David and Aguayo published a finding that CNS axons can grow from a lesion site in the spinal cord into a peripheral nerve graft (David and Aguayo, 1981). This finding showed that, in fact, CNS axons can regenerate. This fundamental study is an underpinning for current CNS regeneration research, which has blossomed to include foci of inhibitors of regeneration, enhancers of regeneration, and alternative strategies. Recently, it was discovered that deletion of the gene *PTEN*, phosphatase and tensin homolog, results in regeneration of axons in the optic nerve within the CNS (Park et al., 2008), and our group has also shown that deletion of *PTEN* results in regeneration of corticospinal tract (CST) axons in the spinal cord (Liu et al.,
2010). These results imbue optimism for potential means to restore function following SCI, and mark significant progress since Ramón y Cajal’s proclamation that in the CNS “nothing may be regenerated.”

The budding field of CNS regeneration in the spinal cord has not been without faults, however, and numerous studies have shown promising results but many of them have lacked follow-up and/or replication. In fact, a replication initiative from the National Institutes of Health (NIH) has yielded successful replication of findings for only 2 of 12 originally promising pre-clinical SCI studies (Steward et al., 2012). Thus, while it is critically important that progress be made toward clinical recovery of function, it is likewise critical that positive laboratory results are veritable scientific advancement. To serve the field in guidance on using a model for assessing regeneration, this dissertation includes a critical assessment of the utility of the CST-YFP mouse, a mouse designed to have genetic labeling of the CST and proposed to be useful for studying regeneration of the CST.

Regeneration of CST axons is expected to be important for reestablishing control of voluntary motor circuitry (Tuszynski and Steward, 2012). For regenerated axons to contribute to circuitry, it is critical that they form synapses (Aubert et al., 1995; Woolf, 2003; Bradbury and McMahon, 2006; Deng et al., 2013). In consideration of the findings that PTEN deletion promotes substantial CST regeneration, this dissertation also addresses whether these regenerated axons form synapses. Further, it is reasonable to intuit that it would be optimal for CST axons to regenerate so as to have lateralized input into caudal circuitry similar to that of the intact CST. Toward understanding CST regeneration that may contribute to circuitry, this dissertation also
assesses the laterality of regenerative growth of the CST that results from PTEN deletion.

**Part I: The Corticospinal Tract**

*The Corticospinal Tract: Development and Anatomy*

The corticospinal tract descends from the cortex to the spinal cord and is recognized to control voluntary motor function (Kuypers, 1982, 2011; Lemon and Griffiths, 2005; Martin, 2005). The CST of one cortical hemisphere descends via the internal capsule to the ipsilateral cerebral peduncle, continuing through the basis pontis and into the medullary pyramids (Armand, 1982; Stanfield, 1992; Gianino et al., 1999; Kuypers, 2011). Here, most CST axons cross in the pyramidal decussation to descend in the contralateral spinal cord to finally synapse onto interneurons and α and γ motoneurons in the spinal grey matter (Kandel et al., 2000; Gazzaniga et al., 2002; Squire et al., 2002; Lemon and Griffiths, 2005; Lemon, 2008). For the CST, also referred to as the pyramidal tract, it is worth noting that essentially all CST axons pass through the medullary pyramids (Armand, 1982; Squire et al., 2002; Nielson et al., 2010). One medullary pyramid has approximately 1,000,000 axons in humans, 186,000 in cats, 70,000 in rats, and 30,000 in mice (Lassek and Rasmussen, 1940; Armand, 1982; Nielson et al., 2010). While the CST carries axons from the primary motor cortex (Brodmann area 4), premotor cortex and supplementary motor area (Brodmann area 6), the CST also carries projections from the primary somatosensory cortex (Brodmann areas 1-3) and somatosensory association cortex (Brodmann areas 5 and 7) (Armand, 1982; Gazzaniga et al., 2002; Lemon and Griffiths, 2005; Lemon, 2008).
Development of the CST is not complete until after birth. Based on experiments using anterograde tracing with *Phaseolus vulgaris leucoagglutinin* (PHA-L) or retrograde tracing with fast blue, the CST in mice has been shown to extend axons to the cervical enlargement at postnatal day 2 (P2), has branching of cervical CST axons at P4, extends pioneering axons to the lumbar enlargement by P7, and has extensive branching extending along the spinal cord by P14 (Gianino et al., 1999; Hsu et al., 2006; Richter and Roskams, 2009). The P14 mouse is only a juvenile; mice that have reached 8 weeks of age are regarded as adults. Similar results have been attained in rats (Donatelle, 1977; Jones et al., 1982; Joosten et al., 1989; Stanfield, 1992), and maturation of the CST also continues postnatally in cats, monkeys, and humans, though on a longer time-course (Martin, 2005).

The course and laterality of the CST has similarities between species, but differences remain even between similar species. Humans have ~75-90% of CST axons projecting contralaterally into the spinal cord, with most descending in the posterior lateral column, and a minor component of mostly ipsilateral axons coursing in the anterior column (Armand, 1982; Nathan et al., 1990; Kandel et al., 2000; Martin, 2005; Engle, 2010). Monkeys have ~87-88% of CST axons coursing contralaterally and ~10% coursing ipsilaterally in the same lateral location as humans, with most remaining axons coursing ipsilaterally in the anterior column (Armand, 1982; Lacroix et al., 2004; Rosenzweig et al., 2009, 2010). In rats, however, the main component of CST axons course contralaterally in the ventral aspect of the dorsal funiculus, the dorsal CST (dCST) (Armand, 1982). An additional contralateral component is in the dorsal lateral column, the dorsolateral CST (dICST), and a much more sparse ipsilateral component
has been observed in the ventral column, the ventral CST (vCST) (Brösamle and Schwab, 1997). Mice have the same contralateral dCST and dICST components (Inman and Steward, 2003; Steward et al., 2004, 2004), but existence of a ventral component is disputed. A ventral component of the CST has been reported in some strains of mice (Uematsu et al., 1996; Sicotte et al., 2003; Lang et al., 2012), but an ipsilateral vCST has not been detected by tracing in wild-type C57Bl/6 mice or genetically-modified mice on a C57Bl/6 background. Existence of a vCST may, in fact, vary between mice of different strains and even between mice within a strain (Tuszynski and Steward, 2012). The established locations of CST axons are important for defining normal locations of CST axons in intact animals, which is significant for defining criteria to be used in studies of CST regeneration (discussed in the section below).

Regeneration, sprouting, and sparing

To enhance clarity in interpreting studies in the field of axon regeneration, it has been proposed that the terms regeneration and sprouting be reserved for specific phenomena. “Regeneration” is proposed to have reserved use for describing growth from a cut axon that extends into or beyond a lesion (Tuszynski and Steward, 2012). “Sprouting” has been proposed for describing new growth from uninjured axons. The prototypical example of sprouting is that of collateral sprouting: growth of intact collateral axons into denervated areas (Woolf, 2003; Tuszynski and Steward, 2012). Whereas regeneration may recapitulate uninjured circuitry to effect recovery of function, sprouting effects recovery via anatomical reorganization and remodeling, and thus is
thought of as a form of plasticity (Weidner et al., 2001; Woolf, 2003; Bareyre et al., 2004; Bradbury and McMahon, 2006).

A critical pitfall in regeneration studies in the CNS is mistaking spared axons for ones that have regenerated, potentially leading to incorrect conclusions. To this end, several criteria have been put forth that can be used to identify and establish that an axon has regenerated (Steward et al., 2003). 1) The axon extends into a lesion or 2) other non-host environment, such as an implanted graft. 3) The axon extends from near the lesion site. 4) The axon takes an unusual course such as coursing in a location where axons of this type are not established, or coursing on the “wrong side” for a lateralized tract. 5) The distance of axon extension can be accounted for by a plausible growth rate. It is estimated that axons may take 3 weeks to grow beyond a spinal cord lesion, with further maximal extension of 1mm/day. 6) The axon has a growth cone. 7) The axon’s morphology is unusual for normal axons of its type (e.g. unusual branching and tortuous trajectory).

Notably, lesions that leave no intact tissue where axons could be spared, such as a complete transection, make it easier to establish that axons beyond a lesion could not have been spared. However, this lesion type is further disabling for the animal, and the lack of tissue sparing presents a higher barrier to regeneration (Tuszynski and Steward, 2012). Indeed, with a lesion that extends the entire cross-section of the spinal cord, there is no aspect of the cross section where axons could avoid the lesion and the resultant inhibitory glial scar (Silver and Miller, 2004; Yiu and He, 2006). Further, a complete transection model has less clinical relevance because most spinal cord
injuries in humans are crush or contusion injuries with at least some spared tissue (Tuszynski and Steward, 2012).

Reconstructions of the CST

The course of the CST is 3-dimensional, but anatomical CST studies are most often based on essentially 2-dimensional sections mounted on microscope slides. One way to derive the 3-dimensional course of axons, such as axons extending caudal to a lesion, is by reconstructing the axons from segments in serial sections. With tracings of segments from every section in a series, large arbors of axons can be constructed with 3D encoding from each section. (For an example, see Fig. 5 of Tuszynski and Steward, 2012.) Reconstructions from tracings can also provide additional useful information for regeneration studies of the CST. First, tracing an axon caudal to a lesion back to origins of a cut parent tract provides evidence that the axon had regenerated. Second, tracing an axon back to the main CST indicates that the axon is of the CST, which may be particularly useful in the presence of non-CST labeling. Of course, it is advantageous to have sparse labeling for tracing back axons in this manner. Disadvantages of creating reconstructions from sections include that it is a laborious process, and physical cutting of tissue and non-uniform stretching of tissue sections creates some degree of mis-matching of segments in the reconstruction.

An alternative method of making 3D reconstructions that avoids making tissue sections is with 3D imaging based on recent advancements in tissue clearing such as 3-DISCO (Ertürk et al., 2012a), CLARITY (Chung et al., 2013; Tomer et al., 2014), and others (Hama et al., 2011; Ertürk et al., 2012b; Chung et al., 2013; Ke et al., 2013;
Kuwajima et al., 2013; Tomer et al., 2014). In clearing-based 3D imaging of the spinal cord, a large block of unsectioned spinal cord is cleared and then optically sectioned such as with 2-photon microscopy (Ertürk et al., 2012b; Spence et al., 2014). As the volume of tissue is imaged en bloc, clearing-based 3D imaging can be used to produce a detailed and spatially continuous reconstruction of the fluorescently-labeled axons within. Further, some subsequent analysis of the imaging data can be automated (Ertürk et al., 2012b). Some protocols producing rapid clearing (Ertürk et al., 2012a, 2012b) may also be useful for screening regenerative responses in animals with fluorescently-labeled axons.

**CST labeling for studying regeneration**

Studies of regeneration following spinal cord injury often focus on the CST, and methods of visualization of CST axons have included tract tracing and histological staining. General tissue staining, like the Golgi stain (Golgi, 1873), is not specific to any axon tract, and there is no antibody that can be used to specifically label the CST. PKC$_\gamma$ can be used as a marker for bulk CST axons, but it is not specific to the CST, and it does not label CST axon terminals (Mori et al., 1990; Barritt et al., 2006; Hughes et al., 2008). The only method thus far that can be made to be specific to the CST within the spinal cord is anterograde tract tracing. The CST originates in the cortex, and thus by definition, an axon labeled with a tracer that has been transported down an axon from the cortex to the spinal cord without leaking or crossing synapses is a CST axon. Fitting these conditions is the 10,000 molecular weight biotinylated dextran amine (BDA) (Fritzsch and Wilm, 1990; Nance and Burns, 1990; Schmued et al., 1990; Reiner
et al., 2000; Ferguson et al., 2001), which has become a standard for labeling intact CST axons as well as regenerated CST axons. With the tracer’s limited diffusion volume and lack of synaptic crossing, a stereotactic injection of the tracer into the motor cortex will result in labeling of CST axons throughout their extent.

There are drawbacks to tract tracing, however, including: 1) labeling requires an injection of tracer, which means a second manipulation. 2) BDA labeling is inherently variable leading to a variable number of labeled axons in the tracts (Zheng et al., 2006). 3) Transport of tracer requires time and the tracer dissipates over time, which means that the tracer must be injected at defined time periods prior to the animal being killed (Zheng et al., 2006). 4) Tracers are transported in a wave, so in long axons, different parts of the axon may be labeled at different times. Finally, 5) injections of BDA at the time of a spinal cord injury can lead to artifactual labeling of non-CST axons near the injury site (Steward et al., 2007; Lee et al., 2009). As previously mentioned, this artifactual labeling has previously led to an erroneous report of extensive regeneration (Steward et al., 2007).

The importance of specific labeling

Misinterpretation of findings can result in misleading a field, potentially misdirecting time and resources on experiments based on faulty conclusions. With consideration of the consequences of misdirection and a lack of replication of findings for 10 of 12 studies (Steward et al., 2012) and lack of replicated findings of enhanced regeneration with deletion of Nogo (Steward et al., 2007; Lee et al., 2009), it is critical that axons are confirmed to have definitively regenerated to conclude the presence of
regeneration. To follow the definition of regeneration as being growth of a severed axon that extends into or beyond a lesion (Tuszynski and Steward, 2012), it becomes necessary to demonstrate that this growth has occurred, and to confirm that the axon was severed in the first place. The problem of non-specific labeling in this case is actually twofold for studying regeneration of the CST. First, regeneration of the CST is not limited to expected CST locations. In fact, as mentioned above one of the criteria that can be used for identifying a regenerated axon is that it takes on an unusual course (Steward et al., 2003). Thus, labeling of a fiber in a non-CST location fulfills one of the criteria for establishing that an axon is regenerated, and hence labeling of non-CST axons presents a confound in assessing CST regeneration. Second, to ascertain that regeneration observed is CST regeneration, it must be possible to conclude that the regenerated axons are actually CST axons. With non-specific labeling, this conclusion might not be possible in all regions of the spinal cord outside of the densely bundled dCST. As well, the CST is particularly refractory to regeneration in comparison to other tracts (Blesch and Tuszynski, 2009; Liu et al., 2010), so it remains possible that regeneration of non-CST axons may occur without regeneration of CST axons, and thus non-specific labeling presents the confound that regeneration observed may not be of the CST.

**Part II: CST-YFP mice**

*CST-YFP mice: design and expression*

For the limitations presented by tract tracing to label the CST (“CST labeling”, above), having the CST genetically labelled would be advantageous. Such is the
rationale for Bareyre et al. in creating transgenic mice that express yellow fluorescent protein (YFP) in the CST—the CST-YFP mice. Beyond the advantages over the aforementioned limitations with tract tracing, additional proposed advantages of CST-YFP mice are a near complete labeling of the CST, and a lack of labeling variability between mice (Bareyre et al., 2005).

CST-YFP mice are designed to have a *Thy1* promoter drive expression of YFP only in cells that express Emx. The parental strains to produce the CST-YFP mouse are *Thy1-STOP-YFP* mice and *Emx-Cre* mice, both of a C57Bl/6 background (Bareyre et al., 2005). Thy1 is thymus cell antigen 1, and in mice is found in cells of the immune systems and neurons (Vidal et al., 1990 p.1; Feng et al., 2000). Non-neural sequences were deleted from a *thy1* vector that was used to create *Thy1-STOP-YFP* mice, in which the Thy1 promoter will only drive expression of YFP in neurons following excision of the stop sequence by Cre recombinase (Cre) (Feng et al., 2000; Nagy, 2000; Buffelli et al., 2003). The gene *Emx1* is *empty spiracles homeobox protein 1*, which encodes a transcription factor that has been found in the mouse embryonic cortex and olfactory bulbs (Simeone et al., 1992). Thus, in a mouse containing both gene sequences *Emx-Cre* and *Thy1-STOP-YFP*, expression of Emx1 in any cell should result in excision of the STOP sequence by Cre, and if the same cell expresses Thy1, it should result in labeling of this cell with YFP. This approach was taken by Bareyre et al. to restrict YFP labeling to neurons of the forebrain to yield specific labeling of the CST in the spinal cord. One caveat of this approach, however, is that it relies on the assumption made by Bareyre et al. that *Emx1* and hence Cre is “specifically expressed in the forebrain” (Bareyre et al., 2005). A previous study, in fact, reported expression of *Emx1* in the
additional locations of the developing diencephalon and mesencephalon (Briata et al., 1996). Thus, based on the lack of specificity of Emx1 for the forebrain, it is expected that Thy1-expressing cells in the mesencephalon are also labeled with YFP, and it is plausible that some of these cells project to the spinal cord (e.g. projections from the red nucleus) and result in YFP-labeling of non-CST axons. Labeling of non-CST axons would present a confound that would greatly limit the utility of CST-YFP mice for studies of CST regeneration.

Bareyre et al., however, report that YFP-labeling in CST-YFP mice is both complete and specific for the CST. They report labeling of 92% of layer V pyramidal cells in the cortex, indicating near-complete labeling, and in the spinal cord they report YFP-labeled axons in the ventral aspect of the dorsal funiculus and the dorsal aspect of the lateral funiculus, locations consistent with the respective dCST and dlCST. However, they also report YFP-labeling in the ventral column, where a mouse vCST is disputed to exist, and the mid-lateral column, where the CST is not known to exist in mice. As well, they report the existence of YFP-labeled cell bodies and projections in the spinal grey matter, indicating labeling in the spinal cord that is not specific to the CST (Bareyre et al., 2005).

