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Research Paper

Long-term consequences of conditional genetic deletion of *PTEN* in the sensorimotor cortex of neonatal mice



E.A. Gutilla ^{a,b,e}, M.M. Buyukozturk ^{a,b,e}, O. Steward ^{a,b,c,d,e,*}

^a Reeve-Irvine Research Center, University of California, Irvine, United States

^b Departments of Anatomy and Neurobiology, University of California, Irvine, United States

^c Neurobiology and Behavior, University of California, Irvine, United States

^d Neurosurgery, University of California, Irvine, United States

^e School of Medicine, University of California, Irvine, United States

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ABSTRACT

Targeted deletion of the phosphatase and tensin homolog on chromosome ten (PTEN) gene in the sensorimotor cortex of neonatal mice enables robust regeneration of corticospinal tract (CST) axons following spinal cord injury as adults. Here, we assess the consequences of long-term conditional genetic PTEN deletion on cortical structure and neuronal morphology and screen for neuropathology. Mice with a LoxP-flanked exon 5 of the PTEN gene (PTENf/f mice) received AAV-Cre injections into the sensorimotor cortex at postnatal day 1 (P1) and were allowed to survive for up to 18 months. As adults, mice were assessed for exploratory activity (open field), and motor coordination using the Rotarod®. Some mice received injections of Fluorogold into the spinal cord to retrogradely label the cells of origin of the CST. Brains were prepared for neurohistology and immunostained for PTEN and phospho-S6, which is a downstream marker of mammalian target of rapamycin (mTOR) activation. Immunostaining revealed a focal area of PTEN deletion affecting neurons in all cortical layers, although in some cases PTEN expression was maintained in many small-medium sized neurons in layers III-IV. Neurons lacking PTEN were robustly stained for pS6. Cortical thickness was significantly increased and cortical lamination was disrupted in the area of PTEN deletion. PTEN-negative layer V neurons that give rise to the CST, identified by retrograde labeling, were larger than neurons with maintained PTEN expression, and the relative area occupied by neuropil vs. cell bodies was increased. There was no evidence of tumor formation or other neuropathology. Mice with PTEN deletion exhibited open field activity comparable to controls and there was a trend for impaired Rotarod performance (not statistically significant). Our findings indicate that early postnatal genetic deletion of PTEN that is sufficient to enable axon regeneration by adult neurons causes neuronal hypertrophy but no other detectable neuropathology.

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1. Introduction

The phosphatase and tensin homolog (*PTEN*) gene has emerged as a promising target for enhancing axon regeneration in the mature central nervous system (CNS). The initial finding was that deletion of *PTEN* enabled regeneration of axons of retinal ganglion cells following optic nerve crush (Park et al., 2008) and reduced the retrograde degeneration of axotomized RGCs that otherwise occurred. This study used transgenic mice with a floxed *PTEN* gene that was deleted by local injections of AAV-Cre. A subsequent study used the same approach to show that conditional genetic deletion of *PTEN* in the sensorimotor cortex of neonatal mice enabled regeneration of corticospinal (CST) axons following spinal cord injury (SCI) in adulthood. CST regeneration was seen following

both dorsal hemisection and complete crush injury (Liu et al., 2010). Follow-up studies using similar approaches reported CST regeneration with conditional genetic deletion of *PTEN* in adult mice at the time of a spinal cord injury (Danilov and Steward, 2015) as well as in the chronic post-injury period (Du et al., 2015). These results, coupled with PTEN's upstream and non-redundant negative regulation of the PI3K/AKT pathway, make it a potentially important therapeutic target following CNS injury (Don et al., 2012).

The *PTEN* gene has been extensively studied for its role as a tumor suppressor and mutations are seen in several human cancers and cancer syndromes (Ali et al., 1999 Goffin et al., 2001; Shi et al., 2012). Also, the PTEN/mammalian target of rapamycin (mTOR) pathway is well known for its ability to regulate cell growth and proliferation (Laplante and Sabatini, 2012). mTOR is up-regulated by activation of phosphoinositide 3-kinase (PI3K), which converts phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-triphosphate (PIP3) (Georgescu, 2010). Via its action as a phosphatase, PTEN opposes

^{*} Corresponding author at: Reeve-Irvine Research Center, 837 Health Sciences Dr., University of California Irvine School of Medicine, Irvine, CA 92697, United States. *E-mail address*: osteward@uci.edu (O. Steward).

PI3K activity by converting PIP3 into PIP2, thus damping activation of downstream pathway components, such as phosphorylated ribosomal protein S6 (pS6), limiting cell growth (Park et al., 2010). Deleting *PTEN* activates mTOR, which presumably leads to downstream changes in mRNA translation regulated by mTOR signaling. *PTEN* mutations in humans have been associated with neurological abnormalities, including macrocephaly and autism (Goffin et al., 2001; Waite and Eng, 2002).

Previous studies have explored the consequences of deleting *PTEN* using transgenic promoter-driven Cre expression in *PTEN*^{*f*/*f*} mice, including Ca²⁺/calmodulin-dependent protein kinase II (CamKII), (Sperow et al., 2012), dopamine active transporter (DAT), (Diaz-Ruiz et al., 2009), glial fibrillary acid protein (GFAP), (Backman et al., 2001; Fraser et al., 2008; Fraser et al., 2004; Kwon et al., 2001; Wen et al., 2013; Yue et al., 2005), and neuron specific enolase (NSE), (Kwon et al., 2006; Takeuchi et al., 2013). The expression of each promoter (and therefore Cre recombinase) varies, with the earliest expression beginning at embryonic day 12.5 (E12.5) for NSE (Forss-Petter et al., 1990), E13.5 for GFAP (Brenner et al., 1994), E15 for DAT (Smits and Smidt, 2006), and postnatal day 4 for CamKII (Burgin et al., 1990).

Deletion of *PTEN* in neurons via transgenic promoter-driven Cre expression *in vivo* leads to increased brain mass, cerebellar enlargement, disruption of cortical lamination, and neuronal hypertrophy (Kwon et al., 2001; Marino et al., 2002; van Diepen and Eickholt, 2008; Yue et al., 2005). In addition to the gross and microscopic anatomical changes following *in vivo PTEN* deletion, neuropathologies including seizures, hydrocephalus, and ataxia were observed (Fraser et al., 2004; Kwon et al., 2001; Pun et al., 2012).

