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Research paper

Self-reinforcing effects of mTOR hyperactive neurons on dendritic growth

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A B S T R A C T

Loss of the mTOR pathway negative regulator PTEN from hippocampal dentate granule cells leads to neuronal hypertrophy, increased dendritic branching and aberrant basal dendrite formation in animal models. Similar changes are evident in humans with mTOR pathway mutations. These genetic conditions are associated with autism, cognitive dysfunction and epilepsy. Interestingly, humans with mTOR pathway mutations often present with mosaic disruptions of gene function, producing lesions that range from focal cortical dysplasia to hemimegalanecephaly. Whether mTOR-mediated neuronal dysmorphogenesis is impacted by the number of affected cells, however, is not known. mTOR mutations can produce secondary comorbidities, including brain hypertrophy and seizures, which could exacerbate dysmorphogenesis among mutant cells. To determine whether the percentage or "load" of PTEN knockout granule cells impacts the morphological development of these same cells, we generated two groups of PTEN knockout mice. In the first, PTEN deletion rates were held constant, at about 5%, and knockout cell growth over time was assessed. Knockout cells exhibited significant dendritic growth between 7 and 18 weeks, demonstrating that aberrant dendritic growth continues even after the cells reach maturity. In the second group of mice, PTEN was deleted from 2 to 37% of granule cells to determine whether deletion rate was a factor in driving this continued growth. Multivariate analysis revealed that both age and knockout cell load contributed to knockout cell dendritic growth. Although the mechanism remains to be determined, these findings demonstrate that large numbers of mutant neurons can produce self-reinforcing effects on their own growth.

1. Introduction

Genetic lesions that impact the mechanistic target of rapamycin (mTOR) signaling pathway cause a range of human diseases. Examples include tuberous sclerosis complex (TSC1 and TSC2), focal cortical dysplasia (AKT3, TSC1, PTEN, PIK3CA, mTOR), hemimegalencephaly (AKT3, PIK3CA, mTOR) and Cowden syndrome (PTEN) (Crino, 2011; Wong and Crino, 2012; Krueger et al., 2013; Lasarge and Danzer, 2014; Marsan and Baulac, 2018). These aptly named "mTORopathies" can result from germline or somatic mutations. Intriguingly, somatic mutations can impact widely varying numbers of cells. In hemimegalancephaly, for example, an entire hemisphere may be affected, while mutations may be present in only a small region of cortex in focal cortical dysplasia. This variability raises the possibility that neurons with mTOR mutations may follow different pathological trajectories depending on the number of surrounding cells that also exhibit the mutation. Excess mTOR signaling profoundly disrupts the morphology and function of neurons exhibiting the mutation, and widespread mutations can alter the gross structure of the brain, increase inflammation, alter network behavior and produce secondary pathologies, such as seizures (Ogawa et al., 2007; Zeng et al., 2008; Pun et al., 2012; Parker et al., 2013; Matsushita et al., 2016; Barrows et al., 2017; Wesseling et al., 2017). mTOR-mediated disruption of neuronal growth may precede independently of these secondary effects, or secondary changes may produce feedback effects, whereby mTOR mutant cells become increasingly pathological over time and as a function of the "load" of surrounding mutant cells.

To assess the impact of altering the load of mTOR mutant cells on the pathological development of these same cells, we developed a conditional, inducible PTEN knockout mouse model of epilepsy in which PTEN can be deleted from variable numbers of postnatally-generated hippocampal granule cells (Pun et al., 2012; LaSarge et al., 2015, 2016; Santos et al., 2017). At the single cell level, PTEN loss induces somatic hypertrophy, increases dendrite length and complexity (Kwon et al., 2001, 2003; Zhou et al., 2009; Urbanska et al., 2012; Sperow et al., 2012) and leads to the de novo appearance of hilar basal dendrites on hippocampal granule cells (Kwon et al., 2006; Lasarge and Danzer, 2014). At the systems level, PTEN loss can lead to gross brain hypertrophy, inflammatory changes, behavioral abnormalities and epilepsy (Kwon et al., 2001, 2006; Amiri et al., 2012; Pun et al., 2012; Lugo et al., 2014; Nguyen and Anderson, 2018).

Animals lacking PTEN from variable numbers of granule cells were generated in two cohorts. In the first, PTEN deletion rates were held at around 5%, and knockout cell growth over time was assessed. Previous studies have demonstrated that PTEN deletion leads to the rapid appearance of abnormalities over weeks (Luikart et al., 2011; Williams

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et al., 2015), but whether changes become progressively worse over months or eventually plateau is not known. In the second cohort, knockout cell deletion rates ranged from 2 to 37%, and the impact of both age and knockout cell load were assessed. These experiments reveal whether and how the mosaic nature of mTORopathies might impact the development of individual morphological abnormalities.