**Considerations for utility**

The labeling of non-CST projections in the grey matter alone requires distinction of CST axons from these non-CST projections for CST studies. As regenerated CST axons are known to course through grey matter and are not limited to white matter locations (Steward et al., 2008; Liu et al., 2010), labeling of some non-CST projections
in the grey matter presents an initial hurdle for the assessment and analysis of CST regeneration. The non-CST labeling in grey matter further raises suspicion for the potential of labeling non-CST axons in the white matter, particularly as YFP-labeled axons were observed in non-CST locations. In consideration of CST-YFP mice for studying CST regeneration, it is important to determine if the CST in these mice extends into locations not established for the mouse CST, or if YFP-labeled axons in non-established CST locations are a result of non-CST labeling. Further, if non-CST axons coursing longitudinally are labeled with YFP, it may be impossible to discern which axons are CST axons outside the main CST as minor CST components overlap with axons of longitudinal tracts (e.g. the dICST and rubrospinal tract) (Inman and Steward, 2003; Steward et al., 2004; Liang et al., 2012; Watson and Harrison, 2012). A further confound is that the CST is particularly refractory to regeneration (Blesch and Tuszynski, 2009; Liu et al., 2010), so it is possible that regenerative responses of other tracts may be observed and incorrectly attributed to being of the CST.

An additional consideration with CST-YFP mice is that extensive labeling may impede analysis for regeneration. As mentioned above, tracing axons is valuable in axon regeneration studies because tracing axons back to a cut parent tract can be used to support that the axons had regenerated (Tuszynski and Steward, 2012). Further, tracing axons back to the main CST bundle could be used to confirm that individual axons are of the CST, which would be useful among the non-CST axons in the grey matter (Bareyre et al., 2005).

A final consideration of CST-YFP mice is that due to having extensive fluorescent labeling, they might be useful for 3D imaging following rapid tissue clearing to screen for
regenerative responses following injury. Using these mice for screening could be advantageous for indicating interventions promoting regenerative growth before embarking into more focused and laborious studies.

Accordingly, in this dissertation CST-YFP mice are evaluated for utility in studying CST regeneration based on specificity of labeling for the CST, the traceability of YFP-labeled axons, and potential for screening with 3D imaging.

Part III: PTEN and wiring of CST regeneration

PTEN

The phosphatase and tensin homologue gene (PTEN) is a tumor suppressor gene that has a principal function of dephosphorylating phosphatidylinositol-3,4,5-triphosphate (PIP₃), an upstream activator of growth and cell survival pathways (Song et al., 2012). PTEN acts to counter phosphoinositide 3-kinase (PI3 kinase or PI3K), which converts phosphatidylinositol-4,5-bisphosphate (PIP₂) to PIP3. PIP3 can then activates AKT, in turn activating the mammalian target of rapamycin (mTOR) (Guertin and Sabatini, 2007; Song et al., 2012), and signaling further downstream pathways for cell growth. The roles of PTEN are myriad, with normal functions including regulation of adhesion, chemotaxis, invasion, growth cone guidance, and apoptosis (Yamada and Araki, 2001; Iijima and Devreotes, 2002; Henle et al., 2013).

Regeneration from PTEN deletion

In 2008, Park et al. demonstrated that deleting PTEN promotes robust axon regeneration in the mouse optic nerve, and hence promotes axon regeneration in the
CNS (Park et al., 2008). This study used PTEN<sup>fl/fl</sup> mice that had PTEN flanked by loci of recombination (floxed, fl), so that PTEN could be deleted by Cre recombinase. This study was conducted by administering intra-vitreal injections of an adeno-associated viral (AAV) vector expressing Cre (AAV-Cre) in adult mice to delete PTEN, or a vector expressing green fluorescent protein (AAV-GFP) for controls, and then performing an optic crush 2 weeks later. Two or four weeks following injury, over 1000 retrogradely-labeled axons were observed to extend 1 mm beyond the lesion in mice that had received AAV-Cre, whereas only a few axons were observed to extend past the lesion in controls.

The regenerative response was demonstrated to be mostly dependent on mTOR as shown in 2 experiments. First, administration of the mTOR inhibitor rapamycin mostly abolished the regenerative effects from PTEN deletion. Second, tuberous sclerosis complex 1/2 (TSC1/2) negatively regulates mTOR, and loss of either TSC 1 or 2 results in constitutive mTOR activation (Garami et al., 2003; Inoki et al., 2003; Tee et al., 2003). Deletion of TSC1 resulted in substantial axon regeneration in the optic nerve, though to a lesser extent than that from PTEN deletion (Park et al., 2008). Thus, these experiments suggest that mTOR has a substantial role in this regenerative response, and part of the regenerative response due to PTEN deletion acts independently of mTOR. Overall, this study demonstrated that manipulation of mTOR such as via PTEN can enhance regeneration in the CNS, and it served as proof-of-principle that manipulation of PTEN is a valid therapeutic target.

In a following study, collaborative work by Liu et al. demonstrated that deletion of PTEN results in enhanced regeneration of the CST following spinal cord injury (Liu et
al., 2010). In this study, at P1 $PTEN^{fl/fl}$ mice were injected with either AAV-Cre to delete $PTEN$ in the cortex or AAV-GFP as controls, and as adults these mice were given a thoracic dorsal hemisection or thoracic crush injury and survived for 2 and 3 months, respectively. CST axons were anterogradely traced by cortical injections of BDA. In mice that had received cortical injections of AAV-Cre, numerous CST axons extended beyond both lesion types, whereas no axons were observed to extend into or beyond a lesion in mice injected with AAV-GFP. Thus, PTEN deletion enhances regeneration of CST axons.

**Wiring of regenerated axons**

Synaptic transmission is the basis of communication between neurons that underlies all aspects of neural function (Jessell and Kandel, 1993; Purves et al., 1997; Schwartz, 2003). Thus, for regeneration to be functionally meaningful, it is critical that the regenerated axons form synapses (Aubert et al., 1995; Woolf, 2003; Bradbury and McMahon, 2006; Deng et al., 2013). Synaptic contacts can be indicated by synaptically-marked appositions at the level of light microscopy, and electron microscopy can be used to confirm definitive anatomical synapses based on the presence of synaptic structures. Accordingly, in this dissertation CST axons regenerated as a result of PTEN deletion were assessed for synapses caudal to the lesion based on the presence of synaptic structures at the level of electron microscopy.

As regenerated CST axons may represent input to caudal circuitry, the laterality of these axons may also be important for resultant function. As previously mentioned, the CST is a crossed tract, having both a predominantly contralateral course and
terminations as demonstrated in rats and primates (Rouiller et al., 1991; Lacroix et al., 2004; Rosenzweig et al., 2009, 2010). The importance of laterality of the CST is evident from cases of aberrant ipsilateral or bilateral CST projections and synkinetic mirror movements in mice and humans. Some of these cases have been tied to dysfunction of guidance molecule signalling. The Eph/ephrin receptor-ligand system primarily serves axon guidance through contact-mediated repulsion and ephrin B3 is expressed in the spinal cord midline (Harel and Strittmatter, 2006; Iwasato et al., 2007; Omoto et al., 2011). In mice with dysfunctional ephrin B3/A4 ligand-receptor signalling, the CST projects bilaterally within the spinal cord, and mice have a resultant kangaroo-like hindlimb hopping gait (Dottori, 1998; Coonan et al., 2001; Yokoyama et al., 2001). Netrin is a diffusible ligand chemotropic in axon guidance and is expressed at midline ventral to the developing pyramidal decussation (Aubert et al., 1995; McFarlane, 2000; Canty and Murphy, 2008). A mutation in the netrin-1 receptor gene deleted in colorectal carcinoma (DCC) in mice results in a lack of CST decussation at the pyramids and a hopping gait (Finger et al., 2002), and a mutation in this gene in humans has been identified as the cause of congenital mirror movements (CMM) (Srour et al., 2010). Further, 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). Additionally, in humans synkinetic mirror movements are present in Joubert Syndrome, and individuals have a decreased or lack of CST decussation (Friede and Boltshauser, 1978; Maria et al., 1999; Engle, 2010). Thus, laterality of CST axons is presumed to be important for asymmetrical motor control.
Based on the extent and consistency of laterality of the CST in primates and rats, the mammalian CST can be regarded to have a general contralateral rule. Regenerated CST axons, however, may not follow this rule. Toward understanding CST regeneration that may contribute to caudal circuitry for recovery of function, this dissertation also assesses whether regenerative growth of the CST that results from PTEN deletion maintains the contralateral rule of the intact mouse CST.

Summary

Both understanding of tools and fundamental facets of regeneration are important for research and researchers working to make advances toward recovery of function following SCI. CST-YFP mice have been proposed to be useful for assessing CST regeneration, and it is important to evaluate aspects that affect assessing regeneration of CST axons. To serve the field in guidance on using CST-YFP mice for regeneration studies, the first aim of this dissertation was to critically evaluate the utility of CST-YFP mice for studying of CST regeneration.

For regenerated axons to contribute to circuitry, it is critical that they form synapses (Aubert et al., 1995; Woolf, 2003; Bradbury and McMahon, 2006; Deng et al., 2013). Toward understanding whether CST regeneration that results from PTEN deletion may contribute to circuitry beyond a lesion, the second aim of this dissertation was to assess whether CST axons that regenerated due to PTEN deletion formed synapses.

In consideration of CST regeneration that may contribute to circuitry, it is reasonable to intuit that it would be optimal for CST axons to regenerate so as to have
lateralized input into caudal circuity similar to that of the intact CST. Accordingly, the third aim of this dissertation was to assess the laterality of regenerative growth of the CST that results from PTEN deletion. Overall, this dissertation serves to guide and advance the field of CST regeneration.

REFERENCES


CHAPTER 1

Non-specific labeling limits the utility of Cre-Lox bred CST-YFP mice for studies of corticospinal tract regeneration
ABSTRACT

Studies of axon regeneration in the spinal cord often assess regeneration of the corticospinal tract (CST). Transgenic mice have been created that selectively express yellow fluorescent protein (YFP) in cortical neurons leading to labeling of CST axons in the spinal cord—CST-YFP mice, and it was suggested that these mice would be useful for studies of CST regeneration. Because regeneration past a lesion may involve only a few axons, the presence of labeled non-CST axons compromises interpretation. We show here that in CST-YFP mice, some YFP-labeled axons are not from the CST. Specifically, YFP-labeled axons are present in regions beyond those with anterogradely-labeled CST axons, most YFP-labeled axons beyond established CST locations do not undergo Wallerian degeneration following a large lesion of the sensorimotor cortex, some rubrospinal and reticulospinal neurons are labeled with YFP, and some YFP-labeled cells in the spinal grey matter have YFP-labeled projections into the spinal cord white matter. We further demonstrate that the density of YFP-labeled axon arbors hinders tracing of single axons to their point of origin in the main descending tracts. In light of recent advances in 3D imaging for visualizing axons in un-sectioned blocks of spinal cord, we also assessed CST-YFP mice for 3D imaging and found that YFP fluorescence in CST-YFP mice is faint for clearing-based 3D imaging in comparison to fluorescence in Thy1-YFP-H mice and fluorescence of mini-ruby BDA. Overall, the non-specific and faint YFP labeling in CST-YFP mice limit their utility for assessments of CST axon regeneration.
INTRODUCTION

Studies of axon regeneration in the spinal cord often assess regeneration of the corticospinal tract (CST) following injury. These studies have commonly used anterograde tracers, which can specifically label CST axons but produce variable and incomplete labeling of the total complement of CST axons. Axonal labeling with tracer injections is transient, so the timing between tracer injection and euthanasia is critical, which complicates studies of axon regeneration after injury. As an alternative to anterograde tracing, Bareyre et al. (Bareyre et al., 2005) used a Cre-Lox breeding system to create a mouse model in which cortical neurons express yellow fluorescent protein (YFP), leading to labeling of CST axons in the spinal cord—the CST-YFP mouse. Bareyre et al. proposed that these mice would be useful for studies of regeneration of CST axons because the permanent genetic labeling is independent of tracer injections and all CST axons in these mice are labeled with YFP.

For unambiguous interpretation of CST regeneration, it is important that genetic labeling be specific to the CST. Regeneration may involve only a few axons, and these may extend into locations outside the territory of normal CST axons (Steward et al., 2003). For this reason, the presence of even a few YFP-labeled non-CST axons can compromise interpretation. In CST-YFP mice, Bareyre et al. report YFP-labeling of axons in the spinal cord in locations where the mouse CST had not been previously described (Bareyre et al., 2005). Though these axons might be CST axons outside of the main tracts (ectopic axons), another possibility is YFP labeling of non-CST axons. If so, this non-specific labeling would limit the utility of these mice for studying CST regeneration.
We show that in CST-YFP mice, YFP is not specific to the CST. First, some YFP-labeled axons in the spinal cord do not degenerate following cortical lesions; second, some neurons in the spinal cord gray matter have YFP-labeled projections into the white matter; and third, some rubrospinal and reticulospinal neurons are labeled with YFP.

In addition, although it is advantageous that most or all CST axons express YFP for assessing the entire population of CST axons, we show that the density of labeled axon arbors in the spinal cord makes it difficult to trace individual axons. This dense labeling hinders tracing axons back to the main CST bundle to determine their point of origin, an important approach for concluding that the axons actually branch from injured axons [see (Tuszynski and Steward, 2012)].

Finally, in light of recent advances in 3D imaging allowing visualization of axons in unsectioned spinal cords (Ertürk et al., 2012b), we assessed CST-YFP mice using clearing-based 3D imaging and found that nascent YFP fluorescence in CST-YFP mice is faint for 3D imaging in comparison to fluorescence in Thy1-YFP-H mice and fluorescence of mini-ruby BDA-labeled axons. Overall, our results reveal limitations of CST-YFP mice for studying CST regeneration, and highlight issues that may limit the general usefulness of genetic labeling for assessing tract-specific axons.

**MATERIALS AND METHODS**

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, Irvine.
**Mice**

CST-YFP mice were created by crossing Emx1-Cre mice [B6.129S2-Emx1\textsuperscript{tm1(cre)Krj}/J, Jackson Labs, Bar Harbor, ME] to Thy1-STOP-YFP mice [B6.Cg-Tg(Thy1-EYFP)15Jrs/J, Jackson Labs] as described by Bareyre et al. (Bareyre et al., 2005). The progeny were thus heterozygotes for both Emx1-Cre and Thy1-STOP-YFP, with the transgenic design to yield YFP expression in all cells expressing both Emx1 and neuronal Thy1. A total of 25 adult CST-YFP mice of both sexes were analyzed for this study including 4 mice that had no labeling or injury, 4 that had cortical lesions, 3 that had received Fluoro-Gold (FG) injections into the spinal cord to identify cells of origin of descending spinal pathways, and 14 that received intra-cortical injections of BDA to trace the CST. Two Thy1-YFP-H mice (one homozygous and one hemizygous) [B6.Cg-Tg(Thy1-YFP)HJrs/J, Jackson Labs] and one PTEN-floxed (Pten\textsuperscript{Pten\textsuperscript{f/f}}) mouse [C;129S4-Pten\textsuperscript{tm1Hwu}/J, Jackson Labs] with BDA labeling of CST axons were also used in imaging analysis.

**Motor cortex lesions**

CST-YFP mice (n=4) received unilateral cortical lesions to assess anterograde degeneration of CST axons in the spinal cord. Lesions were unilateral in order to detect the laterality of degenerating axons from one cortical hemisphere. Cortical lesion extent was guided by mapping CST origins based on retrograde labeling with Fluoro-Gold (see “Mapping CST origins”, below). Mice were anesthetized with an intraperitoneal injection of mixed ketamine (100 mg/kg) and xylazine (10 mg/kg). The scalp was incised and the skull overlying the dorsal right cortex was removed from near midline to
the temporals muscle. Care was taken to avoid damaging the sagittal and transverse
sutures and underlying venous sinuses. The cortex was gently aspirated using a pulled
Pasteur pipette with a tip diameter of approximately 300 µm, down to the level of sub-
cortical white matter. The ablated area extended approximately 4 mm medio-laterally
and 5-6 mm rostro-caudally. The scalp was sutured with 5-0 silk, and mice survived for
1 week before being humanely killed by an overdose of Euthasol®. Mice were
transcardially perfused with 4% paraformaldehyde in phosphate buffer (PFA), and
brains and spinal cords were post-fixed overnight and stored in 27% sucrose.

*Tract tracing*

Biotinylated dextran amine (BDA) was injected to anterogradely label axons of
the CST. Unilateral intracortical injections of mini-ruby BDA (10,000 MW, 10% in dH₂O,
Invitrogen) were made in stereotaxic coordinates into the left or right sensorimotor
cortex. Coordinates for injections were 0.5 mm deep, 1.0 mm lateral, and 0.5 mm
rostral, 0.2, 0.5, and 1.0 mm caudal to bregma (0.4 µl per site). Mice were humanely
killed and transcardially perfused as above, 2-3 weeks post-injection.