Previous studies involving conditional postnatal *PTEN* deletion in neurons using transgenic promoter-driven Cre expression have limited their analyses primarily to young adult mice (up to 30 weeks old) (Kwon et al., 2006; Luikart et al., 2011; Takeuchi et al., 2013; Williams et al., 2015). The consequences of longer-term *PTEN* deletion have not been assessed. Also, there have not yet been assessments of the consequences of early postnatal AAV-mediated *PTEN* deletion in the sensorimotor cortex, in the way that enables robust regeneration of adult CST axons (Liu et al., 2010).

The goal of the present study was to determine the consequences of long-term *PTEN* deletion in the sensorimotor cortex, focusing on the effects on cortical structure, neuronal size, functional consequences (if any), and whether there is evidence of tumors or other neuropathology. We show that AAV-Cre injections at postnatal day 1 lead to focal deletion of *PTEN* but with selective maintenance of *PTEN* expression in some neuronal types (small–medium sized neurons). There were increases in cortical thickness in the area of *PTEN* deletion, and cortical lamination was altered, with increases in the ratio of neuropil to cell bodies. Cortical motoneurons lacking *PTEN* identified by retrograde labeling were hypertrophied. There was no evidence of tumors, necrosis, inflammation, or other evident neuropathology. Maintained *PTEN* expression by some neurons may be a result of selective tropism of AAV-Cre for certain neuron types in the neonatal stage of development.

2. Methods

2.1. PTEN deletion in the motor cortex

All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of California, Irvine. Transgenic mice with a floxed exon 5 of the *PTEN* gene (*PTENf/f*) were from our local breeding colony that was established from founders obtained from Jackson Laboratories (Strain: C;129S4-Pten^{tm1Hwu}/J, Stock number: 004597). The colony was maintained by intercrossing mice homozygous for the floxed allele.

PTENf/f mice were injected on postnatal day 1 with AAV-Cre (n = 22) or AAV-GFP (n = 12). The vectors were AAV serotype 2, and were obtained from Vector Bio Labs $(1 \times 10^{13}$ genome copies/mL, Catalog number 7011). The virus was diluted with sterile-filtered PBS and 5%

glycerol for a final concentration of 1×10^{12} genome copies/mL. To perform the injections, 1-day-old pups were anesthetized by hypothermia (up to 5 min) and placed on a clay body mold attached to the bite bar of a stereotaxic apparatus. Injections were made using a Hamilton microsyringe with a pulled glass micropipette tip. 6 mice received injections as in Liu et al. with two unilateral injections of AAV-Cre (0.5 mm lateral, 0.5 mm deep to the cortical surface, and +0.5 and 0.0 mm with respect to bregma) for a total volume of 2 µl (Liu et al., 2010). 23 mice were injected using a procedure developed in Dr. Zhigang He's lab involving three unilateral injections (0.5 mm lateral, 0.5 mm deep to the cortical surface, and +0.5, 0.00, and -0.5 mm with respect to bregma) for a final total injection volume of 1.5 μ l of vector (n = 11 AAV-Cre and 12 AAV-GFP). After injection, pups were dipped in a few drops of sesame oil and returned to their home cages. After weaning, mice were maintained under standard vivarium conditions for 12-18 months. Table 1 summarizes animals and experimental conditions.

2.2. Rotarod assessment of motor coordination

Animals with *PTEN* deletion (n = 9) and AAV-GFP injected controls (n = 12) were tested for motor coordination when they were 48 to 53 weeks of age (51.6 weeks of age, on average). Mice were placed individually in each of the four chambers on the rod of a Rotarod® (San Diego Instruments) and were allowed to acclimate to the stationary rod for 30 s before starting every trial. Initial speed of the rotorod was set to 3 RPM and acceleration was set at 1 RPM/s. Mice were allowed to stay on the rod until they fell off or up to a maximum of two minutes, for a total of 7 trials/day. The first two trials were considered training trials, and the next 5 were testing trials. The time at which the mice fell from the rod during the testing trials (if at all) was recorded. *PTEN* deletion was assessed by immunocytochemistry (see below) and data from 3 mice with *PTEN* deletion located outside of the motor cortex were excluded from the statistical analysis, leaving a final n = 6 for the *PTEN* deletion group.

2.3. Open field test

The same mice that were tested using the Rotarod were also tested for general locomotion and spatial learning in the open field (9 animals with PTEN deletion and 12 animals with AAV-GFP). A 24-in. by 24-in. dark plexiglass box was used for the open field test. A 3-in. by 3-in. grid was drawn onto the bottom of the plexiglass box. The grid had four different colored zones, a center square, and the corner squares marked. The four differently colored zones were determined based on their proximity to the center square. The lines that comprised the outer two zones were considered "outer", while the lines of the inner two zones and the center square were considered "inner" lines. Individual mice were placed in the center of the box and allowed to explore freely for 5 min. One observer recorded total grid line crossings by forepaws, noting whether the lines crossed were "outer" or "inner" lines. The second observer recorded other events such as defecation, urination, grooming, and rearing. Open field activity was tested once a day for four consecutive days.

2.4. Retrograde tracing of the cells of origin of the CST with Fluorogold

At approximately 1 year of age, mice that had received AAV-Cre (n = 6) or AAV-GFP (n = 2) received bilateral injections of the retrograde tracer Fluorogold (FG) into the spinal cord. Mice were anesthetized with isoflurane and a laminectomy was performed to expose the spinal cord at C5. $0.2 \,\mu$ l of 1% FG was injected with a Hamilton syringe bilaterally from the midline (0.5 mm lateral, 0.5 mm deep) over 1 min. The syringe was left in place for an additional minute. After completing the injections, the skin was closed with sutures and staples, and mice were kept on a 37 °C water circulating heating pad until they recovered from the anesthetic. Mice received subcutaneous injections of

