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Research Paper

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

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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.

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

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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 Ca^{2+}/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 (1×10^{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 μl 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 μl 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