*Retrograde labeling*

To identify supraspinal neurons that project to the spinal cord, Fluoro-Gold
(Fluorochrome, LLC) was injected into the spinal cord of CST-YFP mice (n=8). Briefly,
mice were anesthetized with isofluorane, and a laminectomy was performed at cervical
level 5 (C5) to expose the underlying spinal cord. Injections of 4% Fluoro-Gold (FG)
were made bilaterally 0.5 mm lateral to midline and 0.6 mm deep using an
electronically-controlled injection system (Nanoliter 2000 injector and Micro4 pump controller, World Precision Instruments) with pulled glass micropipettes. Initially mice received injections of 0.4 µl per site. During recovery from anesthesia, mice exhibited unusual symptoms involving tonic limb extension with trembling, fixed postures and dorsiflexion of the tail, and one of the first 2 mice with 0.4 µl injections died the same afternoon. The spasms and death following injections of FG into the cervical spinal cord are an animal welfare issue. In this regard, it is noteworthy that there have been previous reports of neurotoxicity from FG (Schmued et al., 1993; Naumann et al., 2000). To address this issue, 5 mice were injected with smaller volumes of FG (0.2 µl per site). Four of the 5 mice exhibited spasms though they were less pronounced, and one died later the same day. To test whether the spasms were due to the method of using the electronically-controlled injection system, an additional mouse was injected with 0.4 µl FG by Hamilton syringe tipped with a pulled glass micropipette, and it also had subsequent spasms. Mice that recovered did not exhibit obvious behavioral abnormalities. One week following injections, mice were transcardially perfused with 4% PFA, and brains and spinal cords were dissected and post-fixed in 4% PFA overnight before being equilibrated and stored in 27% sucrose. Two mice with 0.4 µl injections and 3 mice with 0.2 µl injections had robust labeling, and the larger cohort (with 0.2 µl injections) was used for analysis.

As a technical aside, we have carried out follow-up studies testing lower concentrations of FG, which demonstrated that 6 out of 6 mice injected with lower concentrations of FG (1%) exhibited less intense and shorter spasms during recovery from anesthetic, and there were no deaths.
**Histology**

**Sectioning.** Tissue was cryoprotected via equilibration in 27% sucrose, frozen in TissueTek O.C.T. (Sakura Finetek), and sectioned on a cryostat at 30 µm for the retrograde tracing and degeneration studies and at 20 µm for all other studies. Spinal cord cross sections were collected from all intact specimens and those injected with BDA. Sagittal sections were also collected from 6 mouse spinal cords with BDA tracing. Horizontal sections were collected from all of the spinal cords of mice with cortical lesions and 2 spinal cords with BDA tracing. Coronal brainstem sections were collected from mice with retrograde FG labeling.

**Signal amplification.** Native YFP fluorescence in CST-YFP mice appears weak for individual axons, so sections were immunostained using antibodies raised in rabbit against green fluorescent protein (GFP) and secondary antibodies conjugated to Alexa-488, as described (Bareyre et al., 2005). Sections were washed three times in phosphate-buffered saline (PBS), blocked in 5% normal goat serum (Vector Biolabs, Philadelphia, PA) in PBS, and then incubated overnight in a 1:250 dilution of rabbit anti-GFP (Millipore, Temecula, CA) in 5% normal goat serum and PBS. Sections were washed 3 times in PBS, incubated in a 1:250 dilution of Alexa Fluor® 488 (Life Technologies) in PBS for 2 hours, then washed a final 3 times before slide mounting and cover slipping with VectaShield® (Vector Biolabs) or Kaiser’s glycerol jelly mounting medium.

In select slides of specimens with mini-ruby BDA injections, BDA was amplified by staining with a tyramide signal amplification (TSA) kit with Cy3 conjugation (PerkinElmer, Waltham, MA). Endogenous peroxidases were quenched with 3%
peroxide in tris-buffered saline (TBS; 100mM Tris, 150mM NaCl, pH 7.4) for 15 minutes and then washed twice in TBS before blocking in TSA-kit blocking buffer for 30 minutes. Slides were then incubated with a 1:100 dilution of streptavidin-horseradish-peroxidase (SA-HRP) for 2 hours, then washed 3 times in TBS with 0.05% Tween® 20 (Sigma Aldrich, St. Louis, MO). TSA detection was performed by adding a 1:50 dilution of Cy3-tyramide in substrate amplification diluent for 30 minutes in humid incubation, followed by washing TBS with 0.05% Tween® and cover-slipping using Kaiser’s mounting medium.

Fluorescent Nissl-like staining. To identify cytoarchitectural borders in midbrain sections of specimens with FG labeling, selected brain sections were stained with NeuroTrace® (Life Technologies). Sections were washed in PBS with 0.1% Triton X-100 (Sigma Aldrich), 2x in PBS, then incubated in 1:50 dilution of blue NeuroTrace® for 20 minutes with agitation. Sections were again washed in PBS with Triton and then 2x in PBS before being mounted and coverslipped with VectaShield® (Vector Biolabs).

Epifluorescent and confocal imaging

Epifluorescent imaging was performed on an Olympus AX-80 microscope, and confocal imaging was performed on an Olympus Fluoview FV1000. For creating a montage of images wider than the viewfield, overlapping images were collected and stitched together in ImageJ (Rasband, 1997) using the linear blending fusion method of the Preibisch stitching plugin (Preibisch et al., 2009). In confocal images of longitudinal spinal cord sections from mice with cortical lesions, background speckling noise was
reduced to enhance the distinction of degenerated axon fragments by using ImageJ’s remove outliers function (radius 0.5 px, threshold 50).

\textit{Mapping CST origins}

A map of CST origins was created based on cortical FG labeling in 30 µm coronal sections spaced ~420 µm apart. The lateral spans of retrogradely labeled cells were plotted for each series of sections for each of the 3 analyzed mice injected with Fluoro-Gold, relative to bregma. Bregma was estimated based on comparison to the mouse brain atlas by Paxinos (2004), and rostro-caudal distance from bregma was extrapolated from section number and spacing. Based on a minimum of 2 strongly-labeled cells within 0.1mm in a section, the spans of labeled cells for each spaced section was plotted on one side of a cortex diagram adapted from Tennant et al. (2011) with permission, with the cortical pair of span groups plotted in a different pair of shaded colors for each specimen.

\textit{Clearing and 3D imaging}

3D imaging was performed on blocks of spinal cord from CST-YFP mice, Thy1-YFP-H mice, and a PTEN-floxed (\textit{Pten}\textsuperscript{fl/fl}) mouse with CST axons labeled with mini-ruby BDA. The block of spinal cord from the \textit{Pten}\textsuperscript{fl/fl} mouse was taken rostral to a dorsal hemisection spinal cord injury at T12 and had received cortical injections of AAV-Cre (as described in (Liu et al., 2010)), as part of a prior CST regeneration experiment. The area analyzed was far rostral to the injury and presumably reflects normal CST axon distribution. Blocks of spinal cord were progressively dehydrated and
defatted in tetrahydrofuran (50%, 80%, and 3x 100%, Sigma Aldrich) and
dichloromethane (Sigma Aldrich) before clearing in a mixture of benzyl alcohol (Sigma
Aldrich) and benzyl benzoate (Sigma Aldrich) in a 1:2 ratio (BABB), as described (Ertürk
et al., 2012b). Specimens were placed in the BABB clearing solution in a chamber
made by silicone isolators (Grace Bio-Labs, #664207) sandwiched by a glass
microscope slide and coverslip. Specimens were then imaged in transverse and
horizontal orientations on a 3i Vivo 2-photon microscope equipped with a Zeiss 20x
objective with 1.0 numerical aperture. Imaging was of nascent fluorescence, without
antibody enhancement or any signal amplification.

Quantification of YFP-labeled axons after unilateral cortical ablation

In specimens with cortical ablations (n=4), we characterized and quantified YFP-
labeled axons in the dorsolateral white matter (DL) based on morphology. Axons in the
DL that were visibly continuous and lacked distributed swellings were categorized as
intact. Discontinuous axons appearing as ovoids (Lubińska, 1977; Griffin et al., 1992),
or with breaks and swellings were categorized as fragmented. As axonal beading has
been documented in the progression of Wallerian degeneration in the central nervous
system (Kerschensteiner et al., 2005; Beirowski et al., 2010) including of the CST (Kalil
and Schneider, 1975), continuous axons with distributed swellings were categorized
separate from intact axons, as being beaded.

In horizontal sections, confocal image stacks at 40X magnification of dorsolateral
white matter extending from a cut edge of the mid-cervical horizontal sections were
analyzed from every 3rd section from the dorsal extent of the lateral column down to the
level of the central canal. One specimen was cut off-axis, and was excluded from this quantification. In ImageJ, axons crossing a line drawn at 200 µm from the cut edge were categorized and quantified through the stack. Only axons distinctly in the white matter were quantified, consequentially omitting any dICST axons that remained in fascicles in the adjacent grey matter in the mid-cervical sections (Steward et al., 2004). An optical dissector method (Guillery, 2002) was employed by excluding axons present in a lookup section within each stack. The number of dorsolateral axons was then extrapolated by multiplying the raw counts by 3. The mean number of dorsolateral axons ipsilateral vs. contralateral to the lesion for each categorized type were assessed by Bonferroni post-hoc tests following a 2-way mixed-model ANOVA using GraphPad Prism® (GraphPad Software).

In the ventral spinal cord, quantification of axons was performed in cross sections to maintain regularity in quantification throughout the spinal cord’s ventral and lateral extremes. This avoided adjusting parameters of quantification in oval or irregularly shaped sections that are commonly produced through longitudinal sectioning at or near the circumferential edges of the spinal cord (an imperfect cylinder). Cross sections of mid-cervical spinal cords from specimens with cortical ablations (n=4) and uninjured controls (n=3) were analyzed at 20X magnification in stitched image stacks in ImageJ. Stitched images were rotated to align midline to the vertical axis, and a horizontal line was overlaid through the central canal to define the dorsal vs. ventral spinal cord. A perpendicular line was overlaid ventrally from the central canal to laterally divide the ventral column (VC), and 2 additional lines were overlaid extending from the central canal 30° bilaterally from midline approximately along the medial border of each ventral
horn to delineate the ventral lateral white matter (VL) from the ventral column on either side (Fig. 2F). Analysis in confocal image stacks permitted virtual focusing along the z-axis and quantification of axons appearing to be continuous within each section. Ipsilateral and contralateral axons and axons from one side of controls in each respective ventral region were averaged from 3 sections per animal. Quantified axons from the VL and VC regions were each analyzed by ANOVA using GraphPad Prism®.

Quantification of identified rubrospinal and reticulospinal YFP-labeled neurons.

Coronal midbrain sections of CST-YFP mice injected with FG were examined for cells labeled with FG in the red nucleus and other brain regions, and FG-labeled cells in these sections were analyzed for co-labeling with YFP. Co-labeled cells in the red nucleus and reticular formation were quantified in every 7th section through a standardized volume of midbrain encompassing the red nucleus, beginning at the supramamillary decussation and extending 1.5 mm caudally. The red nucleus and other midbrain regions were identified by cytoarchitectural borders from blue fluorescent Nissl-like staining (NeuroTrace®), neuroanatomical landmarks, and reference to the Allen Mouse Brain Atlas, ©2012 Allen Institute for Brain Science [internet, available from: http://mouse.brain-map.org/]. Estimates of the total number of co-labeled cells were made by multiplying raw counts by the inverse of the fraction of sections quantified and the Abercrombie correction factor (Abercrombie, 1946; Guillery, 2002).

Axon tracing assessment

YFP-labeled axons were assessed for traceability in grey matter by using an
axon tracing task performed by 4 individuals. Stacks of confocal images from horizontal sections of spinal cord imaged at 40X and with 0.5 µm spacing were analyzed in ImageJ. Three BDA-labeled axons with long discontinuous segments and YFP co-labeling were selected for tracing in the densely YFP-labeled grey matter, and an additional axon was selected to familiarize individuals with the task. Following the familiarization with the task, 4 individuals were instructed to trace each axon within a stack of images from a designated starting point. Two individuals had experience studying labeled axons in the spinal cord and two did not (novice). None of the individuals had seen the selected images before, and each were presented with each image stack to trace each axon based on only YFP-labeling first, and BDA-labeling second. A tracing’s extent was marked with the cursor point tool in ImageJ. A line was drawn from the cursor point to the starting point, and the length of this line was measured in ImageJ and recorded as the distance that the axon was traced. The BDA vs. YFP tracing lengths were analyzed by paired t-test using GraphPad Prism®.

RESULTS

In their original publication, Bareyre et al. report complete and specific YFP-labeling of CST axons in the spinal cord, but also document YFP-labeled axons in locations previously not established for the mouse CST (Bareyre et al., 2005). These locations include the ventral column, where the CST is established in rats but not in mice, and the ventral lateral white matter. To estimate the number of YFP-labeled axons in these non-CST locations, we counted the number of YFP-labeled axons that were ventral to the central canal in mid-cervical sections on one side, and multiplied by
2 to account for both sides. This yielded an estimate of 128 ± 22 (mean ± SEM) YFP-labeled axons in non-CST locations.

**YFP labeling vs. anterograde CST tracing**

YFP-labeled axons in unexpected locations might be ectopic CST axons, or non-CST axons from other neurons that express YFP. To begin to test these possibilities, we injected mini-ruby biotinylated dextran amine (BDA) into the right sensorimotor cortex to anterogradely label CST axons.

Intra-cortical BDA injections in CST-YFP mice, which have a mixed C57Bl/6 background, produced a pattern of CST labeling in the spinal cord comparable with what has been previously reported in C57Bl/6 mice (Inman and Steward, 2003; Steward et al., 2004, 2008) and mice on a C57Bl/6 background (Coonan et al., 2001). Figure 1 illustrates the pattern of BDA and YFP labeling in the low cervical and upper thoracic spinal cord. In panels A-F, BDA signal was amplified with tyramide signal amplification (TSA) and imaged with confocal microscopy. Large numbers of BDA-labeled axons (red) are present in the dorsal column on the left and a smaller number of BDA-labeled axons are present in the dorsal part of the lateral column on the same side (Fig. 1A,E), corresponding to the dorsal CST (dCST) and dorsolateral CST (dICST), respectively (Steward et al., 2004, 2008). In one of 14 mice, a few BDA-labeled axons coursed longitudinally in the ventral column contralateral to the labeled dCST (ipsilateral to the injected cortex), in the location of the ventral CST seen in rats (Fig. 1G,N, without BDA amplification) (Brösamle and Schwab, 1997; Steward et al., 2008; Lang et al., 2012).
YFP-labeled axons were also present in the dorsal, lateral, and ventral columns, (Fig. 1B,M). In the dorsal lateral column, however, YFP-labeled axons were present farther lateral and ventral than the BDA-labeled dlCST axons (Fig. 1E,F). Some of these YFP-labeled axons appeared distinctly thicker than those in the region of dlCST axons labeled with BDA. YFP-labeled axons were also present in the ventral lateral columns (Fig. 1B,C,H,J) and sparsely distributed in the ventral column. BDA-labeled axons were not observed coursing in these non-CST locations (Fig. 1A,D,I,K). Thus, BDA labeling of CST axons in CST-YFP mice is comparable to patterns previously reported for the mouse CST, whereas YFP-labeled axons were present beyond the regions of BDA-labeled axons in locations where CST axons have not been reported.

Ablation of the sensorimotor cortex does not lead to Wallerian degeneration of YFP-labeled axons outside the expected distribution of the CST

Conceivably, our BDA injections may have missed some part of the sensorimotor cortex that gives rise to the YFP-labeled axons in atypical locations. To provide a complementary approach to testing whether all YFP-labeled axons are of the CST, we assessed whether large cortical lesions led to Wallerian degeneration of YFP-labeled axons in non-CST locations.

We first confirmed locations of CST origins in 3 CST-YFP mice by retrograde labeling of cortical neurons following bilateral injections of Fluoro-Gold (FG) into the spinal cord at the 5th cervical level (C5). Previous studies in rats have shown that injections at C5 retrogradely label neurons throughout the portions of the sensorimotor cortex representing both forelimb and hindlimb (Nielson et al., 2011). Retrograde
labeling of neurons in the hindlimb cortex presumably reflects uptake of FG by CST axons of passage projecting to caudal segments, consistent with previous evidence of FG labeling axons of passage (Dado et al. 1990).

In each of the CST-YFP mice, retrogradely-labeled neurons were present bilaterally in a pattern that was generally similar between the two sides of the cortex. The sections in Figure 2 illustrate the case with the largest number of retrogradely-labeled cells. Retrogradely-labeled neurons were found in layer V in the primary motor, secondary motor and primary somatosensory cortical areas defined in the Paxinos mouse brain atlas (Paxinos, 2004). We refer to this hereafter as the “sensorimotor cortex”.

To determine the rostro-caudal distribution of retrogradely-labeled cells, we identified the location of bregma based on the appearance of the sections compared to the Paxinos mouse brain atlas (Paxinos, 2004). The rostro-caudal distribution of retrogradely labeled cells was calculated based on the distance between sections in the series. In the mouse illustrated in Figure 2A-I, FG-labeled cells were present from approximately 2.3 mm rostral to 1.1 mm caudal to bregma. There were a few labeled cells in an additional caudal section (not shown).