 Table 1

 Summary of experimental groups

Vector	# of injections	Volume/ injection	# of mice	Age (ave)	Fluorogold	Behavior	Histology
AAV-Cre	2	1 µl	6	415 days	n = 1	-	H&E, $n = 6$
							pS6, n = 6
							PTEN, $n = 6$
AAV-Cre	3	0.5 µl	11	406 days	n = 5	Rotarod, open field, $n = 9$	Cleaved caspase-3, $n = 2$
							Cresyl violet, $n = 2$
							GABA, $n = 3$
							GFAP, $n = 6$
							H&E, $n = 11$
							Cell density and cortical thickness analysis in H&E, $n = 5$
							Ki-67, n = 3
							pS6, $n = 12$
							PTEN, $n = 12$
AAV-GFP	3	0.5 µl	12	388 days	n = 2	Rotarod, open field, $n = 12$	Cleaved caspase-3, $n = 1$
							Cresyl violet, $n = 2$
							GABA
							GFAP, $n = 2$
							GFP, $n = 12$
							H&E, $n = 12$
							Ki-67, n = 2
							pS6, n = 12
							PTEN, $n = 12$

buprenorphine, Baytril, and lactated Ringer's solution twice a day for 3 days post surgery, and were allowed to remain in their home cages for 3 (n = 7) to 4 (n = 1) days before tissue collection.

2.5. Tissue collection and immunohistochemistry

At 12–18 months of age, mice received intraperitoneal injections of Euthasol (1 mL/300 g), and were perfused transcardially with 4% paraformaldehyde (PFA). The brain and spinal cord were dissected and post-fixed in PFA overnight, then placed in 30% sucrose solution in PBS overnight for cryoprotection. Brains were embedded in OCT compound and frozen in a container of methyl butane surrounded by ethanol/dry ice slush. 20 μ m thick coronal sections were collected at 200 μ m intervals and stored in PBS with 0.1% sodium azide at 4 °C until staining.

Sections were either directly mounted for Fluorogold visualization under fluorescent microscopy, stained for H&E or cresyl violet, or immunostained (see Table 1 for summary). To visualize GFP signal in the control animals, we examined unstained sections for GFP fluorescence and immunostained sections for GFP.

For immunostaining for cleaved caspase-3, Ki67, pS6, and PTEN, an antigen retrieval protocol was used in which sections were placed in 1.7 ml Eppendorf tubes with 1 mL 10 mM citrate buffer, pH 8.80. Tubes were then immersed in boiling water for 5 min, followed by immediate immersion in an ice bath for 10 min. Sections immunostained for GFP were incubated in 1% H₂O₂ for 20 min to quench endogenous peroxidase activity. Primary antibodies and dilutions were as follows: PTEN (Cell signaling, 1:250), pS6 (Cell signaling, 1:250, #4858), GABA (1:500), GFAP (Sigma, 1:1000), GFP (Lifetech, 1:1500), Ki-67 (Thermo Scientific, 1:200), Cleaved caspase-3 (BD Biosciences, 1:250), GFP (Lifetech, 1:1000). Antibodies were diluted in Blocking Reagent for cleaved caspase-3, pS6, and PTEN (PerkinElmer), 5% secondary antibody host serum in PBS with 0.5% Triton (GABA, GFAP, Ki67), or 5% secondary host serum in TBS with 0.1% Triton (GFP).

Sections were incubated in primary antibody dilutions overnight at room temperature, with the exception of GABA, which was incubated for 36–48 h at 4 °C. Secondary antibodies were diluted in the same blocking solution as the respective primary, and applied for two hours at room temperature. For immunofluorescence, Alexa Fluor 488 or Alexa Fluor 594 (ThermoFisher Scientific, both at 1:250 dilution) were used. For sections undergoing DAB detection and fluorescent amplification, a biotinylated IgG secondary antibody was used (Vector Laboratories, 1:250 dilution). Following secondary incubation, sections were washed $3 \times$, incubated for 1 h with avidin and biotinylated horseradish peroxidase (Vectastain ABC kit, Vector Laboratories) and reacted with DAB (DAB Substrate Kit for Peroxidase, Vector Laboratories) or FITC (made in house, 1:250 in 0.003% H₂O₂ borate buffer).

2.6. Layer V pyramidal cell size measurements

To determine the effect of *PTEN* deletion on pyramidal neurons that give rise to the CST, cortical sections from AAV-Cre injected animals that had received intra-spinal FG injections were analyzed. Immunostaining for PTEN using a DAB reaction quenched FG fluorescence in PTENpositive CST neurons whereas fluorescence was still prominent in CST neurons lacking PTEN. We then measured the cross sectional area of 50 PTEN-negative/FG positive CST neurons per animal. For control cells we used the nearest neighboring unstained section and visualized FG signal in the contralateral cortex in unstained sections. This allowed us to specifically measure PTEN-positive layer V pyramidal cells.

2.7. Measurement of cortical thickness and assessment of cortical lamination

It has previously been reported that promoter-driven *PTEN* deletion in the forebrain results in cortical enlargement. To assess this, we measured the thickness of the cortex (distance from the edge of the corpus callosum to the pial surface) in H&E stained-sections within the area of deletion (n = 5 mice). The region of *PTEN* deletion was verified in a nearby section that had been immunostained for PTEN. To examine changes in cortical laminar patterning, we screened H&E-stained sections from animals with *PTEN* deletion and compared the region of deletion to the homotopic contralateral control cortex.

2.8. Measurement of cell density

To determine whether conditional postnatal *PTEN* deletion results in altered cell density in the motor cortex we performed point-counting analysis. H&E stained sections within the region of *PTEN* deletion were imaged at $20 \times$ along with images from the control cortex contralateral to the *PTEN* deletion. These images were then overlaid with a 50 µm grid and analyzed in ImageJ for the number of grid crosshairs overlying cells vs. cell-free neuropil (154 maximum). This value was then used to calculate the relative area of neuropil vs. cell bodies.

3. Results

3.1. Pattern of PTEN deletion in the sensorimotor cortex with AAV-Cre injections on postnatal day 1

To define the area of *PTEN* deletion, sections were immunostained for PTEN. On the non-injected control side, neurons in all cortical layers stained positively for PTEN. There was also diffuse staining in the neuropil layers (Fig. 1B, right cortex). Higher magnification views reveal that large pyramidal neurons in layer V are robustly stained (Fig. 1C, arrows). PTEN immunostains of sections from mice that received intracortical AAV-GFP injections appeared similar to the contralateral control cortex of AAV-Cre injected mice.

On the side of the AAV-Cre injection, the area of *PTEN* deletion is easily identified by the decrease in PTEN immunostaining (Fig. 1A, left cortex). Higher magnification views reveal a characteristic immunostaining pattern in which neurons lacking PTEN stood out in negative relief as "ghost cells" (Fig. 1B, arrows). Although these cells resemble holes in the tissue, visualizing the same section immunostained for PTEN using phase contrast microscopy confirms that the ghost cells are neuronal cell bodies (Fig. 1E–F).

Ghost cells were especially evident in layer V (the location of the cells of origin of the CST), and large PTEN-negative dendrites could be seen emerging from the apex of the pyramid-shaped cell bodies and extending through more superficial layers of the cortex (Fig. 2A). The area of deletion was defined as the total region containing ghost cells. Although neurons lacking PTEN were evident, in some cases there were many PTEN-positive cells throughout the area of deletion. In these cases, PTEN-positive neurons were especially evident in cortical layers II and IV and these layers contained fewer ghost cells than layers III and V (Fig. 2A). Many of the PTEN-positive medium-sized neurons in the AAV-Cre injected cortex had the morphology typical of cortical interneurons (Fig. 2A).

To assess whether neurons with preserved PTEN expression were inhibitory interneurons, two nearby sections were immunostained for PTEN or γ -Aminobutyric acid (GABA) (Fig. 2B and C). GABA-positive neurons were present in all cortical layers within the area of deletion, but their distribution did not align with the distribution of PTEN-positive



Fig. 1. Area of *PTEN* deletion in the sensorimotor cortex following AAV-Cre at postnatal day 1. A.) PTEN immunostain of AAV-Cre injected cortex (left side) and contralateral cortex. Roman numerals indicate cortical layers. B.) Higher mag view of PTEN-negative neurons in left cortex of A. C.) Higher mag view of control cortex in A (right) showing neurons positive for PTEN. D.) A second case with *PTEN* deletion, showing preserved PTEN expression in neurons in layers II and IV. E.) High mag view of PTEN negative ghost cells (black arrowheads) and nearby PTEN-positive neurons (white arrowheads) centered over layer V. F.) Same microscopic field as in panel E viewed with phase microscopy to verify that PTEN-negative areas are neurons. Scale bar in A = 800 µm, in B and C = 80 µm, in D = 300 µm, in E and F = 20 µm.



Fig. 2. *PTEN* expression is preserved in small-medium sized neurons within the deletion region. A.) PTEN immunostain illustrating preservation of PTEN expression in neurons in layers IV and VI. Note PTEN-negative dendrites (arrows), some of which can be seen emanating from PTEN deleted ghost cells in layer V (arrows with asterisks) B.) PTEN immunostain from mouse with maintained PTEN expression in multiple cortical layers, especially layers IV and VI. C.) GABA immunostain of nearby section to section in panel B, note that GABA positive cells in panel C do not account for the pattern of maintained PTEN expression in B. Scale bar in A, B, and $C = 300 \,\mu\text{m}$.

neurons within the area of deletion; thus, neurons that are PTEN-positive are not exclusively inhibitory interneurons (Fig. 2B and C).

3.2. Variations in the size of the region of deletion

All mice injected with AAV-Cre had areas with ghost cells characteristic of successful *PTEN* deletion. The location and size of the area of *PTEN* deletion varied across cases, however, even when comparing cases that had used identical AAV-Cre titers, injection volumes, number of injections, and injection coordinates. By visual comparison, we found that

3.3. Neurons lacking PTEN have increased phosphorylation of ribosomal protein S6

Deletion of PTEN results in the activation of AKT, which in turn activates mTOR. One downstream marker for mTOR activation is phosphorylation of ribosomal protein S6 (Ma and Blenis, 2009). Confirming previous findings (Liu et al., 2010), immunostaining with antibodies specific for S6 phosphorylated at ser235/236 revealed increased S6 phosphorylation in neurons within the area of PTEN deletion, even a year after deletion. Fig. 3A illustrates a neighboring section to the section immunostained for PTEN in Fig. 1A, B, E. In the area of PTEN deletion, individual neurons exhibited elevated levels of immunostaining in comparison to the same region on the control side (Fig. 3A, left vs. right cortex). Pyramidal neurons in layer V with elevated levels of pS6 appeared larger than neurons in the homologous part of the cortex on the contralateral side (compare Fig. 3B with 3C). There was also increased pS6 immunostaining of ascending apical and basal dendrites of the large layer V pyramidal neurons (Fig. 3B) in comparison to the contralateral control side (Fig. 3C). As a technical aside, co-labeling sections for pS6 and PTEN was not attempted because both primary antibodies were generated in rabbit.

In the control cortex and areas where PTEN expression was maintained in the AAV-Cre injected cortex, neurons in all cortical layers stained positively for pS6 with the highest levels of staining exhibited by large pyramidal neurons in layer V (Fig. 3C). Levels of immunostaining were highest in neuronal cell bodies and proximal dendrites.

An important technical point is that levels of immunostaining for pS6 depend on the animals' experience during the time just before death, varying in a way that is similar to immediate early gene (IEG) expression. For this reason, it is important to have an internal control, provided here by comparisons of immunostaining in areas with maintained PTEN expression (for example, the side contralateral to the AAV-Cre injection).

3.4. No evidence of neuropathology with long-term PTEN deletion

Previous studies have reported neuronal and cortical hypertrophy when PTEN is deleted in developing mice using promoter-driven CRE expression (Fraser et al., 2004; Kwon et al., 2003; Kwon et al., 2001; Sperow et al., 2012). To assess whether focal PTEN deletion led to detectable changes in the histological appearance of the cortex, 20 µm sections taken every 200 µm throughout the brain were stained with hematoxylin and eosin (H&E). Areas of PTEN deletion were evident in several respects. First, in most mice, the cortex was noticeably thicker, and in some mice, there was actually a bulge in the cortex corresponding to the area of PTEN deletion (Fig. 3D, left vs. right cortex). Second, cortical cytoarchitecture was somewhat different in that cortical layers were less distinct and cell-packing density appeared lower with increased spacing between neuronal cell bodies (particularly in layer V, compare Fig. 3E and F). There was no evident neuropathology, such as accumulations of basophilic (basic) immune cells. Note that the tear in the tissue in Fig. 3D is a result of tissue processing damage and not damage due to the injection, as was seen in some mice (see below).