Retrogradely-labeled cells were also present in the dorso-medial frontal cortex (centered at about 2.8 mm anterior to bregma) in a region ~750 µm lateral to midline and ~600 µm deep. The area between this population of labeled neurons and the main population in the more caudal motor cortex had few labeled neurons. There was also a population of FG-labeled cells in the dorsal lateral cortex adjacent to the S1 barrel field.
The area between this subpopulation of labeled neurons and the main population of labeled neurons contained few labeled neurons.

To provide a summary diagram of the distribution of retrogradely labeled cells in the 3 cases, we measured the medio-lateral distance over which labeled cells were found in each section and then represented this for each mouse on cortical map similar to that in Tennant et al. (2011) (Figure 1J). The medio-lateral distribution of cells at different rostro-caudal locations in each mouse is represented by the different colored lines over the right hemisphere of the cartoon in Figure 1J. The rostro-caudal locations of the distribution varied slightly relative to bregma in the different mice, but the rostro-caudal extent was similar with the main distribution spanning ~3.5 mm and the lateral distribution spanning ~1 to 1.5 mm in each cortex (Fig. 1J). The medio-lateral extent of the labeled population of layer V neurons varied by anterior-posterior location. In anterior regions of the sensorimotor cortex, labeled neurons were present from about 0.75-1.25 mm lateral. Near bregma, labeled neurons were present from about 0.75-2.75 mm lateral. In posterior regions, labeled neurons were present from about 1.25-2.25 mm lateral. The separate population of FG-labeled neurons in the dorso-lateral cortex was present in all 3 mice, and the area between the main population of FG-labeled neurons and the separate population in the dorso-lateral cortex lacked retrogradely labeled neurons. This map was used to guide our unilateral cortical lesions of CST origins.

Figure 3A illustrates the extent of unilateral cortical ablation in one mouse. Wallerian degeneration of YFP-labeled axons was assessed in the mid-cervical spinal cord (approx. C3-C4) in the areas indicated in Fig. 3B. The dorsal column (DC), dorsal
part of the lateral column (DL) and ventral column (VC) contain the dCST, dICST, and vCST respectively. The part of the lateral column ventral to the central canal (VL in Figure 3) contains YFP-labeled axons, but is not known to contain CST axons. Panels C-H illustrate confocal images of horizontal sections through the respective areas. One week after lesions of the right sensorimotor cortex, YFP-labeled axons exhibiting signs of Wallerian degeneration were present in the expected locations of CST axons including the left DC and DL (Fig. 3C&D). Wallerian degeneration was evidenced by axonal fragmentation and beading which are characteristic of degenerating CNS axons (Waller, 1850; Lubińska, 1977; Kerschensteiner et al., 2005; Beirowski et al., 2010) including of the CST (Kalil and Schneider, 1975). YFP-labeled axons appeared intact in the right dorsal column (Fig. 3C), and no intact YFP-labeled axons were seen in the left dorsal column.

Although fragmented axons were evident in the dorsal part of the lateral column on the left, there were also intact YFP-labeled axons in the same region, especially just lateral to the ones that were fragmented (Fig. 3D, arrow). Most YFP-labeled axons in the dorsolateral column on the right appeared intact (Fig. 3E), although a few fragmented axons were seen, consistent with degeneration of the small number of uncrossed dCST and dICST axons (Brösamle and Schwab, 1997; Steward et al., 2004, 2008; Zheng et al., 2006). Occasional YFP-labeled axons also appeared as beaded in non-injured control mice (not shown). The number of intact, beaded, and degenerated axons were not significantly different between the left and right sides (all p > 0.05, Bonferroni post-hoc tests following 2-way mixed-model ANOVA, n=3; Fig. 3I).
There were a few beaded and fragmented YFP-positive axons in the right ventral column in the position of the vCST, but most YFP-positive axons in the ventral column and the ventral lateral columns appeared intact (Fig. 3F-H). There were no significant differences between sides in the number of intact YFP-labeled axons in the lateral column ventral to the central canal (p=0.671, ANOVA, n=4; Fig. 3J) or ventral column (p=0.627, ANOVA, n=4; Fig. 3K) or relative to one side of non-injured control mice (n=3).

Because unilateral cortical lesions deplete CST axons on one side, the remaining YFP axons on the side contralateral to the lesion provide an estimate of non-CST axons on one side. Adding the intact axons on the left from the DL in Fig. 3I to those in the left VL and VC from the same 3 of the 4 animals assessed in Fig. 3J & 3K yields 136 ± 16 (mean ± SEM) intact YFP-labeled axons on the left side. Based on this, the total on both sides is estimated to be 272 ± 32 (mean ± SEM) YFP-labeled non-CST axons.

**Some non-CST cells that project to the spinal cord express YFP**

If non-CST axons are labeled with YFP in the spinal cord, then these axons must arise from neurons outside of the cortex. As reported by Bareyre et al. (2005), a few YFP-positive neurons were present in the spinal gray matter in our tissue (Figure 1G, arrowhead). Other examples are shown in Figure 4 both in cross section (Fig. 4A) and horizontal sections (Fig. 4B-K), with some imaged by confocal microscopy (Fig. 4I-K). YFP-labeled axons could be followed from some of these neurons into the white matter in both uninjured mice (Fig. 4A-H) and mice with cortical lesions (Fig. 4I-K), and some
projections extended both rostrally (Fig. 4B,K), and caudally (Fig. 4G,I,J). Projections of YFP-labeled neurons were observed in the ventral medial and lateral white matter. Additionally, one axon extending from the lateral white matter was identified coursing in a ventral root and exiting the spinal cord (Fig. 4D), suggesting YFP-labeling of a motoneuron axon. Hence, some neurons in the spinal grey matter contribute YFP-labeled projections into the white matter of the CST-YFP spinal cord.

To identify other possible sources of YFP-labeled non-CST axons in the spinal cord, we first examined brain sections for YFP-labeled neurons outside of the cortex. Scattered YFP-labeled cells were present in most brain regions examined, including the red nuclei, superior colliculi, reticular formation, and vestibular nuclei, which are brain nuclei with major descending projections to the spinal cord.

To test the hypothesis that descending non-CST axons express YFP, we injected the retrograde tracer Fluoro-Gold (FG) into the cervical spinal cord of CST-YFP mice, and examined FG-labeled brain cells outside of the cortex for co-labeling with YFP. In midbrain sections of each of the 3 mice examined, YFP-labeled cells co-labeled with Fluoro-Gold were identified by confocal microscopy in the red nucleus and the reticular formation (Fig. 5). A very small number of co-labeled cells were also present in the periaqueductal grey (not shown), consistent with previous studies (Mantyh and Peschanski, 1982; Liang et al., 2011). A raw average of 22.7 co-labeled rubrospinal and 17.0 co-labeled reticulospinal neurons (StdDev 11.6 and 14.4, respectively) were identified per mouse, with an estimated total average of 89 rubrospinal and 66 reticulospinal co-labeled neurons in 1.5 mm of midbrain for the 3 animals (Fig. 5G). This quantification does not present an absolute number of YFP-labeled rubrospinal and
reticulospinal neurons, but rather confirms that some rubrospinal and reticulospinal neurons are labeled with YFP. These results confirm YFP-labeling of some non-CST neurons that project to the spinal cord in CST-YFP mice.

**Dense and faint YFP labeling impedes individual axon visualization and tracing**

With some YFP-labeled non-CST axons, one way to confirm CST origins is by tracing axons back to the main tract. Tracing axons back to a cut parent tract can also support that the axons had regenerated (Tuszynski and Steward, 2012). In CST-YFP mice, however, the density of YFP-labeled axons is high especially adjacent to the dorsal column, which is the location through which axons would need to be traced to confirm origins from CST axons in the dorsal column. Figure 6 illustrates the grey matter adjacent to the dorsal column. YFP-labeling of many axons is also faint, such that under epifluorescence microscopy at 20X magnification, YFP-labeling was not evident in some CST axons labeled with BDA (Fig. 6A-B), though the same CST axons were confirmed to have YFP co-labeling under confocal microscopy at 40X magnification (Fig. 6C-E).

To compare the ability to trace YFP vs. BDA-labeled axons, 4 individuals were asked to trace selected co-labeled axons in a stack of confocal images from uninjured mice, first using YFP and then BDA imaging. One of these axons extending from the dCST into the grey matter was traced by all 4 individuals into the grey matter based on BDA labeling, but none of the individuals traced the axon beyond the grey matter interface based on labeling with YFP (Fig. 6F-H). Other axons were also traced incompletely under visualization by YFP as compared to BDA (Table 1). In three
instances, individuals’ tracing ended on incorrect YFP-labeled axons, whereas this only occurred in one case based on BDA-labeling (Table 1). Individuals with experience in tracing axons were generally able to trace YFP-labeled axons farther than the novices. Overall tracings based on visualization by YFP were significantly shorter than tracings by visualization by BDA ($p = 0.0402$, two-tailed paired t-test), as were tracings in which incorrectly traced axons were excluded ($p = 0.0491$, two-tailed paired t-test). These results indicate that visual tracing of YFP-labeled CST axons is impeded in the densely YFP-labeled grey matter adjacent to the main CST.

**Nascent YFP fluorescence in CST-YFP mice is faint for 3D imaging relative to fluorescence in Thy1-YFP-H mice and mice with mini-ruby BDA labeling**

Recent advances in tissue clearing have allowed imaging and 3D reconstructions of fluorescent axons deep in un-sectioned tissue (Hama et al., 2011; Ertürk et al., 2012a, 2012b; Chung et al., 2013; Ke et al., 2013; Kuwajima et al., 2013; Tomer et al., 2014; Yang et al., 2014). 3D imaging is especially promising for regeneration studies as it enables evaluation of candidate regenerated axons along their continuous course, rather than relying on reconstructions from axon segments. With the extensive genetic labeling in CST-YFP mice, this approach could be useful for initial screening of axon growth following injury. To evaluate the utility of CST-YFP mice for 3D imaging, we used the protocol of (Ertürk et al., 2012b), which can rapidly render spinal cords clear (in a few hours) and has been used for imaging axons in the spinal cord following injury. Blocks of spinal cord from CST-YFP mice, Thy1-YFP-H mice, and a mouse with mini-ruby BDA-labeling of CST axons were cleared and imaged as in (Ertürk et al., 2012b),
using 2-photon microscopy. To maintain consistency in comparison, a hemizygous Thy1-YFP-H mouse was also assessed as CST-YFP mice are hemizygous for Thy1-driven YFP expression.

As demonstrated previously in rat (Ertürk et al., 2012b), mini-ruby BDA-labeled CST axons are evident in 3D reconstructions (Fig. 7A, arrow); individual BDA-labeled axons are evident in the dorsal column in a thin projection (Fig. 7B), and one labeled axon can be seen in the gray matter (arrow). This is from a block of cervical spinal cord rostral to a low-thoracic lesion in a PTEN-floxed mouse that was injected with AAV-Cre as in Liu et al. (2010). After empirically determining 2-photon laser excitation wavelength to be optimal at ~950 nm for our YFP-labeled specimens, cleared blocks of spinal cord from CST-YFP and Thy1-YFP-H mice were imaged using this laser wavelength and the same scan settings. In a hemizygous Thy1-YFP-H mouse, labeled axons were evident in the dorsal column and distributed through the white matter (Fig. 7C-D). Labeled neurons and axons were also present in the grey matter. The overall appearance was similar in a homozygous Thy1-YFP-H specimen (not shown). In contrast, in CST-YFP mice the bundle of YFP-labeled axons in the dorsal column was only faintly visible, and was less intense than the background fluorescence in the grey matter (Fig. 7E). Individual labeled axons were also not readily discernable in thin horizontal projection (Fig. 7F). Thus by the approach that we used, in contrast to labeling with mini-ruby BDA and the axonal labeling in Thy1-YFP-H mice, labeling intensity in CST-YFP mice is not sufficient for axon imaging in cleared tissue.
DISCUSSION

In their original study, (Bareyre et al., 2005) suggested that CST-YFP mice would be useful for evaluating regeneration of CST axons because most or all CST axons are labeled without requiring tracer injections. However, regenerative growth distal to a spinal cord injury can involve small numbers of axons extending along routes not normally taken by CST axons (Steward et al., 2003). For definitive identification, it is important that labeling be specific to the CST. Here we show that some YFP labeled axons extending longitudinally along the spinal cord are not CST axons. YFP-labeled axons were seen outside of regions containing CST axons anterogradely labeled by BDA and most YFP-labeled axons outside the main CST fail to degenerate following a large cortical lesion. Also, following FG injections into the cervical spinal cord, some YFP-positive neurons were retrogradely labeled in the red nucleus and reticular formation. These could account for YFP-labeled non-CST axons in the lateral column.

We further demonstrate that axon visualization and tracing, such as could be used to confirm axons are of the CST, are impeded by dense YFP-labeling in CST-YFP mice. Finally, in evaluation of CST-YFP mice for 3D imaging, we found that in comparison to Thy1-YFP-H mice and mice with mini-ruby BDA labeling, the YFP signal from CST-YFP mice is not sufficient for axon imaging in cleared un-sectioned tissue. Overall, these caveats limit the utility of CST-YFP mice for assessing regeneration of CST axons.

YFP labeling of axons and anterograde CST labeling

The pattern of BDA labeling we show in CST-YFP mice is comparable to what has been described previously in mice. The main components of labeled axons are the...
dCST in the dorsal column, and the dICST in the dorsal part of the lateral column (Inman and Steward, 2003; Steward et al., 2004). As in our previous studies involving mice, there were few if any BDA-labeled axons in the ventral column in the position of the ventral CST that has been characterized in rats (Brösamle and Schwab, 1997). Thus, CST axons do not follow an unusual course in CST-YFP mice.

YFP-labeled axons were present in the same regions as BDA-labeled CST axons but were also sparsely distributed through the dorsal to ventral lateral column that contains rubrospinal and reticulospinal axons (Jones and Yang, 1985; Inman and Steward, 2003; Ballermann and Fouad, 2006; Liang et al., 2012; Watson and Harrison, 2012). Some of these axons in the DL also appeared thicker than other YFP-labeled axons in the region of BDA-labeled dICST axons; this is noteworthy because rubrospinal axons are thicker (Powers et al., 2012) than CST axons (Sima and Sourander, 1978). Thus, one possibility is that the YFP-labeled axons outside the normal territory of CST axons are of brainstem origin.

**Limited degeneration of YFP-labeled axons outside the dorsal column**

The conclusion that some YFP-labeled axons are not part of the CST is also supported by the results following cortical ablation. Large unilateral cortical lesions of CST origins led to Wallerian degeneration of YFP-labeled axons in the expected locations for the CST (the DC and DL contralateral to the lesion and the ipsilateral VC). In contrast, YFP-labeled axons outside the normal territory of CST axons did not exhibit signs of Wallerian degeneration.
It is noteworthy that there were surprisingly few degenerating YFP-labeled axons in the region of the dlCST contralateral to the lesion. In this regard, there are points to consider. First, the rostro-caudal location selected for analysis and quantification of axons was the mid-cervical spinal cord (approx. C3-C4), and dlCST axons caudally course in fascicles in the dorsolateral grey matter before joining the white matter in the first few cervical levels (Steward et al., 2004). Our counts only included axons in the white matter so dlCST axons in fascicles in the gray matter would not have been counted. Second, dlCST axons might degenerate more slowly than dCST axons. This seems unlikely, however, because signs of beading and fragmentation generally appear by 3-4 days (Lubińska, 1977; Griffin et al., 1992, 1996). Our results are consistent with results of Galley and Clowry (2010) who found that only a few of large YFP-labeled axons in the DL were lost after neonatal cortical lesions. However, our results and those from Galley and Clowry differ from those of Bareyre et al. (2005), who report that 10% or fewer of YFP-labeled axons in the DL and VC remain after bilateral pyramidotomy in adult CST-YFP mice.

**YFP labeling of rubrospinal and reticulospinal neurons**

Following Fluoro-Gold (FG) injections into the spinal cord, some retrogradely-labeled YFP-positive neurons were present in the red nucleus and reticular formation. Similarly, Galley and Clowry (2010) also found YFP-labeled neurons in the red nucleus and other non-forebrain regions in juvenile CST-YFP mice. However, Bareyre et al. (2005) reported that no YFP-labeled cells were present in the red nucleus, reticular formation, vestibular nucleus, or superior colliculus in adult CST-YFP mice. There is no
obvious explanation for this discrepancy, though it is generally recognized that genetic modifiers can change transgenic expression over generations (Allen et al., 1990).

The number of FG and YFP co-labeled neurons varied between mice, ranging from approximately 45-140 rubrospinal neurons and ~20-140 reticulospinal neurons in 1.5 cm of midbrain. Most rubrospinal axons originate from the magnocellular part of the red nucleus, which in mice may have ~2,200 neurons (Liang et al., 2012). In mice, one red nucleus has ~3,200 neurons, and ~2,100 of these have been labeled following injections of FG at C2 (Liang et al., 2012). If there are bilaterally ~4,200 rubrospinal neurons, about 1-3% are YFP-positive in CST-YFP mice. This could account for the YFP-labeled axons in the region of the rubrospinal tract in CST-YFP mice that don’t degenerate after cortical lesions. Further, as there may be ~272 non-CST axons labeled with YFP based on our specimens with cortical lesions, YFP-labeled brainstem neurons could account for the majority of non-CST axons labeled with YFP.