In AAV-GFP injected controls native GFP fluorescence was not detectable one year following injection. Immunostaining for GFP revealed scattered GFP positive astrocytes and a few neurons in the area of the injection (Fig. 4A and B). In contrast to the situation with AAV-Cre-mediated *PTEN* deletion, cortical cytoarchitecture appeared essentially normal in cases injected with AAV-GFP (Fig. 4C). As expected,



Fig. 3. Activation of phosphorylation of ribosomal protein S6 and overall cytology in area of *PTEN* deletion. A.) Immunostain for phosphorylated ribosomal protein S6 (pS6, Ser234/235) on the side of the *PTEN* deletion cortex (left) and contralateral control cortex (right). This section is nearby the one illustrated in Fig. 1A. B.) Higher mag view of pS6 immunostain in the *PTEN* deletion region in A (left cortex). Note the highly stained apical and basal dendrites. C.) Higher mag view of pS6 immunostain of control cortex in A (right cortex). D.) H&E stain of a nearby neighboring section to the section in A, *PTEN* deletion region in the left motor cortex and the homotopic control cortex on the right. E.) Higher mag view of layer V in the left cortex in D; note the decrease in cell packing compared to F. F.) Higher mag view of layer V in the right motor cortex in D (control). Scale bar in A and D = 600 µm, in B, C, E, and F = 100 µm.

PTEN immunostaining was intact in this region (Fig. 4D) and there were no increases in immunostaining for pS6 in comparison to the contralateral, non-injected side (Fig. 4E and F).

PTEN is a tumor suppressor, so it was important to screen for any evidence of tumors. No unusual cell clusters were evident in H&E stained sections. For five cases, 20 µm sections taken every 200 µm were immunostained with GFAP in order to detect areas of astrogliosis indicative of neurodegeneration or any astroglial tumors. GFAP stained sections within the *PTEN* deletion region had GFAP-positive astrocytes in the glia limitans and in white matter tracts, similar to what we

found in the contralateral control cortex (Fig. 5A and B). No abnormal accumulations of GFAP-positive cells were identified in the area of *PTEN* deletion.

We noticed that one animal that received AAV-Cre injections and two animals that received AAV-GFP injections had cortical defects in the region of the injection. Sections through the defect were immunostained for GFAP (Fig. 5C and D); examination of these revealed that the defects were lined with GFAP positive stellate astrocytes. The defects are probably areas of cortical injury produced at the time of the P1 injection, although other explanations cannot be excluded.



Fig. 4. No obvious alterations in cortical morphology in AAV-GFP injected controls. A.) GFP immunostained section from a mouse injected with AAV-GFP. GFP labeling is present in scattered astrocytes and a few neurons. B.) High mag view of region in A, Arrows point to neurons; arrowheads point to astrocytes. C.) A nearby section to A–B stained for H&E. AAV-GFP was injected into the left cortex, and the right cortex was un-injected. Arrows indicate tissue defects that are likely needle tracts from the injections. D.) PTEN immunostain of a nearby section to A–C. No PTEN negative neurons were seen in the region of AAV-GFP injection. E.) pS6 immunostain of the cortex ipsilateral to AAV-GFP injection in a nearby section to A–D. F.) pS6 immunostain of the contralateral uninjected cortex from the same section in E. Scale bar in A, B, C, and D = 600 µm, in B = 150 µm.



Fig. 5. Immunostaining for GFAP in the area of *PTEN* deletion. A.) Representative PTEN immunostain from one of the five mice used to assess the distribution of GFAP positive cells following *PTEN* deletion. B.) GFAP immunostained of a neighboring section to the section in panel A. No qualitative increase in GFAP positive cells or clusters of GFAP cells were found in the *PTEN* deleted motor cortex or in any other brain regions. C.) Cresyl violet stain of section from an animal with a cortical lesion caused by intracortical injections. D.) GFAP immunostain of a nearby neighboring section to C, Astrocytes surrounding the cortical defect shown in C. Scale bar in A and B = 300 µm, C and D = 600 µm.

To screen for ongoing abnormal cell proliferation in areas of *PTEN* deletion, sections were immunostained for Ki-67. As a positive control, Ki-67 positive cells were evident in the subventricular zone (SVZ) lining the lateral ventricles. There were no Ki-67 positive cells in the area of *PTEN* deletion (Fig. 6B and C). Note that small fluorescent particles evident in Fig. 5C were also present in the contralateral *PTEN* intact cortex immunostained for Ki-67 (Fig. 6D). These particles have a different appearance than the Ki-67-positive cells in the SVZ (Fig. 6E), and thus are likely immunostaining artifacts. To assess whether the decreased ratios of cell bodies relative to neuropil following *PTEN* deletion might be due to apoptotic cell death, we immunostained sections for cleaved caspase-3 since cleavage of caspase-3 reflects activation of proapoptotic pathways. We did not find any cells within the *PTEN* deleted or control motor cortex that were positive for cleaved caspase-3 (Fig. 6F).

3.5. Changes in the cell density and lamination within the region of deletion

Previous studies have reported alterations in cortical lamination and cell packing following promoter-driven *PTEN* deletion during early development (Wen et al., 2013; Yue et al., 2005). Similar though less severe effects were qualitatively evident in the area of *PTEN* deletion.

In H&E stained sections, the hexilaminar patterning of the cortex appeared disorganized (Fig. 7A vs. B). To quantify cell-packing density in the region of *PTEN* deletion, we used a point-counting method to determine the relative area occupied by cell bodies vs. neuropil in the *PTEN*-deleted area of the motor cortex vs. the contralateral non-injected cortex. The percentage of neuropil in the control motor cortex was $71.76\% \pm 3.47\%$ vs. $79.52\% \pm 2.73\%$ in the area of *PTEN* deletion (paired t-test 12.70, p < 0.0002, see Fig. 7C). The percentage of neuropil is the inverse of cell-packing density.

3.6. Increased cortical thickness following long-term PTEN deletion

Previous studies have also reported cortical hypertrophy following neonatal *PTEN* deletion (Fraser et al., 2004; Kwon et al., 2006). Since AAV-CRE injections were unilateral, we compared cortical thickness in the area of maximal *PTEN* deletion vs. the contralateral control. In the area of deletion, cortical thickness ranged from 1171.1 μ m to 1527.9 μ m (1332.7 μ m average, \pm 139.6), vs. 980.2 μ m to 1290.9 μ m (1177.6 μ m average, \pm 123.1) on the contralateral un-injected side (two-way ANOVA: F = 12.69 for vector type, p < 0.002, Fig. 8). In mice that received injections of AAV-GFP, the injected region of the cortex was somewhat thinner than the contralateral cortex. In the



Fig. 6. No ongoing cell division or apoptotic cell death in the region of *PTEN* deletion. A.) PTEN immunostain of a nearby section to panels B–F. Arrows indicate boundaries of the region of *PTEN* deletion. Cortical layers are labeled. B.) Low mag view of Ki-67 immunostain in the region of *PTEN* deletion (region indicated by arrows). C.) High mag view of the section in B. Arrowheads point to large layer V neurons in the region of *PTEN* deletion (likely *PTEN* negative ghost cells). D.) Ki-67 immunostain of contralateral control cortex from the same section as B. Fluorescent particles in B and D have a different appearance than bona fide Ki-67 positive cells in the subventricular zone (SVZ, E). E.) Ki-67 positive cells in the subventricular zone of the same section as B–D. F.) Cleaved caspase-3 immunostain from a nearby section to B–E in the *PTEN* deletion region contains no apoptotic (cleaved caspase-3) cells. Scale bar in A, B, and F = 300 µm, in C, D, and E = 150 µm.



Fig. 7. Decreased cell density the area of *PTEN* deletion. A.) H&E stained section of motor cortex in the area of *PTEN* deletion. B.) H&E stained section of control motor cortex contralateral to panel A. C.) Data from 5 animals used for measurement of cell density. The percentage of crosshairs overlying cell bodies vs. neuropil is represented in the graph (paired t-test = 12.70, p = 0.0002). Scale bar in A = 100 µm.

area of AAV-GFP injection, cortical thickness ranged from 913.4 μ m to 1242.2 μ m (1087.5 μ m average, \pm 105.9), vs. 967.2 μ m to 1160.1 μ m (1104.6 μ m average, \pm 67.1) on the contralateral un-injected side (two-way ANOVA: F = 0.6280, p = 0.4413).

3.7. Hypertrophy of retrogradely-labeled PTEN-negative cortical motoneurons

To identify PTEN-negative cells of origin of the CST, 6 mice that had received unilateral injections of AAV-Cre at P1 received bilateral injections of Fluorogold (FG) at C5 as adults. In 5 mice with sufficient FG labeling, FG-positive neurons were evident bilaterally in layer V of the sensorimotor cortex (Fig. 9A and B). FG-positive dendrites could be seen extending from the labeled cell bodies, and in some cases their dendritic processes and axonal projections were also labeled with FG.

FG-labeled neurons in layer V within the region of *PTEN* deletion (Fig. 9A) appeared larger than FG-labeled layer V neurons in the homologous part of the contralateral cortex (Fig. 9B). To identify retrogradely-labeled neurons lacking *PTEN*, sections from mice that had received FG injections were immunostained for PTEN using DAB for detection. The DAB reaction quenched the FG signal in any neurons that stained positively for PTEN, leaving FG fluorescence intact in neurons that did not stain for PTEN (Fig. 9C). For comparison, retrogradely-labeled neurons in the cortex contralateral to the *PTEN* deletion were identified in near-adjacent sections that were not immunostained for PTEN. The cross-sectional areas of *PTEN* negative vs. *PTEN* positive cell bodies were measured in NIH ImageJ. Fig. 9E-I illustrate the cell body size



Fig. 8. Increased cortical thickness in the area of *PTEN* deletion. Data from 5 animals were used to measure cortical thickness. The thickness of the *PTEN* deleted and contralateral control motor cortex were measured by drawing a line from the corpus callosum to the pial surface in ImageJ. The first pair of bars illustrate data from mice injected with AAV-GFP; the second pair of bars illustrate data from mice injected with AAV-Cre. Gray bars indicate average thickness of the uninjected cortex contralateral to the AAV injections; red bars are data from the injected side. Asterisks indicate p = 0.002 (repeated measures two way ANOVA, F = 12.69 for vector type). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

distributions in 5 mice; values of t-tests for cell size data in each individual animal are illustrated in each graph; two way AVOVA of data of individual cell sizes from all 5 cases: F = 178.9, p < 0.0001, df = 392. In Fig. 9J, data points are the average size of neurons in the 5 individual animals. The overall average cross-sectional area for FG-positive, *PTEN* negative neurons was 484.0 \pm 125.7 μ m² vs. 291.3 \pm 35.1 μ m² for FG-positive neurons in the contralateral cortex (paired t-test = 5.47, n = 5 pairs, p = 0.005).

We also prepared tissue from 2 mice that had received vector control injections of AAV-GFP and intra-spinal Fluorogold injections. Examination of GFP immunostained sections from these two cases revealed only a few GFP labeled neurons consistent with the findings above, which precluded meaningful cell size measurements. Thus, we were not able to compare the size of neurons that had been transfected with AAV-GFP with controls.

3.8. Mice with PTEN deletion do not differ significantly from controls in the rotorod or open field tasks

Casual observation revealed no noticeable behavioral differences between mice with *PTEN* deletion in the cortex vs. control mice. Mice grew to normal size, appeared as healthy as controls even up to 18 months of age, were active, seizures were not observed, and there was no premature death.

To screen for major deficits in overall motor function of mice with *PTEN* deletion, motor coordination was assessed by Rotorod on 4 nonconsecutive days in mice that had received AAV-Cre vs. AAV-GFP (Fig. 10A). Behavioral testing was carried out when the mice were 48 to 53 weeks old. For both groups, the average amount of time spent on the rod increased over the testing period, reflecting motor learning. Mice with *PTEN* deletion did not achieve the same level of performance as controls, but the difference was not statistically significant (p = 0.3764, df = 16, repeated measures two way ANOVA).

Activity in an open field is considered to be a measure of overall locomotor activity, exploratory activity, spatial memory, and anxiety. Decreases in exploration with repeated exposure are considered to be a measure of familiarity with the open field (and thus spatial memory). During the initial exposure to a novel environment, mice typically remain close to the walls of the enclosure (thigmotaxis) (Simon et al., 1994) and with repeated exposure, tend to increase time spent in the center of the open field. To examine these behaviors in our mice, we tested them over four consecutive days. Open field performance was measured in the same mice used for Rotorod assessment, and was carried out when the mice were around 51.6 weeks of age.