YFP-labeled neurons in the spinal cord also extend axons into white matter, accounting for another source of non-CST axons in CST-YFP mice. Tracing axons from these neurons often required superimposing images from adjacent sections. Bareyre et al. (Bareyre et al., 2005) also reported YFP-labeled somata in the grey matter but did not report axons extending into the white matter.

**Complete tract labeling: too many axons?**

As regeneration of the CST may involve few if any axons extending beyond a lesion, complete labeling of the CST optimizes chances to detect rare regenerated axons. YFP-labeling of non-CST axons could mitigate this advantage, but origin from
the CST can be confirmed if individual axons can be traced back to the main CST bundle. In CST-YFP mice, however, tracing individual YFP-labeled axons through the dense YFP-labeled arbors is impeded relative to tracing by BDA. This is also noteworthy for regeneration studies as tracing axons back can indicate that potentially regenerated axons branched from injured axons (Tuszynski and Steward, 2012).

**Faint labeling limits deep imaging of axons in cleared tissue**

Two-photon imaging of rapidly-cleared blocks of spinal cord from CST-YFP mice revealed that individual axons were difficult to resolve relative to those in Thy1-YFP-H mice and mice with mini-ruby BDA labeling. This is probably due to the level of YFP expression in the CST-YFP mice in comparison to Thy1-YFP-H mice. Even immunostaining for YFP produces signal more faint than nascent mini-ruby BDA (Fig. 7). The approach we used permitted rapid clearing (a few hours) appropriate for screening through the unsectioned spinal cord, but immunohistological methods with clearing such as CLARITY may solve the problem of inadequate axon visualization, though with a much longer processing time-course (Spence et al., 2014; Tomer et al., 2014; Yang et al., 2014).

**Caveats of Emx1 and breeding in the Cre-Lox system for specific labeling**

CST-YFP mice are generated in a Cre-Lox breeding system as the progeny of one parent mouse with Emx1-driven Cre recombinase (Emx-Cre) and one parent mouse with a floxed STOP cassette following a neuronal Thy1 promoter and preceding YFP (Thy1-STOP-YFP). In offspring CST-YFP mice, YFP expression is achieved by
Cre-mediated excision of the STOP cassette driven by Emx1 in Thy1-STOP-YFP neurons (Briata et al., 1996; Buffelli et al., 2003; Bareyre et al., 2005). As Emx1 is a forebrain transcription factor in development (Simeone et al., 1992; Briata et al., 1996), this mouse is designed to yield permanent labeling of forebrain neurons, including the cells of origin of the CST.

In a Cre-Lox breeding system, however, transient Cre expression can result in permanent genetic excision. This may explain labeling of non-CST axons in CST-YFP mice. Additionally, Emx1 is not specific to the embryonic forebrain; Emx1 has also been identified in the developing embryonic mesencephalon (Briata et al., 1996), which could account for YFP-labeled cells in the red nucleus and midbrain reticular formation.

In future generation of genetically-labeled mice for experiments requiring stringent tract or tissue specificity such as is needed for studying CST regeneration, we anticipate that it will be necessary to avoid genetic drivers that result in permanent non-specific labeling.

REFERENCES


**Figure 1.** YFP-labeled axons are in areas of the spinal cord that do not contain anterogradely-labeled CST axons. **A-F,** Confocal images of a spinal cord cross section with amplified BDA signal. BDA labeled CST axons (**A**) are illustrated relative to YFP labeling (**B**) following BDA injections into the right sensorimotor cortex. The ventral lateral white matter (arrowhead, **B**) is enlarged in **C** and **D**; note the absence of BDA-labeled axons in **D.** The dorsolateral column (boxed region in **B**) is shown in higher magnification in **E-F;** arrowheads indicate YFP-labeled neurons in the grey matter; arrows indicate the region of the rubrospinal tract, which overlaps with the dlCST. Note that some YFP-labeled axons in this region are distinctly thicker in appearance. **G,** Cross section with mini-ruby BDA labeling and indicated locations of sections shown in **H-N.** **H-K,** Sections in ventral lateral white matter with YFP-labeled axons (arrows in **H,J**) and as shown for BDA labeling (**I,K**); note the bilateral absence of BDA-labeled axons. **L-M,** BDA (**L**) and YFP labeling (**M**) in the ventral column (boxed region from **G**). **N,** A BDA-labeled axon coursing longitudinally in the ventral column ipsilateral to the injected cortex, as seen in one of 14 mice. The inset in **N** has been further enhanced for contrast. dCST, dorsal corticospinal tract; dlCST, dorsolateral CST. Scale bars, 200 µm (**A-B, G-M**), 100 µm (**C-D, N** and inset), 50 µm (**E-F**).
Figure 2. Distribution of FG-labeled cell bodies in the cortex following injections of Fluoro-Gold as shown in coronal sections from one mouse (A-I) and a composite cortical map from 3 mice (J). **A-C**: A clustering of FG-labeled cells is present in the anterior cortex centered at ~1.9 mm rostral to bregma (B) and sparsely present ~0.4 mm further rostral (A). At ~1.5 mm rostral to bregma a few labeled cells have further lateral
positioning than the more rostral sections and there is a general paucity of labeling (C). 

D-I: A moderately dense collection of labeled layer V neurons extends from ~1.0 mm rostral to ~1.1 mm caudal to bregma in the dorsal aspect of the cortex, indicating the main population of CST neurons. Labeling is most dense and prominent centered +/- 0.6 mm around bregma (E-H). Labeling in the same region is more sparse +1.0 mm (D) and -1.0 mm from bregma (I). Near the rostral aspect of the hippocampus, a small population of cells is also labeled with FG lateral to the lateral ventricles (H, I). A few labeled cells remained in this region and in the region of the main dorsal population in an additional section 1.5 mm caudal to bregma (not shown). J, The spans of regions with labeled cells are represented by colored bars on one side of the cortical map for 3 mice. Each pair of shaded colors represents the span of labeled cells in a pair of cortices of one specimen. Map adapted from Tennant et al. (2011) with permission. Scale bars = 400 µm in F (applies to A-F), and I (applies to G-I).
Figure 3. Ablation of the sensorimotor cortex does not lead to degeneration of YFP axons outside the main CST. **A**, Sections from a representative specimen with a large lesion to ablate CST origins from the right cortex; arrowheads show lesion extent. Rostro-caudal distances of each section from bregma are indicated. **B**, Confocal cross section of cervical spinal cord 1 week after unilateral cortical ablation. The overlaid yellow lines distinguish the different white matter regions where axons were quantified. **C-H**, Longitudinal sections of dorsal (C-E) and ventral (F-H) cervical spinal cord, by
regions as indicated in B. Purple arrows indicate beaded axons; arrowheads indicate fragmented axons; white arrows indicate intact axons. Note the intact axons in the left DL (D) and right VC (G), as well as bilaterally in the VL (F, H). I, Quantification of categorized axons in the DL in longitudinal sections. Differences between sides for the intact, beaded, and fragmented axons were not statistically significant (all p > 0.05, Bonferroni post-hoc tests following 2-way mixed-model ANOVA). J, K, Quantification in cross sections of the overall number of right or left axons or axons of control mice in the VL (J) and VC (K), with no respective significant differences (both p>0.6, ANOVAs).

The image in C is a single-plane confocal capture and D-H are confocal projections. DC, dorsal column; DL, dorsolateral white matter; VC, ventral column; VL, ventral lateral column. Data are mean + SEM. Scale bars, 1 mm (A), 400 µm (B), 100µm (C-H).
Figure 4. Some YFP-labeled cells in the spinal cord project into the white matter. 
A, Cross section of cervical spinal cord showing a YFP-labeled cell in the ventral grey matter with projections extending into the ventral white matter. B-H, Examples of YFP-labeled cells in horizontal sections extending projections into the ventral medial and lateral white matter of the cervical (B, F-H) and thoracic (C-E) spinal cord. Arrowheads highlight some YFP-labeled projections. Each image panel is a projected overlay of 2
adjacent tissue sections except for the images of single tissue sections in inset panels E and G. Inset panels were further adjusted for brightness and contrast to enhance detail. In D, also note the YFP-labeled axon coursing in the ventral root (arrow) and lateral white matter. I-K, Selected confocal projections from mice with large cortical lesions showing YFP-labeled cells in the spinal cord with projections extending both caudally (I-J), and rostrally (K) in the white matter of the ventral column. VC, ventral column; Lat, lateral white matter. Rostral-caudal diagram applies to B-K. Scale bars, 100µm.
**Figure 5.** Some brainstem neurons that project to the spinal cord express YFP.  
**A,** Fluorescent Nissl-like labeling (NeuroTrace®) in the midbrain in a mouse that had FG injected into the spinal cord to retrogradely label supraspinal neurons.  **B,** FG and YFP labeling in the midbrain (same section as **A**).  **C-F,** Merged FG and YFP imaging (**C,E**) and YFP imaging alone (**D,F**) in the red nucleus (**C,D**) and reticular formation (**E,F**).  
Note the FG-labeled rubrospinal and reticulospinal neurons that are YFP-positive (arrows).  **G,** Quantification of sampled YFP and FG co-labeled rubrospinal and reticulospinal neurons through 1.5 mm of midbrain in 3 mice.  **A-F** are single plane confocal images.  FG, Fluoro-Gold; Ret. formation, reticular formation.  Scale bars, 200 µm (**A-B**), 100 µm (**C-F**).
Figure 6. Dense and faint YFP labeling impedes individual axon visualization and tracing. **A-B.** Epifluorescent images at 20X magnification showing individual BDA-labeled CST axons in the white matter (arrowhead, A) and grey matter (arrows, A) and YFP labeling in the same section (B). Note the faint labeling of individual axons with YFP. **C-E.** Confocal images at 40X magnification showing CST axons labeled with BDA from A (C, arrowhead, arrow and inset) are co-labeled with YFP (D, E). **F-H,** Confocal projections of one axon originating from the dCST and traced by experienced (X) and novice (+) axon tracers. Individuals were instructed to trace the axon from the arrowhead to the best of their ability within the stack of 40X confocal images, as visualized first by YFP and then BDA. Note that none of the YFP-based tracings extend
beyond the grey matter interface (F), whereas all the BDA-based tracings extend into the grey matter (H). One novice tracer continued tracing to an incorrect axon segment under visualization with BDA (+* in H). dCST, dorsal corticospinal tract; gm, grey matter; wm, white matter. Scale bars, 100 µm (A-B), 50 µm (C-H), 10 µm (C-E insets).
Figure 7. 3D imaging of cleared blocks of spinal cord from a mouse with BDA-labeled CST axons, a Thy1-YFP-H mouse, and a CST-YFP mouse. A-B, A 3D view through 482 µm of cervical spinal cord (A) and horizontal projection through 6µm of the dorsal column (B) show visible BDA-labeled axons in the dorsal CST (arrows), rostral to a spinal cord injury. This tissue is from a PTEN*/* mouse that had received cortical injections of AAV-Cre and mini-ruby BDA injections into the left cortex. Note the visible individual axons in horizontal projection in the dorsal column (arrow, B), and in the grey matter (inset, B). C-D, A 3D view through 450 µm of spinal cord (C) and 6µm horizontal projection in the dorsal column (D) from a hemizygous Thy1-YFP H mouse. Note the strong signal intensity of axons in the dorsal column (arrows) as well as individual axons distributed through the spinal cord. E-F, A 3D view through 400 µm of a block of thoracic spinal cord (E) and 6 µm horizontal projection though the dorsal column (F) from a CST-YFP mouse. Note that the signal from axons in the dorsal column (arrows) is lower than that of the grey matter background. Scan settings were the same as for the block of spinal cord from the Thy1-YFP-H mouse shown in C-D. Laser excitation was 850 nm for the specimen with BDA (A-B), and 950 nm for the CST-YFP and Thy1-YFP-H mouse (C-F). Grid spacing, 500 µm (A, C), 100 µm (E). Scale bar, 200 µm (B, D, F), 50 µm (B, inset).
### Table 1. Axon Tracing Task Results

<table>
<thead>
<tr>
<th>Tracer 1: experienced</th>
<th>YFP tracing</th>
<th>BDA tracing</th>
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<tbody>
<tr>
<td></td>
<td>Axon 1</td>
<td>Axon 2</td>
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<tr>
<td></td>
<td>15.9⁺</td>
<td>96.6⁺</td>
</tr>
<tr>
<td>Tracer 2: novice</td>
<td>12.2</td>
<td>8.5⁺</td>
</tr>
<tr>
<td>Tracer 2: novice</td>
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<tr>
<td>Tracer 4: experienced</td>
<td>32.1</td>
<td>78.7</td>
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</tbody>
</table>

*Table 1.* Lengths of tracings of designated axons as visualized first by YFP, then by BDA. In three cases individuals’ tracing ended on incorrect YFP-labeled axons (⁺), and this occurred in one case based on BDA-labeling, from a novice axon tracer. Overall tracings based on visualization by YFP were significantly shorter than tracings by visualization by BDA (p = 0.0402, two-tailed paired t-test), as were tracings in which incorrectly traced axons were excluded (p = 0.0494, two-tailed paired t-test).
CHAPTER 2

Corticospinal tract axons that regenerate as a result of PTEN deletion form synapses caudal to a spinal cord lesion
ABSTRACT

Deletion of PTEN (phosphatase and tensin homolog) in retinal ganglion cells of mice has previously been demonstrated to promote robust axon regeneration after optic nerve injury. In more recent studies, our group has demonstrated that genetic deletion of PTEN in the sensorimotor cortex enables CST axons to regenerate past even complete crush injuries of the spinal cord. Here, we assess whether corticospinal tract (CST) axons that have regenerated following spinal cord injury (SCI) form synapses in caudal segments. Mice homozygous for the floxed PTEN gene (PTEN<sup>fl/fl</sup>) were injected with Cre-expressing adeno-associated virus (AAV-Cre) at P1 to delete PTEN from the right sensorimotor cortex. At 2 months of age mice were given a complete crush injury at the 8<sup>th</sup> thoracic level (T8), then at 10 weeks were injected with biotinylated dextran amine (BDA) into the right sensorimotor cortex to anterogradely label CST axons. Mice were terminated a total of 12 week after injury. Spinal cord sections through the lesion were embedded in resin for analysis under light microscopy to identify BDA-labeled axonal swellings suggestive of synaptic boutons. Subsequent processing and analysis under electron microscopy (EM) was used to confirm synaptic structures. Of 13 bouton-like structures examined by electron microscopy, 3 are definitive synapses based on the presence of a contact zone and post-synaptic density. These results indicate that axons from neurons with PTEN deleted can regenerate past and form synapses caudal to a spinal cord lesion, indicating that manipulation of the PTEN pathway can produce a robust and potentially meaningful regenerative response.
INTRODUCTION

Permanence in paralysis from spinal cord injury is thought to result from a lack of regenerative capacity of axons within the spinal cord (Case and Tessier-Lavigne, 2005; Tuszynski and Steward, 2012). Indeed, Ramon Y Cajal stated that of the central nervous system, “nothing may be regenerated” (Ramón y Cajal, 1928). Failure of regeneration may be attributed to extrinsic factors such as inhibitory molecules or the glial scar (Silver and Miller, 2004; Yiu and He, 2006), as well as intrinsic factors such as a loss of regenerative competency of mature neurons (Case and Tessier-Lavigne, 2005; Park et al., 2008). Recently it was discovered that phosphatase and tensin homolog (PTEN) is an intrinsic inhibitor of regeneration in the central nervous system (CNS), and its deletion results in robust regeneration within the CNS (Park et al., 2008; Yang and Yang, 2012). In this previous study robust regeneration due to PTEN deletion was demonstrated by Park et al. in the optic nerve, and it has been of interest to see if manipulation of PTEN may also promote axon regeneration in the spinal cord after injury.

In the spinal cord, a long axon tract that is particularly refractory to regeneration is the corticospinal tract (CST) (Blesch and Tuszynski, 2009; Liu et al., 2010). The CST is particularly important in humans for controlling voluntary motor function (Lemon and Griffiths, 2005; Martin, 2005; Lemon, 2008; Tuszynski and Steward, 2012). Recently, our group has discovered that PTEN deletion in the cortex results in robust regeneration of CST axons beyond a spinal cord lesion. As neural circuitry is governed by its synapses, a critical question for whether regeneration may be functionally meaningful is whether or not the regenerated axons form synapses. Here, we assess whether CST
axons that regenerate due to PTEN deletion form structural synapses caudal to a spinal
cord injury. The results we present here were published in part previously (Liu et al.,
2010).

MATERIALS AND METHODS

In this collaborative study, the live animal portion of this experiment was
performed by others at the Children’s Hospital in Boston. The data presented here
involved preparation and analysis of tissue that was performed by our lab at UC Irvine.
All live animal procedures were approved by the Institutional Animal Care and Use
Committee (IACUC) at Children’s Hospital, Boston.