Over the testing period the overall activity of both groups declined (measured as a decrease in total number of lines crossed during the testing period) (Fig. 10B). Both groups also demonstrated a preference for the perimeter of the arena throughout the testing period (determined by perimeter line crossings), and a progressive decline in the total number of perimeter lines crossed over the four days of testing



Fig. 9. Hypertrophy of CST neurons following *PTEN* deletion. A.) Fluorogold (FG)-labeled CST neurons in the area of *PTEN* deletion. Inset is a high magnification view of the area indicated by the rectangle. B.) FG signal in contralateral control cortex C.) *PTEN* deletion region immunostained for PTEN, bright field microscopy. D.) Same field as in panel C, UV microscopy E–I.) Distribution of cell body cross sectional area from 5 cases. J.) Data points indicate the average size of PTEN-negative vs. PTEN-positive CST neurons in the 5 individual cases (paired t-test = 5.47, n = 5 pairs, p = 0.005). Scale bar in A = 300 \,\mu\text{m}, in C = 30 μm .

(Fig. 10D). For both *PTEN* deleted and AAV-GFP injected controls, the change in total lines crossed/day, inner lines crossed/day, and outer lines crossed/day were only significant between day 1 and day 2 (repeated measures two way ANOVA, p = 0.0007, df = 16 for total lines, p = 0.0006, df = 16 for inner lines, p = 0.0001, df = 16 for outer lines). From day 2 to day 4 both *PTEN* deleted and control animals crossed a stable number of inner lines, illustrating an increase in exploratory behavior across testing sessions (Fig. 10C). Comparing the *PTEN* deleted and AAV-GFP injected controls, there were no significant differences on any of the measures (repeated measured two way ANOVA, p = 0.7380, df = 16 for total lines crossed/day over four days, p = 0.8455, df = 16 for inner lines crossed/day over four days).

4. Discussion

The present study was undertaken to define the long-term consequences of postnatal deletion of *PTEN* in the sensorimotor cortex, which previous studies have shown enables cortical motoneurons to regenerate their axons after an adult spinal cord injury (Liu et al., 2010; Zukor et al., 2013; Lewandowski and Steward, 2014; Danilov and Steward, 2015). These findings, together with previous findings in other systems (Park et al., 2008), establish *PTEN* deletion as an effective way to enable axon regeneration following injury. We show here that conditional deletion of *PTEN* in the motor cortex of early postnatal mice results in robust hypertrophy of layer V cortical motoneurons and of the cortex itself, decreased cell density in the motor cortex, and



Fig. 10. Functional assessments of mice with unilateral postnatal *PTEN* deletion in the motor cortex. A) Average time spent on accelerating Rotarod over four testing days. Data are from mice in which *PTEN* deletion was verified by immunohistochemistry (n = 6) vs. AAV-GFP-injected controls (n = 12). Difference in time spent on the rod was not significantly different between *PTEN* deleted and control mice (repeated measures two way ANOVA, p = 0.3764, df = 16, F = 0.8279). B.) Open field activity of *PTEN* deleted and control mice over four testing days as measured by total lines crossed/day (repeated measures two way ANOVA for total lines cross/day p = 0.7380, df = 16, F = 0.1159). C.) Open field inner lines crossed/day over four days (repeated measures two way ANOVA for total lines cross/day p = 0.3455, df = 16, F = 0.03922). D.) Open field outer lines crossed/day over four days (repeated measures two way ANOVA for outer lines crossed/day p = 0.3455, df = 16, F = 0.03922). D.) Open field outer lines crossed/day over four days (repeated measures two way ANOVA for outer lines crossed/day p = 0.3455, df = 16, F = 0.03358).

maintained mTOR pathway activation a year after *PTEN* deletion. There was no evident neuropathology, and behavioral assessment revealed normal open field activity and somewhat impaired motor coordination on the Rotarod task that was not statistically significant.

Our results extend previous studies reporting structural and functional phenotypes resulting from promoter-driven genetic deletion of *PTEN* in early development. Other groups have observed a variety of neuropathologies following the loss of *PTEN* in multiple brain regions during development including macrocephaly, alterations in cortical lamination, hydrocephalus, seizures, premature death, and autism phenotypes (Backman et al., 2001; Fraser et al., 2008; Fraser et al., 2004; Kwon et al., 2006; Kwon et al., 2001; Sperow et al., 2012; Wen et al., 2013; Yue et al., 2005). Most of these previous studies examined the effects of *PTEN* deletion after only a few months. Indeed, morbidity and premature death would have precluded longer-term analyses. It is noteworthy that we did not observe any of the major neuropathologies, perhaps because *PTEN* deletion was postnatal and focal.

4.1. Cell-type specificity of PTEN deletion

Immunostaining for PTEN in mice that received AAV-Cre intracortical injections confirmed *PTEN* deletion in neurons in layer V that give rise to the CST as well as neurons in other cortical layers. *PTEN* expression was maintained in some neurons in layers II and IV that had the morphological features of cortical interneurons. Immunostaining for GABA revealed that GABAergic neurons did not account for the *PTEN* positive neurons within the deletion region.

It is noteworthy that intracortical injections of AAV-PTEN shRNA (shPTEN) in adult rats create a focal region in which there is a complete loss of PTEN immunostaining in all cell types (Lewandowski and Steward, 2014). The same is true of AAV-Cre injections into the cerebral cortex of adult *PTEN*^{f/f} mice (Danilov and Steward, 2015). Thus, selective preservation of expression in some neuron types following injections at P1 is likely to be due to the stage of development and not differential promoter-driven expression of the AAV in different neuron types.

Selective preservation of *PTEN* expression in some neurons of mice with neonatal deletion of *PTEN* may be due to neuron type selectivity of AAV transfection at the age of deletion or a developmentally time sensitive expression of the necessary receptors and co-receptors for AAV transfection. AAV-2 is known to use heparan sulfate proteoglycan, $\alpha V\beta 5$ integrin, and the laminin receptor as receptors for transfection (Akache et al., 2006; Summerford et al., 1999; Summerford and Samulski, 1998). Onset of expression of these necessary receptors may vary by cell type so that a subpopulation of neurons is preferentially transfected by AAV-Cre in neonatal mice.