AAV injection

AAV-Cre, serotype 2, was prepared as previously described (Park et al., 2008).
Neonatal PTEN-floxed (Pten\(^{f/f}\)) mice [C;129S4-Pten\(^{tm1Hwu}\)/J, Jackson Labs] were
cryoanesthetized and injected with 2 \(\mu\)l of AAV-Cre via a nanoliter injector into the right
sensorimotor cortex to delete PTEN in cells of origin of the CST.

T8 Spinal Cord Crush

Eight weeks after receiving cortical injections of AAV-Cre, mice were given a T8
crush spinal cord injury with modification from what has been previously described
(Fujiki et al., 1996; Inman and Steward, 2003). Briefly, a midline incision was made
over the thoracic vertebra and a laminectomy was performed at T8. A crush injury was
made by clamping the spinal cord with modified no. 5 jewelers forceps for 2 seconds,
keeping the dura intact. Muscle layers were sutured and the skin closed with wound clips. Bladders were manually expressed for urine twice daily for the duration of the experiment.

**CST labeling**

At 10 weeks post-injury, mice received cortical injections of BDA to anterogradely trace the CST. A total of 1.6 µl BDA was stereotactically injected into 4 sites in the right sensorimotor cortex. (AP coordinates from bregma in mm: AP 1.0/1.5, 0.5/1.5, -0.5/1.5, -1.0/1.5, all at a depth of 0.5 mm into the cortex.) Mice were transcardially perfused with 4% paraformaldehyde 2 weeks later, a total of 12 weeks after injury. Two of these mice were used for analysis in this study.

**Histology**

An approximately 8 mm block of spinal cord containing the lesion was sectioned in the sagittal plane on a Vibratome® at 50 µm. BDA labeling of CST axons was then detected by chromogenic staining with diaminobenzidine (DAB), as has been previously reported (Steward et al., 2008). Briefly, sections were incubated for 1 hr with avidin and biotinylated horseradish peroxidase (Vectastain ABC kit, Vector Laboratories), washed in PBS and then reacted with DAB in 50mM Tris buffer, pH 7.6, 0.024% hydrogen peroxide and 0.5% nickel chloride. While wet, sections were examined for BDA-labeled axons extending caudal to the lesion, and a collection of serial sections from both mice were selected for embedding into resin.
Resin embedding

Sections were rinsed in 0.1 M cacodylate buffer and then post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hr. Sections were rinsed in nanopure water for 2x 10 min, and then dehydrated through a series of graded ethanols (70%, 85%, 90% and 100% 2x) for 10 min each. Sections were immersed in propylene oxide (intermediate solvent) for 2x 10 min 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 overnight at 60ºC.

Imaging and analysis

Images of each section were captured under light microscopy. To enhance visualization of axons through the extent of the thick resin-embedded sections, a stack of images was collected at 10X magnification for each section and then each made into a projection using ImageJ. Tracings were made of the BDA-labeled axons in each projection using Adobe Photoshop, and the tracings were superimposed and aligned to reveal the coursing of BDA-labeled axons within the collection of sections for both mice.

One of the sections having axon segments several hundred microns caudal to the lesion was selected for examination under electron microscopy (EM). A collection of bouton-like axonal swellings was identified in advance under light microscopy and marked within an image stack. Ultrathin 60-nm sections were cut and mounted onto copper grids and viewed on a JEOL 1400 electron microscope. Individual BDA-labeled swellings were then located and assessed at the level of EM.
RESULTS

The CST courses in established locations far rostral to a lesion in PTEN-deleted mice

In this experiment, PTEN\textsuperscript{f/f} mice were injected with AAV-Cre on the first postnatal day (P1) to delete PTEN from neurons in the right cortex. At birth, however, the CST only has an incomplete projection into the spinal cord, with extension and maturation continuing in the mouse for several days after birth (Gianino et al., 1999; Hsu et al., 2006; Canty and Murphy, 2008). As PTEN knock-out embryos have cephalic malformation (Suzuki et al., 1998), it was possible that deletion of PTEN would result in an altered course of the adult CST. To address this issue, we analyzed sections of spinal cord far rostral to a spinal cord lesion in a PTEN\textsuperscript{f/f} mouse that was injected with AAV-Cre. The CST in sections far rostral to a lesion in wild-type mice has been previously reported to be similar to the non-injured CST (Steward et al., 2008).

Figure 1 illustrates the distribution of BDA-labeled CST axons in the upper thoracic spinal cord in a PTEN-deleted mouse, far rostral to a complete crush injury at T8. BDA injected into the right sensorimotor cortex labeled axons on the left in the ventral part of the dorsal column and the dorsal part of the lateral column, the established locations of the respective dorsal CST (dCST) and dorsolateral CST (dlCST) on the left side (Inman and Steward, 2003; Steward et al., 2004, 2008). A few axons were also labeled in the opposite dorsal column and dorsal lateral column, consistent with previous reports (Inman and Steward, 2003; Steward et al., 2004; Zheng et al., 2006). In this specimen, axon arbors are mostly in the dorsal part of the grey matter, which has been previously observed for tracer injections that preferentially
target primary sensory cortex over primary motor cortex (Bareyre et al., 2002; Tuszynski and Steward, 2012). Overall, these results indicate that the CST far rostral to a lesion in PTEN-deleted mice is consistent with the established distribution of the mouse CST.

**CST regeneration through a complete crush lesion in PTEN-deleted mice**

In PTEN<sup>f/f</sup> mice injected with AAV-Cre that were given a complete crush injury as adults at T8, some CST axons extended through and caudal to the lesion. Figure 2 shows CST axons extending caudal to a lesion, in a reconstruction of color-coded axon tracings through 400 µm of resin-embedded serial sagittal sections. Rostral to the lesion, CST axons have an expansive spread extending into the ventral spinal cord, indicating a bloom of CST growth not present in the spinal cord several segments farther rostral as shown in Fig. 1. A number of axons extend into the lesion, and some axons extend caudally from the lesion, consistent with the definition of regenerated axons as has previously been proposed (Tuszynski and Steward, 2012). Some axons also appear to be branched within the lesion.

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 tracings in light green are of the background section which has the central canal; the light green and dark green tracings are closest to midline. The tracings progress to purple on the same side as the dorsal CST, and progress to red on the side opposite the dorsal CST. Notably, the arbor includes tracings from sections on both sides of the central canal (red through purple tracings), indicating bilateral extension.
While most of the tracings caudal to the lesion can be followed as being part of the large arbor, at least some axon segments (arrowheads) from the lateral extreme of this reconstruction appear as distinct from the reconstructed arbor, reflecting that they likely have rostral origins in adjacent tissue that was not part of this reconstruction.

**Regenerated CST axons form synapses**

As synaptic transmission is the basis of communication between neurons, for regeneration to contribute to circuitry, it is important that the regenerated axons form synapses (Jessell and Kandel, 1993; Aubert et al., 1995; Schwartz, 2003; Woolf, 2003; Bradbury and McMahon, 2006; Deng et al., 2013). From the specimen in Fig. 2, we selected the resin-embedded section with axon segments traced in red for analysis of whether the regenerated axons formed synapses at the ultrastructural level.

In the resin-embedded section, various axon swellings were identified as putative synapses before analysis by electron microscopy (EM). Of the 13 swellings analyzed under EM (Fig. 3), 3 were confirmed to have a contact zone with synaptic vesicles and an apposing post-synaptic density, thus indicating that these boutons formed structural synapses. Fine detail within the labeled boutons was obscured by the nickel-enhanced BDA reaction product, partially obscuring vesicles. A nearby unlabeled synapse is shown for comparison (Fig. 3E). Bouton #3, which appeared to have less-dense labeling from the reaction product, appeared to have an asymmetrical synapse based on the relative thickness of the post-synaptic density and the dense material on the pre-synaptic membrane. These results confirm that CST axons that regenerate due to PTEN deletion form structural synapses caudal to a spinal cord injury.
DISCUSSION

CST regeneration that results from PTEN deletion is the first reported observation of robust regeneration of the adult CST. Here, we have presented that CST axons that regenerate due to PTEN deletion form synaptic structures caudal to the lesion. These results indicate that manipulation of the PTEN pathway can produce regeneration that may contribute to caudal circuitry.

Regeneration and not axon sparing

In normal mice, there is essentially no re-growth of axons beyond a spinal cord injury (Tuszynski and Steward, 2012). Here, we have identified synapses on CST axons that extend caudal to a spinal cord lesion. One critical consideration is whether the axons caudal to a lesion could be spared axons. A few lines of evidence indicate that the axon arbor from which synapses were identified is regenerated, and not spared. First, spared axons, such as those that have remained patent in the dorsal column following a partial transection (Steward et al., 2003), would be expected to have a straight longitudinal course consistent with once being unbranched, and would be expected in the white matter. In the arbor reconstruction (Fig. 2), no axons course straight and longitudinally for a long distance, and the axons turn laterally into adjacent thick sections (reflected by each color change) nearly exclusively in the grey matter. Second, the extensive arbor seems to expand from a single axon in the dorsal spinal cord, and it would be fairly unlikely to have a single spared axon rather than having few axons spared that are close together and originate from a larger dense bundle. Third, the tracings extend into sections on both sides of the central canal. This course is
unexpected for spared axons, particularly as sections far rostral to the lesion indicated the CST in this specimen predominantly coursing on one side (Fig. 1.). Finally, axons are seen extending into the lesion, and the axon for which the arbor originates extends out from the lesion, which is compelling evidence of axon regeneration (Steward et al., 2003; Tuszynski and Steward, 2012).

**Synaptic structures vs. function**

We have demonstrated that regenerated CST axons form synapses based on the presence of synaptic structures. One of the synapses appeared to be asymmetrical, which is reflective of a putative excitatory glutamatergic synapse (Gray, 1959; Müller et al., 1992; Klemann and Roubos, 2011; Harris and Weinberg, 2012). That this synapse is putatively glutamatergic is consistent with our parallel analysis that identified regenerated CST axons to have boutons co-labeled with the glutamatergic marker vesicular glutamate transporter 1 (VGlut1), as presented in Liu et al (2010). Thus, some regenerated CST axons appear to make glutamatergic synapses. As CST axons are known to be glutamatergic (Persson et al., 2006; Du Beau et al., 2012), formation of putative glutamatergic synapses suggests that at least some CST axons that regenerate due to PTEN deletion form synapses appropriate for being of the CST.

Though the component structures identified (the post-synaptic density and synaptic vesicles) suggest synaptic function, future studies such as by electrophysiology would be required to validate functionality. Of further interest for function is the resultant circuitry of the regenerated axons, which would have composition by the laterality of the projections and identities of the post-synaptic cells.
A note on laterality

The axon arbor in Fig. 2 extends into sections on both sides of the central canal, indicating bilateral extension. The synapses identified were also on the side ipsilateral to the injected cortex. These points are noteworthy because the CST courses predominantly on the side of the spinal cord contralateral to the cortex of origin in most mammals, including mice (Terashima et al., 1983; Yokoyama et al., 2001; Zheng et al., 2006; Iwasato et al., 2007; Kuypers, 2011), as well as in the presented specimen far rostral to the lesion (Fig. 1).

The functional significance of CST regeneration that extends bilaterally is unknown. Other studies have correlated developmental bilateral CST extension with a hopping gait in mice and mirror movements in humans (Dottori, 1998; Kullander, 2001; Yokoyama et al., 2001; Finger et al., 2002; Srour et al., 2010; Nugent et al., 2012). However, as motor performance from the transected spinal cord can be improved by training (Edgerton et al., 2001; Fong et al., 2005), it may be that circuitry in the spinal cord may adapt with bilateral CST regeneration for meaningful recovery.

As bilateral regeneration was observed here but was not part of a quantitative assessment, we have undertaken a follow-up study to assess the extent and consistency of the laterality of regenerative growth of the CST due to PTEN deletion. Further studies will be required to assess functional recovery and elucidate any correlations with underlying circuitry.
**Translation for human SCI?**

We have shown that manipulation of the PTEN pathway can be used to promote regeneration of CST axons that form synapses caudal to a SCI. This is a proof-of-concept study that involved 1) genetic deletion, and 2) manipulation before injury. For translation into a therapy applicable for humans, it will be necessary to identify an approach yielding functional benefit with administration post-injury and that does not involve deletion of a gene. One such approach may be a temporary knock-down of PTEN, such as with short-hairpin RNA (shRNA). Initial follow-up studies using shRNA have been taken up in our lab and the He lab (Zukor et al., 2013; Lewandowski and Steward, 2014).

REFERENCES


Lewandowski G, Steward O. 2014. AAVshRNA-mediated suppression of PTEN in adult rats in combination with salmon fibrin administration enables regenerative growth


Figure 1. Distribution of labeled CST axons far rostral to a spinal cord lesion. 

A, Schematic indicating the relative position of the cross section in B to the T8 crush injury and BDA injection. B, Cross section illustrating the distribution of BDA-labeled CST axons in the upper thoracic spinal cord rostral to the T8 crush injury. Note that the labeled axon arbors in the grey matter are mostly in the dorsal part, and overall axons course predominantly on the left side. dCST, dorsal corticospinal tract; dlCST, dorsolateral corticospinal tract. Scale bar, 250 µm.
Figure 2. CST regeneration through a T8 crush lesion. Tracings of BDA-labeled axon segments are rainbow color-coded by tissue depth in 50 µm increments and overlaid onto a micrograph of one section containing the central canal. Note that the arbor caudal to the lesion is made of axon tracings in adjacent colors, and is mostly in the dorsal spinal cord. Also note the bloom of axons rostral to the lesion extends ventral to the central canal. The light green tracings are from axons in the background image. The yellow, orange, and red axons are on the right of the spinal cord, opposite the dCST. The tracings progress to purple on the same side as the dorsal CST. The red axon tracings are of the section selected for analysis by electron microscopy; note that these are on the side opposite the dCST. Arrowheads indicate traced segments that appear to not be part of the reconstructed arbor. cc, central canal. Scale bar, 250 µm.
Figure 3. CST axons that regenerate due to PTEN deletion form synaptic structures. 
A, A light micrograph of a resin-embedded section indicating locations of putative synaptic boutons that were analyzed under electron microscopy (arrows). Yellow arrows indicate where synaptic structures were confirmed. Structures from axon swellings #3, #6 and #10 are shown in B-D, respectively. B-E, Electron micrographs of synaptic structures formed by BDA-labeled axons (B-D) and a nearby un-labeled synapse (E). Synapses were confirmed based on having a contact zone with a post-synaptic density (arrows) and synaptic vesicles (arrowheads). m, mitochondrion. Scale bars, 100 µm (A), 500 nm (B-E).
CHAPTER 3

Breaking contralateral rule: Variable laterality of regenerative growth of corticospinal tract axons beyond a spinal cord lesion
ABSTRACT

Laterality is established for many tracts of the nervous system, including the corticospinal tract (CST). That CST axons originating in one cortical hemisphere extend and terminate predominantly in the contralateral spinal cord indicates that the CST has a contralateral rule. Disturbed CST laterality has been correlated with motor abnormalities such as mirror movements in humans and a hopping gait in mice. Recent developments have demonstrated extensive CST regeneration due to PTEN deletion and knock-down, and it is of interest to know how the laterality of regenerative growth compares to the intact CST. Here, we use established methods of PTEN deletion to enhance regenerative growth, and assess the laterality of regenerative growth of the CST extending beyond a dorsal hemisection lesion in comparison to the non-injured CST in control mice. Based on anterograde labeling from one cortical hemisphere in the non-injured mouse, we found that the non-injured CST is 97.9 ± 0.7% contralateral in the white matter and 88.5 ± 1.0% contralateral in the grey matter of the low-thoracic spinal cord. In contrast, laterality of regenerative growth in PTEN<sup>f/f</sup> mice injected with AAV-Cre was variable, with 63.2% ± 13.1% on the contralateral side, and in some cases the regenerative growth was predominantly on the ipsilateral side. These results indicate that regenerative growth of the CST does not maintain the contralateral rule of the non-injured CST, highlighting one aspect for how resultant circuitry from axons that regenerate may differ from that of the uninjured CST.
INTRODUCTION

Organization of the vertebrate nervous system includes lateralization of axonal tracts within the spinal cord. Concerning 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 understood to control fine 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), as has been documented in animals including monkeys (Lacroix et al., 2004; Rosenzweig et al., 2009, 2010) and rats (Rouiller et al., 1991).

The importance of laterality of the CST is evident from cases of aberrant ipsilateral or bilateral CST projections and synkinetic mirror movements in mice and humans. In mice with dysfunctional ephrin B3/A4 ligand-receptor signalling, the CST projects bilaterally within the spinal cord, and mice have a resultant kangaroo-like hindlimb hopping gait (Dottori, 1998; Coonan et al., 2001; Yokoyama et al., 2001). In humans, synkinetic mirror movements are present in Joubert Syndrome, and individuals have a decreased or lack of CST decussation (Friede and Boltshauser, 1978; Maria et al., 1999; Engle, 2010b). 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, 2010b). As well, a mutation in the netrin-1 receptor gene deleted in colorectal carcinoma (DCC) in mice results in a lack of CST decussation at the pyramids and a hopping gait (Finger et al., 2002), and a mutation in this gene in
humans has been identified as the cause of congenital mirror movements (CMM) (Srour et al., 2010). Spinal cord injury (SCI) may interrupt long tracts of motor axons and result in permanent paralysis, 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 large numbers of regenerated CST axons had been achieved (Liu et al., 2010). An approach producing 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).

Knock-down of PTEN in the cortex coupled with injection of salmon fibrin into a spinal cord lesion has also been demonstrated to promote recovery of voluntary motor function (Lewandowski and Steward, 2014).

Toward understanding CST regeneration that may contribute to circuitry for recovery of motor function, it is of interest to determine whether the regenerative growth of CST axons maintains the contralateral rule of the intact CST. Notably, we previously noted that CST axons that grew past a lesion as a result of PTEN deletion extended bilaterally, which was one criterion for identifying the axons as having regenerated (Liu et al., 2010). There was no quantitative analysis of the degree of bilaterality, however. The present study continues use of methods as in Liu et al. (2010) to delete PTEN as means to enhance CST regeneration, and quantitatively assesses the laterality of the resultant regenerative growth in relation to that of the intact CST. We present here that regenerative growth of the CST does not maintain the intact CST’s contralateral rule.
MATERIALS AND METHODS

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, Irvine.

Mice

PTEN-floxed (Pten\textsuperscript{f/f}) mice [C;129S4-Pten\textsuperscript{tm1Hwu/J}, Jackson Labs] were used in this experiment. In selected litters, AAV-Cre was injected into the cortex to promote CST regeneration following spinal cord injury via cortical PTEN deletion. Injections of AAV-Cre (serotype 2, Vector Biolabs) were made into the left cortex in newborn Pten\textsuperscript{f/f} mice on the first postnatal day (P1), in manner adapted from (Liu et al., 2010). Briefly, pups were cryo-anesthetized and then injected with 500 nl of AAV-Cre (10\textsuperscript{12} GC/ml) into each of three sites along the left side of 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.

PTEN\textsuperscript{f/f} 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 were given a dorsal hemisection lesion at T12, using techniques described previously (Steward et al., 2008). Briefly, mice were anesthetized with isofluorane, and the
surgical area was shaved and swabbed with betadine. 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 the estimated depth of the central canal to sever the dorsal and dorsolateral components of the CST. Mice were injected with biotinylated dextran amine (BDA) to label CST axons ~8 weeks post-injury, and were humanely killed ~10 weeks post-injury.

*CST tracing*

Mice with a spinal cord lesion (n=8) and non-injured control mice (n=5) were injected with BDA to trace CST axons of the left sensorimotor cortex. Injections of BDA (10,000 MW, 10% in dH$_2$O, Invitrogen) were made in stereotaxic coordinates in the left sensorimotor cortex. Coordinates for injections were 0.6 mm deep, 1.0 mm lateral, and 0.5 mm rostral, 0.2, 0.5, and 1.0 mm caudal to bregma (0.4 µl per site). Mice were humanely killed and transcardially perfused 2 weeks post-injection.

*Histology*

Mice were euthanized with an overdose of Euthasol® (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 4% PFA before being equilibrated in 27% sucrose. Blocks of cryoprotected spinal cord were frozen in TissueTek O.C.T. (Sakura Finetek) for sectioning with a cryostat. Sagittal sections 30 µm thick were collected through the lesion, and cross sections were
collected ~4mm rostral and caudal to the lesion to use in screening for lesion completeness and adequate labeling. Sagittal and rostral cross sections were collected in comparable regions of spinal cord in non-injured control mice. Coronal sections 20 µm 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 µm.

**Signal amplification**

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% 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 spinal cord cross sections used for resin embedding, BDA was detected by chromogenic staining with diaminobenzidine (DAB), as has been 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 reaction was performed in 50mM Tris buffer, pH 7.6, 0.024% hydrogen peroxide and 0.5% nickel chloride.

Brain sections were stained for BDA using direct conjugation and 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 Signalling) 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 0.5% NDS. Sections were then washed in TBS-Tw before being incubated in a 4 µg/ml dilution of tyramide-conjugated fluorescein in 0.1M 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. Sections were washed a final three times in PBS-Tx and mounted onto gelatin-coated slides.

Resin sections

From non-injured control mice, cross sections with strong DAB staining of BDA were selected for resin embedding as has been 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% ethanol. 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 60ºC. 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® software.

*Quantification*

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.

\[
\text{Laterality index} = \frac{\text{contralateral axons}}{\text{contralateral axons} + \text{ipsilateral axons}}
\]

To quantify CST laterality in grey matter, sagittal sections of the low thoracic spinal cord were visually overlaid with 3 dorsal-ventral lines spaced 400 µm apart beginning 400 µm from the lesion epicenter. The number of axons in the grey matter (GM) crossing these lines was counted for the dorsal and ventral spinal cord, both ipsilateral and contralateral to the injected cortex. Uninjured spinal cords were analyzed similarly.

Laterality of CST axons in the white matter of non-injured mice was quantified in DAB-stained cross sections with DAB staining. DAB-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-stained axons in the dorsal column.

Statistics

Laterality indices were transformed by arcsine to reduce skewness due to being proportions. Transformed data were analyzed with a F test for to assess homoscedasticity. Statistical analysis was performed with GraphPad Prism®.

RESULTS

CST Laterality in the non-injured mouse

Baseline CST laterality was established by assessing the sidedness of CST axons in the low thoracic spinal cord labeled from BDA injections into the left sensorimotor cortex of non-injured PTEN\textsuperscript{ff} mice. Laterality of CST axons in white matter was assessed in cross section, as the distribution of CST axons in white matter has been previously assessed in cross sections 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 laterality in grey matter was assessed in sagittal sections as this approach would be used to analyze regenerative growth of the CST in other specimens.

The laterality of the CST in the uninjured mouse 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. 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. Sparse axons were also in the dorsolateral white matter on the contralateral side, and few labeled axons were present in the remaining white matter. In the grey matter, BDA-labeling was also predominantly contralateral, and extended into the dorsal horn more than the ventral horn (Fig 1A,C-D). Quantification of the laterality of the labeled CST axons is presented for each of the 5 control mice in Fig. 1E. Labeled CST axons were 97.9 ± 0.7% contralateral in the white matter, and 88.5 ± 1.0% contralateral in the grey matter (presented as means ± SEM). These data present the baseline laterality of the CST in the white matter and grey matter of the low thoracic spinal cord, and the degree and consistency of laterality establish the contralateral rule of the CST.

**Deletion of PTEN in the cortex**

As there is typically little or no regeneration of the CST, manipulation of PTEN was used to enhance regenerative growth of CST axons (Liu et al., 2010). PTEN<sup>f/f</sup> mice were injected with AAV-Cre into the left side of the brain at postnatal day 1 (P1) to delete PTEN from the left cortex. As adults, these mice were given a lesion at the 12<sup>th</sup> vertebral level of the thoracic spinal cord (T12) and injected with BDA into the sensorimotor cortex ~8 weeks later, before termination ~10 weeks post-injury.
Figure 2 illustrates deletion of PTEN in the cortex. PTEN antibody staining was generally diffuse, but the left cortex revealed cellular-shaped areas devoid of PTEN staining and appearing as staining “holes” in a laminar pattern (Fig. 2A). In contrast, the non-injected cortex on the right side lacked cellular-shaped areas devoid of PTEN staining (Fig. 2B). Injection of BDA made into the left cortex labeled some cells and fibers, including some cells that lacked PTEN staining (Fig. 2C-E), confirming that the staining “holes” were not actual holes in the tissue. These results indicate that based on PTEN staining, injection of AAV-Cre in neonatal pups can delete PTEN from individual cells in the cortex, and some of these cells can be labeled by cortical injection of BDA.

Laterality of CST regenerative growth

PTEN\textsuperscript{fl/fl} mice injected with AAV-Cre were given a dorsal hemisection lesion at T12 to sever the established components of the mouse 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 excluded based on having insufficient labeling or having an incomplete lesion with spared labeled axons in the dorsal white matter. The remaining six specimens had lesions extending down to the central canal and lacked axons continuing longitudinally in the dorsal part of the lateral white matter. No axons were observed coursing longitudinally in the ventral white matter through the rostro-caudal extent of the sagittal sections. Each of these specimens had CST axons extending caudal to the lesion, described as regenerative growth.
All six mice had regenerative growth with at least some axons extending on each side of the spinal cord. In the descriptions below, we use the terms “contralateral” and “ipsilateral” with respect to the cortex of origin. A selected specimen is shown in Figures 3 and 4. Serial sagittal sections in Figure 3 illustrate the laterality of the CST. Rostral to the lesion, BDA-labeled axons are mostly on the side contralateral to the injected cortex (sections #1-21), in contrast to the ipsilateral side (sections #23-44). The expansive labeling rostral to the lesion is indicative of a bloom of growth of CST axons, consistent with what has been previously reported following SCI in mice with cortical PTEN deletion (Liu et al., 2010). In this specimen, regenerative growth is more prevalent 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, indicating that these axons extending caudally were once cut. Axons extended longitudinally 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 this or the other 5 analyzed specimens with regenerative growth.

As the spinal cord is an imperfect cylinder, longitudinal sections at the lateral extremes of the spinal cord are irregular and often lost, and thus axons coursing in the lateral extremes can be missed. As most axons extending caudal to the lesion were observed 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.
The presented specimen in Fig. 3 & 4 had largely ipsilateral regenerative growth, with only 34.3% of axons in the grey matter caudal to the lesion on the contralateral side. Laterality for the group varied, however, with axons extending caudally predominantly on the ipsilateral side in 3 mice, and on the contralateral side in 3 others, producing a bimodal distribution that was 63.2% ± 13.1% contralateral (mean ± SEM, Fig. 4E). Notably, the variances of the arcsine-transformed laterality indices of axons in the grey matter were significantly different for the group with regenerative growth versus the non-injured controls ($F_{5,4} = 104.4, p = 0.0005$, F test). Thus, laterality of regenerative growth of the CST is variable compared to laterality of the uninjured CST.

**Regenerative growth in the white matter**

Though most regenerative growth was in the grey matter, in some instances axons caudal to the lesion also coursed in the spinal cord white matter. Based on axons extending in the ventral column and occasionally in the dorsal column, we estimate that in some mice as many as 13% of axons caudal to the lesion extend 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 contralateral spinal cord ~30 µm from midline. An axon in this section extends caudal to the lesion in the ventral column, thereby bypassing the lesion in the ventral column as has been described by Steward et al. (2008). This axon extends a short distance farther in the adjacent sections, ending <1 mm caudal to the lesion. Fig. 5C-D are of an ipsilateral section ~210 µm from midline, showing an axon extending caudal to the lesion that enters into the ventral column.
Axons bypassed the lesion via the ventral white matter in 3 of the 6 specimens, with 2 having axons bypassing via the ventral column, and two via the ventral part of the lateral column. In each case the ventral axons bypassed the lesion on the contralateral side. Axons joining 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.

**DISCUSSION**

The present study assesses the laterality of regenerative growth of the CST in follow up to a previous study demonstrating enhanced CST regenerative growth resulting from PTEN deletion. We observed that most axons caudal to the lesion course within the grey matter rather than the white matter. We demonstrate the contralateral rule of the non-injured CST by the degree and consistency of CST laterality, and we show that regenerative growth of the CST is variable, sometimes ipsilateral, and overall does not consistently maintain the contralateral rule. Notably, substantial ipsilateral extension was seen in half the mice; in the rest, regenerated axons had the same preference for the contralateral side as normal axons. This highlights one anatomical aspect of regenerative growth that suggests how resultant circuitry might be different from the circuitry of the intact CST.
**Regeneration vs. regenerative growth**

To enhance regenerative growth of the CST, we used the approach of PTEN deletion, which has been shown to result in regenerative growth and bona fide regeneration of CST axons after SCI (Liu et al., 2010). We used the term “regeneration” as has been previously proposed, with specific reference to regrowth of an injured or transected axon beyond a lesion (Tuszynski and Steward, 2012). We use the term “regenerative growth” to describe new growth extending caudal to a lesion, which includes regeneration and new growth of uninjured axons.

In our study, some axons course into and through the lesion, which is indicative of regeneration and consistent with previous observations (Liu et al., 2010). With our partial lesion model, we also observed axons coursing around the lesion, mostly via the ventral grey matter, and we cannot exclude that some growth caudal to the lesion may originate from axons in the grey matter that were not originally cut. Thus, our analysis of axons extending caudal to the lesion is of regenerative growth, and is not analysis of regeneration alone.

**CST laterality**

In the white matter, we found the CST to be ~98% contralateral in the low thoracic spinal cord of the mouse. This is consistent with previous studies in rats; Rouiller et al. (1991) reported that ~98% of CST axons coursed in the contralateral white matter in the cervical spinal of the rat.

In line with our quantification of CST laterality in the grey matter, Rouiller et al. (1991) also report that based on axon density in the cervical grey matter and retrograde
labeling in the cortex from unilateral injection into the spinal cord, ~96-98% of the CST contribution in rat comes from the contralateral cortex. In the mouse, reports based on unilateral injection of a retrograde label in the lumbar spinal cord indicate ~80% (Yokoyama et al., 2001) and ~98% (Iwasato et al., 2007) of CST contribution coming from the contralateral cortex. Our result of the CST being ~89% contralateral in the low thoracic grey matter fits with these previous studies. However, it is possible that our quantification slightly underrepresents the contralateral CST, as potentially some axons overlapped and were missed in the most densely labeled regions of contralateral grey matter in our longitudinal sections. Potential underrepresentation is unlikely in our assessments of the laterality of regenerative growth as the axons extending caudal to a lesion were comparatively sparse and easily distinguished.

The contralateral rule is not maintained in some mice

The fact that CST axons induced to regenerate following PTEN deletion extend bilaterally in caudal segments has been noted previously (Liu et al., 2010), although the extent of contralateral vs. ipsilateral extension was not quantified. It was noteworthy here that 3 of the mice assessed here exhibited axon extension predominantly on the ipsilateral side. That laterality of regeneration can range from being mostly contralateral to mostly ipsilateral fits with laterality being variable, and indicates that regenerative growth due to PTEN deletion may extend beyond a lesion without predetermined sidedness. These findings indicate that a contralateral rule for the CST is not invariably maintained after spinal cord injury.
That the contralateral rule may lend toward being broken is evident from previous studies. Following a lesion to injure one side of the CST, the non-injured side of the CST has been noted to increase crossing of midline into the denervated side of the spinal cord in wild-type mice and rats (Maier et al., 2008; Ghosh et al., 2009; Lee et al., 2010). This fits in consideration of a contralateral rule established by various guiding cues in development that are not maintained into adulthood. An example of this is repulsion of CST axons by midline expression of ephrin-B3 (Coonan et al., 2001; Kullander, 2001; Yokoyama et al., 2001), which is diminished in the ventral midline of the spinal cord by adulthood (Omoto et al., 2011).

The effect of PTEN deletion in mice given a unilateral pyramidotomy is to enhance compensatory sprouting of the uninjured axons across midline, shifting the ratio of CST axon density in the grey matter toward the ipsilateral side (Liu et al., 2010). As PTEN has been demonstrated to mediate chemorepulsive turning of growth cones (Chadborn et al., 2006; Henle et al., 2013), deletion of PTEN may be rendering axons insensitive to some repellant cues that result in more axons crossing the midline. In further speculation, this same phenomenon provides an explanation for how numerous axons of regenerative growth due to PTEN deletion may end up on the ipsilateral side, as well as how axons may regenerate through a normally repulsive lesion (Pasterkamp and Giger, 2009; Henle et al., 2013).

We do not know what accounts for the bimodal distribution of axons extending caudal to the lesion, but PTEN has been speculated to play strikingly different biological roles under slightly different conditions (Yamada and Araki, 2001), consistent with different phenotypes of PTEN-knockout mouse embryos (Di Cristofano et al., 1998;
Suzuki et al., 1998; Podsypanina et al., 1999). In this line of thought, it is possible that some combination of variables in the injured mice in this experiment results in a binary switching of axons to course predominantly ipsilaterally vs. contralaterally beyond the lesion. Alternatively, what seems to be a bi-modal distribution may simply be a chance observation in our small sample size, and a more continuous distribution of laterality might be seen with larger samples.

Implications for circuitry

As we show that regenerative growth may differ from the intact CST in sometimes being bilateral or even predominantly ipsilateral, it follows that resultant circuitry may also have altered laterality. Evidence that regenerative growth of the CST may contribute to circuitry come from previous studies showing that CST axons that regenerate due to PTEN deletion or knock-down form synapses (Liu et al., 2010; Zukor et al., 2013), including structural synapses on the ipsilateral side (unpublished observations). Additionally, PTEN knock-down that promotes regenerative growth of the CST combined with injection of salmon fibrin into a cervical lesion has been found to enhance fine motor recovery (Lewandowski and Steward, 2014). Motor recovery in this latter study was notably of both forelimbs, despite PTEN knock-down being limited to one cortical hemisphere. However, the recovery could be due to short-distance CST growth and circuit refinement independent of CST regeneration.