In control mice that received AAV-GFP injections, we did not detect native GFP fluorescence in animals one year after viral injection, but immunostaining for GFP revealed GFP-positive astrocytes and a few neurons. Preferential preservation of expression in astrocytes up to 1 year is noteworthy; previous studies have reported maintained AAV-GFP mediated GFP fluorescence up to 3 months following injection (Chamberlin et al., 1998), but to our knowledge this is the first time that AAV-GFP expression has been found up to 12 months after injection,

4.2. Variability in the size of the region of deletion

While *PTEN* deletion was successfully achieved in all experimental animals, the size of the deletion region varied between animals. The inconsistency in size may have implications for SCI research projects that utilize conditional *PTEN* deletion as a therapeutic intervention. If the number of layer V upper motor neurons that have *PTEN* deleted varies between animals, the regenerative capacity of injured CST axons will vary similarly and appear less robust when averaging data across multiple animals. It may be useful in future experiments to determine the relationship between the area of *PTEN* deletion and functional recovery measures.

4.3. Increased mTOR activity following PTEN deletion

Phosphorylation of ribosomal protein S6 (pS6) is a biomarker of mTOR activation, and immunostains for pS6 following *PTEN* deletion

in adult neurons show increased S6 phosphorylation even a year following deletion. The regenerative effect of *PTEN* deletion is thought to be due to increased activity of mTOR, and specifically rapamycin-sensitive mTOR complex 1 (mTORC1) (Park et al., 2008). mTORC1 regulates the translation of a subset of mRNAs that contain either a long unstructured 5' un-translated region (5'UTR), or a 5'UTR with a specific structure (Ma and Blenis, 2009). mTOR regulates the transcription of these transcripts by phosphorylating three downstream targets — the S6 kinases, the inhibitory eIF4E-binding proteins (4E-BPs), and eIF4G initiation factors (Thoreen et al., 2012). It is not yet established how *PTEN* deletion and activation of s6 phosphorylation actually affects mRNA translation in neurons.

4.4. No evidence of neuropathology

Histological assessment revealed no necrosis, abnormal growths, or tumor formation in areas of *PTEN* deletion. There was evidence of cortical injury in some mice, with GFAP positive astrocytes bordering the lesion, but this is most likely a result of damage caused at the time of the injections. Since astrocytes are responsible for repairing damage in the central nervous system, this finding is not surprising.

4.5. Cortical hypertrophy and lamination

Focal increases in cortical thickness could be related to the increased cell body size of *PTEN* deleted neurons. Other groups have found marked cortical hypertrophy in mice with *PTEN* deletion (Fraser et al., 2008; Kwon et al., 2006; Kwon et al., 2001), and in some of our cases, cortical hypertrophy was evident. The laminar disruptions that we observed were more qualitative in nature, in that it was difficult to identify the 6 cortical layers in H&E stains in the *PTEN* deletion region.

4.6. Neuronal hypertrophy and decreased cell density

Layer V cells of CST origin with *PTEN* deletion exhibited considerable cell body hypertrophy. Promoter regulated *PTEN* deletion has been associated with cell body enlargement of dentate and cerebellar granule cells, dopaminergic cells in the substantia nigra, and cortical neurons (Diaz-Ruiz et al., 2009; Fraser et al., 2008; Kwon et al., 2001). Assessment of the size of *PTEN* deleted layer V output neurons in the motor cortex had not been previously assessed, and here we demonstrate that layer V neurons are capable of robust growth. Within the motor cortex we found a statistically significant decrease in cell density (the ratio of neuropil to cell bodies) following *PTEN* deletion. It remains to be determined how neuronal hypertrophy and decreased cortical cell density affect the signaling properties of these neurons or the motor cortex.

4.7. Lack of obvious functional consequences

Despite the presence of markedly hypertrophied layer V pyramidal cells, and in some cases hypertrophy of the cortex itself, we did not observe any adverse behavioral effects of AAV-Cre injection into the sensorimotor cortex of *PTENf/f* mice. Casual observations and our limited testing of exploratory activity did not reveal obvious behavioral differences in mice with *PTEN* deletion, although time spent on the Rotarod was shorter than for AAV-GFP-injected controls. Previous studies of mice in which *PTEN* deletion was regulated by neuron specific enolase-Cre (NSE-Cre) revealed no difference in initial Rotarod performance and an eventual increase in time spent on the rod later in testing compared to controls (Kwon et al., 2006). The lack of major behavioral consequences seen here may be due to the localized deletion of *PTEN* in the motor cortex rather than deletion in widespread brain regions that occurs with promoter-driven Cre expression.

Although we did not monitor animals for seizure activity, the animals were handled throughout the duration of the experiment and never exhibited visible signs of motor seizures. Due to previous findings of seizure activity in animals with conditional transgenic promoterdriver *PTEN* deletion (Backman et al., 2001; Kwon et al., 2001; Ogawa et al., 2007; Pun et al., 2012), the possibility of sub-clinical seizures cannot be excluded without additional studies with electrophysiological monitoring.

The results of the present study provide provisional insights into the potential risks of complete and long-term deletion of *PTEN* in a subpopulation of neurons, as might occur with a therapeutic intervention to enable axon regeneration. We focused our analyses on the long-term effects of *PTEN* deletion induced early in postnatal life in order to closely mimic the method used to promote axon regeneration after injury (Liu et al., 2010; Park et al., 2008). The lack of major negative consequences is noteworthy, although it remains possible that some negative consequences were not detected.

It remains to be determined if *PTEN* deletion performed similarly in adult animals, which has also been shown to enhance axon regeneration and recovery following SCI, will be as benign histologically and behaviorally (Danilov and Steward, 2015; Du et al., 2015). It will also be of interest to explore interactions between the alterations in cortical morphology seen here and responses to axotomy due to spinal cord injury.

Conflict of interest

OS is one of the co-founders of the company "Axonis", which holds options on patents relating to *PTEN* deletion and axon regeneration.

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Author contributions

OS, EG and MB designed research, EG and MB performed research, EG analyzed data, and OS and EG contributed to the writing of the paper.

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