While one may infer that the laterality of circuitry from regenerative growth is abnormal in some mice, we can only speculate as to how bilateral CST regeneration would affect function. Previous studies have correlated aberrant CST laterality with a
hopping gait in mice (Dottori, 1998; Coonan et al., 2001; Yokoyama et al., 2001; Finger et al., 2002), but the hopping gait could also be attributed to misformation of the spinal cord’s central pattern generator (CPG) (Kullander, 2003; Iwasato et al., 2007; Rabe Bernhardt et al., 2012). Further, motor performance in the transected spinal cord can be improved with training (Edgerton et al., 2001; Fong et al., 2005), reflecting that circuitry in the lesioned spinal cord is not static. Thus, it is plausible that bilateral CST regeneration may actually lead to recovery like that observed by Lewandowski and Steward (2014), which would suggest that the resultant circuitry is bilateral, abnormal, and refined for meaningful motor function from a unilateral source. It remains to be seen whether variable laterality of regenerative growth is an unexpected challenge for therapeutic translation, or a regenerative advantage.

REFERENCES

Lewandowski G, Steward O. 2014. AAVshRNA-mediated suppression of PTEN in adult rats in combination with salmon fibrin administration enables regenerative growth


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 staining of BDA-labeled CST axons. Note that the labeled CST axons are predominantly 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<sup>f/f</sup> 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 µm (A, C-D), 50 µm (B).
Figure 2. Deletion of PTEN in the cortex. PTEN staining of cortex from an adult PTEN<sup>fl/fl</sup> mouse reveals areas with cellular shapes devoid of PTEN in the cortex injected with AAV-Cre at P1 (A) in contrast to the right cortex that had no injection (B). Staining for BDA which was also injected into the left cortex (C, enlarged in E) revealed BDA-labeling of some cells devoid of PTEN (white arrows, D-E), and BDA-labeling of other cells with PTEN staining (black arrows, D-E). 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 µm.
Figure 3. Serial sagittal sections of one specimen showing regenerative growth of CST axons extending caudal to a spinal cord lesion predominantly 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. Scale bar, 500 µm.
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 mice with regenerative growth. The variances of arcsine-transformed laterality ratios were significantly different between groups ($F_{5,4} = 104.4, p = 0.0005$). § indicates the specimen depicted. Error bars indicate SEM. 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 µm.
Figure 5. CST axons sometimes extend into white matter caudal to the lesion on either side of the spinal cord. A-B, 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).
CONCLUSIONS

Remarkable gains have been made in the field of CNS axon regeneration in the last decade, though the field is still in adolescence. To serve the field in guidance on using a mouse model with genetic CST labeling for regeneration studies, this dissertation provided a critical evaluation of CST-YFP mice revealing that non-specific and faint labeling limit their utility for studies of CST regeneration. Toward understanding CST regeneration that may contribute to circuitry beyond a lesion, this dissertation also evaluated CST regenerative growth that results from PTEN deletion. In these experiments we found that regenerated CST axons form synapses caudal to a lesion, and the laterality of the intact CST is not maintained by axons extending caudal to the lesion. Overall, this body of work serves to guide and advance the field of CST regeneration.

In consideration of using CST-YFP mice, it is noteworthy that the experiments we performed using PTEN deletion to achieve CST regeneration would likely not have been possible. First, we deleted PTEN by injecting a vector expressing Cre into the cortex of PTEN-floxed mice at P1. Deletion of PTEN after birth was important as PTEN is necessary for development, and PTEN knock-out mice die in utero and have malformations of the cerebral cortex (Di Cristofano et al., 1998; Suzuki et al., 1998; Podsypanina et al., 1999). In CST-YFP mice, however, YFP expression is dependent on expression of Cre, which is driven by Emx1 beginning in prenatal development. Thus, in mice crossed to have floxed PTEN, Thy1-STOP-YFP, and Emx-Cre (theoretical CST-YFP X PTEN mice), the prenatal expression of Cre by Emx would prenatally delete
PTEN, and would likely lead to severe cortical abnormalities and possibly death of the mice. Thus, our study of CST regeneration due to PTEN deletion would likely not have been possible in CST-YFP mice. Additionally, as genetic labeling can not currently be limited to one side of an animal, projections of the CST from both cortical hemispheres would be labeled. Thus, our study of CST laterality would not have been possible with genetic labeling, highlighting one advantage of using anterograde tracers for the study of CST axons. Not being able to perceive bilateral CST regeneration would also eliminate being able to use unusual bilateral extension as evidence that axons have taken an unusual course, a criterion that can be used to support that axons had regenerated (Steward et al., 2003; Tuszynski and Steward, 2012).

A substantial consideration of using CST-YFP mice for studying CST regeneration is the significance of the labeling of non-CST axons. Notably, the CST has a lower capacity for regeneration than other axon tracts, including the rubrospinal and reticulospinal tracts (Blesch and Tuszynski, 2009). In CST-YFP mice with retrograde FG labeling, we estimated that there were ~89 rubrospinal and ~66 reticulospinal neurons co-labeled for YFP and FG in 1.5 mm of midbrain, representing a total of ~155 FG-positive and YFP-positive neurons in 1.5 mm of the brainstem. These co-labeled neurons could account for over half of the estimated ~272 non-CST axons labeled with YFP in the spinal cord. As there may be ~12,000 YFP-labeled CST axons in the cervical spinal cord as calculated by Bareyre et al. (2005), non-CST axons labeled by YFP represent ~2% of all YFP-labeled axons in the CST-YFP mouse spinal cord. Our study of CST regeneration due to PTEN deletion and a subsequent study using PTEN knock-down (Zukor et al., 2013) broadly reflects that ~1-7% of the labeled CST
regenerates due to PTEN manipulation. Thus, the number of non-CST axons labeled with YFP is substantial even in comparison to the robust CST regeneration due to PTEN manipulation.

In consideration of CST-YFP mice for assessment for synapses of regenerated CST axons as we did for BDA-labeled axons in PTEN-deleted mice, could meaning be discerned for a synapse of a regenerated YFP axon? In the CST-YFP mouse, if a YFP-labeled axon is confirmed to have regenerated and formed a synapse, it would indicate that a CNS axon regenerated and may contribute to circuitry beyond the lesion. But based on YFP labeling alone, it may not be possible to conclude that it is a CST axon as some non-CST axons also being labeled with YFP. However, the glutamatergic synaptic marker VGluT1 has some specificity for the CST, with VGluT1 labeling ~96% of CST terminals but <5% of terminals from the other main descending tracts (Du Beau et al., 2012). Thus, identification of a bouton labeled by VGluT1 on a YFP-labeled axon increases the probability that the axon is of the CST, and reflects a putative CST synapse. Though not definitive, this identification would be useful as a preliminary finding in a study on enhancing CST regeneration, and ultrastructural analysis of BDA-labeled CST axons could be used in follow-up to definitively confirm that CST axons that regenerated due to the intervention form synapses.

We expect that labeling of non-CST axons in CST-YFP mice stems from the mice being designed to have permanent labeling resulting from expression of Emx1, which is not restricted to the forebrain (Briata et al., 1996). Wrongfully presuming CST specificity in CST-YFP mice and mice with Emx1-driven labeling may result in incorrect conclusions including from studies outside of CST regeneration. Some previous studies
using CST-YFP mice or mice with Emx1-driven labeling reported findings on CST reorganization following stroke (Liu et al., 2009, 2013) and direct synaptic contacts of CST axons onto motoneurons (Bareyre et al., 2004). Our revealing of non-specific labeling in CST-YFP mice casts doubt on findings from these previous studies, and without follow-up with specific CST labeling, we consider these findings as being provisional.

The ideal genetically-labeled mouse for studying CST regeneration would have labeling specific to the CST. That CST-YFP mice have non-specific labeling reflects that the strategy used in the design of these mice—permanent labeling dependent only on co-expression of Emx1 and neuronal Thy1—is insufficient to produce labeling specific to the CST in the spinal cord. Thus, future generation of mice with CST-specific labeling will require additional and/or different strategies to preclude non-CST labeling. Two approaches that could be used toward this purpose are the requirement of expression of additional genes in the cortex that are not found in the midbrain (Luan and White, 2007), and use of temporally-inducible expression such as with the Tet On/Off system to impart temporal restriction (Schönig and Bujard, 2003). Solving this puzzle may require complicated strategies, and overall is likely to prove to be challenging.

We showed that CST axons that regenerated due to PTEN deletion form synapses caudal to a lesion, indicating that manipulation of the PTEN pathway can result in CST regeneration that may contribute to caudal circuitry. One of the synapses was identified as putatively being glutamatergic (Müller et al., 1992; Klemann and Roubos, 2011; Harris and Weinberg, 2012), which fits with our other findings of glutamatergic bouton-like swellings on regenerated CST axons (Liu et al., 2010).
As intact CST axons are glutamatergic (Persson et al., 2006; Du Beau et al., 2012), the formation of putative glutamatergic synapses by regenerated CST axons is encouraging for potential regain of normal CST function. Additionally, the synapses identified were of axons that had in fact grown through a crush lesion, reflecting that CST regeneration resulting from manipulation of the PTEN pathway can overcome a lesion regarded to have an especially high barrier to regeneration (Inman and Steward, 2003; Liu et al., 2010). Regeneration through a crush lesion is further promising and relevant to humans as most human SCIs are crush or contusion injuries (Tuszynski and Steward, 2012). Thus, these findings of CST regeneration and formed synapses are overall significant in the identification of PTEN manipulation as a therapeutic target that may be useful in producing meaningful regeneration beyond a spinal cord injury in humans.

In consideration of therapeutic translation, the PTEN-deletion experiments we have presented were notably proof-of-concept experiments that involved 1) genetic deletion, and 2) manipulation before injury. For translation into a therapy applicable for humans, it will be necessary to identify an approach yielding functional benefit with administration post-injury, and that does not involve deletion of a gene. One such approach may be a temporary knock-down of PTEN, such as with short-hairpin RNA (shRNA). Initial follow-up studies using shRNA have shown that knockdown of PTEN enhances CST regeneration (Zukor et al., 2013), and that PTEN knock-down coupled with injection of salmon fibrin into the lesion enhances recovery of voluntary motor function (Lewandowski and Steward, 2014).

In our experiments assessing regeneration due to PTEN deletion, we observed that regenerated CST axons coursed predominantly in the grey matter rather than the
white matter. As CNS myelin is known to express various myelin-associated inhibitors of axon growth (Woolf, 2003; Yiu and He, 2006; Silver, 2010), one may postulate that the white matter is highly inhibitory to axons that regenerate due to PTEN deletion. However, PTEN deletion in retinal ganglion cells (RGCs) followed by optic nerve crush resulted in substantial regeneration of axons in the optic nerve, demonstrating axon regeneration in CNS white matter (Park et al., 2008). Thus, CNS white matter is not preclusive to regeneration such as that from PTEN deletion. Still, it remains possible that the spinal cord grey matter is more permissive for regenerating axons than the white matter in the spinal cord (Savio and Schwab, 1989).

The mechanism by which deletion of PTEN promotes axon regeneration was demonstrated to be mostly through the mTOR pathway (Figure 1) (Park et al., 2008). However, as shown by Park et al, administration of rapamycin to quash mTOR signaling did not completely abolish the response, and deletion of TSC1 to result in constitutive mTOR activation did not produce regeneration to the same extent as by PTEN deletion. Thus, PTEN deletion effects regeneration through means beyond the mTOR pathway. Notably, PTEN also mediates chemorepulsion of growth cones in response to repellant cues that signal through phosphatidylinositol 3,4,5-trisphosphate (PIP3) (Henle et al., 2013). Guidance cues implicated to have PIP3 signaling include myelin-associated glycoprotein (MAG), Sema3A, bone morphogenic protein (BMP), growth factors such as brain-derived neurotrophic factor (BDNF), and netrin (Tran et al., 2007; Henle et al., 2011, 2013; Kolodkin and Pasterkamp, 2013). Of these, PTEN has been explicitly demonstrated to act in repulsive signaling in vitro from Sema3A, MAG, and BDNF (Chadborn et al., 2006; Henle et al., 2013). Some chemorepulsive cues are
upregulated in a spinal cord lesion [e.g. Sema3A (Pasterkamp and Verhaagen, 2001; Harel and Strittmatter, 2006; Kaneko et al., 2006)], highlighting that the mTOR-independent regeneration that results from PTEN deletion may be due to insensitivity to some repelling cues.

It is interesting that regenerative growth of the CST that results from PTEN deletion had variable laterality, and overall axons extending caudal to the lesion did not maintain the contralateral rule of the intact CST. This abnormal laterality indicates that the sidedness of CST axons that regenerate due to PTEN manipulation is not predetermined, despite that the intact CST and CST far rostral to a spinal cord lesion have a strong contralateral rule. Thus, our findings of abnormal CST laterality and that CST axons regenerated predominantly in the grey matter reflect that the course of the regenerating CST does not recapitulate the course of the intact CST. A possible explanation for this difference is that the growing CST axons post-injury are in a mature and regenerative state (O’Brien and Sagasti, 2009) which involves different signaling pathways from axons in a developmental growth state (Liu and Snider, 2001), reflecting that regenerating axons may react to cues differently from axons in development. Further, the spinal cord milieu during development is different than that in adulthood and after injury, with different levels and patterns of expression of growth factors and guidance factors (Harel and Strittmatter, 2006). Thus, the cues external to the axons and the signaling within axons differs in the spinal cord during development from the mature spinal cord after injury, reflecting that regenerating mature axons might never totally recapitulate intact spinal cord circuitry in advanced vertebrates (Harel and Strittmatter, 2006).
While abnormal CST laterality has been correlated with synkinetic mirror movements, our anatomical observations do not establish that a detriment would result from bilateral CST regeneration. First, humans withHorizontal Gaze Palsy with Progressive Scoliosis (HGPPS), which results from a ROBO3 mutation, have a predominantly ipsilateral CST but lack synkinetic mirror movements (Nugent et al., 2012), suggesting that CST axons still find their appropriate targets albeit on the incorrect side and that the motor circuitry is still developed for asynchronous motor control. Second, the implication of PTEN in specifically mediating chemorepulsion and not chemoattraction suggests that PTEN-deleted axons may still find appropriate targets albeit potentially via circuitous routes (Henle et al., 2013). Thus, it remains plausible that PTEN-deleted axons have an insensitivity for repulsion that enhances their regeneration beyond an inhibitory lesion, and targeting of the extending axons remains intact for meaningful contribution to caudal circuitry.

That cortical deletion of PTEN results in a robust regenerative response of the CST but only a small fraction of labeled axons extend past the lesion indicates that PTEN-deleted axons are still impeded by the lesion. However, even a small number of regenerated axons may still be greatly significant as it may only require a few axons coursing regenerating a short distance to restore valuable motor function. For a human with tetraplegia, regain of one neurological level in the cervical spinal cord could mean a regain of thumb movement, which could be used to operate a motorized wheelchair and provide a significant gain in the person’s independence for mobility.

Our experiments provide insight into the wiring of axons that regenerate due to PTEN deletion, but a number of questions remain important to our understanding of
CST regeneration and resultant circuitry that may be meaningful for motor function. For instance, what are the identities of the post-synaptic cells that receive inputs from regenerated CST axons? Are the cell types appropriate for the CST and on the appropriate side relative to the intact CST? These questions address aspects of how CST regeneration may or may not recapitulate circuitry of the intact CST. As we identified that the laterality of regenerated CST axons is sometimes abnormal, what is the behavioral consequence of bilateral CST regeneration as opposed to regeneration predominantly contralateral to the cortex of origin? One possibility for the circuitry that results from regenerated CST axons is that it may be initially abnormal but may also be refined and optimized over time as occurs during development and with training (Edgerton et al., 2001; Fong et al., 2005; Harel and Strittmatter, 2006). Thus, does motor function performance improve over extended time in mice with CST regeneration, and does it correlate to anatomical differences in circuitry? Further studies will be required to further delineate the resultant circuitry of CST axons that regenerate due to PTEN manipulation, and to elucidate the effects of the circuitry on motor function.

In these promising times of studying axon regeneration in the spinal cord, the work presented in this dissertation gives guidance, promotes caution, and gives hope for the development of regeneration-based therapies to eventually recover function lost from spinal cord injury.

REFERENCES


**Figure 1.** Scheme of the PTEN/mTOR signaling pathway. In response to receptor tyrosine kinase (RTK) activation, PI3K phosphorylates and converts the lipid second messenger phosphatidylinositol (4,5) bisphosphate (PIP2) into phosphatidylinositol (3,4,5) trisphosphate (PIP3), which recruits and activates phosphatidylinositol-dependent kinase 1/2 (PDK1/2). PDK1/2, in turn, phosphorylates and activates Akt. PTEN catalyzes the conversion from PIP3 to PIP2. Thus, inactivation of PTEN results in the accumulation of PIP3 and the activation of the Akt. Akt controls a host of signaling molecules, including TSC1/2. Downstream of the TSC1/2 complex lays mTOR, which integrates various cellular signals including nutrient availability to control protein translation, cell growth, and other processes. The ribosomal protein S6 kinase (RP-S6) and the eukaryotic initiation factor 4E (eIF-4E) binding protein 1 (4EBP1) are the mTOR effector molecules executing these functions. Cellular stresses such as hypoxia induce expression of Redd1/2, which augments TSC1/2 activity and in turn suppress the mTOR activity. From Park KK, Liu K, Hu Y, Smith PD, Wang C, Cai B, Xu B, Connolly L, Kramvis I, Sahin M, He Z (2008) Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. Science 322:963–966. Reprinted with permission from AAAS.