2. Materials and methods

All animal procedures were conducted in accordance with NIH and CCHMC Institutional Animal Care and Use Committee (IACUC) guidelines. All mice were maintained on C57BL/6 background. The following mouse strains were used for the present study: *Gli1-CreER^{T2}* mice (RRID: IMSR_ JAX STRAIN#007913), PTEN$^{flox/flox}$ mice (RRID: IMSR_JAX STRAIN #006440), and *Gt(ROSA)26Sortm1(CAG-Brainbow2.1)Cle/J* "*Brainbow*" reporter mice (RRID:IMSR_JAX STRAIN#013731). *Gli1-CreER^{T2}* mice express tamoxifen-inducible cre-recombinase in neuronal progenitor cells (Ahn and Joyner, 2005). PTEN$^{flox/flox}$ mice possess LoxP flanked "floxed" PTEN exon 5. Brainbow mice stochastically express GFP, YFP, CFP or RFP upon cre-recombination (Cai et al., 2013).

2.1. Brainbow-expressing PTEN knockout mice

Study animals were generated by crossing *Gli1-CreER^{T2}* hemizygous; *PTEN$^{flox/wt}$*; Brainbow$^{+/+}$ triple transgenic mice to *PTEN$^{flox/wt}$*; *Brainbow$^{+/+}$* double transgenic mice. The cross was used to generate the following genotypes:

1) *Gli1-CreER^{T2}* hemizygous; *PTEN$^{wt/wt}$*; *Brainbow$^{+/+}$* (Control, $n = 9$ male mice).
2) *Gli1-CreER^{T2}* hemizygous; *PTEN$^{flox/flox}$*; *Brainbow$^{+/+}$* (PTEN knockout [KO], $n = 11$ male mice).

All mice were injected with tamoxifen (Sigma Aldrich, T5648; 250 mg/kg dissolved at 20 mg/ml in corn oil, S·C) on post-natal day (P) 21 to delete PTEN and express the brainbow fluorophores. Brainbow control and KO mice were randomly assigned to one of two groups for perfusion 4 (7-weeks-old) or 15 (18-weeks-old) weeks after tamoxifen injection. Final experimental groups were as follows: 7-week-old group, 4 control and 5 KO; 18-week-old group, 5 control and 6 KO.

Brainbow-expressing control and KO mice were anaesthetized with pentobarbital (100 mg/kg, intraperitoneal) and transcardially perfused through the ascending aorta with ice-cold 0.1 M phosphate buffered saline (PBS, pH 7.4) containing 1 U/ml heparin for 1 min at 6 ml/min, immediately followed by a solution of 2.5% paraformaldehyde (PFA) with 4% sucrose in PBS for 10 min at 25 °C. Brains were removed and bisected along the sagittal plane into left and right hemispheres. The left hemisphere of each brain was post-fixed for 24 h and cryoprotected in 10%, 20%, and 30% sucrose in PBS for 24, 24, and 48 h, respectively. Left hemispheres were snap-frozen in 2-methylbutane chilled to −23 °C with dry ice and stored at −80 °C until sectioning. Left hemispheres were sectioned sagittally at a thickness of 60 μm using a cryostat maintained at −20 °C. Sections were thawed in PBS, slide mounted, air dried and stored at −80 °C for immunohistochemical tests. Right hemispheres were used to collect images for neuronal reconstructions for morphological analyses. Right hemispheres were post-fixed overnight and incubated in 10% sucrose in PBS for a minimum of 24 h; tissue was then cut into 1 mm coronal sections using a tissue slicer (Campden/Lafayette Instruments, IN). Sections were incubated for optical clearing in Sca*l*eA2 for 3–4 weeks at 4 °C (Hama et al., 2015; Singh et al., 2015) and preserved in Sca*l*eA2 until imaging.

2.2. Neuronal reconstructions of cells labeled in brainbow-expressing PTEN KO mice

One millimeter thick Sca*l*eA2 cleared tissue sections were imaged using a Nikon A1Rsi inverted microscope equipped with a 40× Plan Apo water immersion objective (NA = 1.15, field size 317 × 317 μm). Yellow fluorescent protein (YFP) and red fluorescent protein (RFP) expressing cell clusters in brainbow positive mice (PTEN KO and control) were imaged. Consistent with prior studies, cyan fluorescent protein (CFP) expressing nuclei were rare in CNS, and green fluorescent protein (GFP) expressing cells were not evident (Singh et al., 2015). Three dimensional z-series confocal image stacks were collected through up to 500 μm of the tissue at 1 μm increments to capture cells in their entirety. Between one and eight cells per animal were traced; except for one KO mouse in the 18-week group. In this animal, no brainbow-expressing PTEN KO cells were found. Cells with proximal dendrites cut at the tissue surface, or with dendrites that could not be clearly visualized were excluded. In KO animals, cells were also excluded if their soma areas were within two standard deviations of the mean of soma areas for control cells. These cells likely reflect brainbow-expressing, PTEN-expressing cells in KO animals resulting from incomplete cre-mediated recombination of the floxed PTEN gene. This criteria has been previously validated to distinguish > 95% of KO cells from control cells (Santos et al., 2017). Cells meeting reconstruction criteria were randomly selected for analysis.

Confocal z-series image stacks were imported to Neurolucida for whole cell tracing. Reconstructions encoded soma area, apical and basal dendrite length, and dendritic branch points. Image stacks were also used to encode the location of the hilar-granule cell body layer border, the granule cell body layer-molecular layer border, and the location of the hippocampal fissure. The molecular layer was further subdivided into inner (IML), middle (MML), and outer (OML) regions, with the inner region being the first 17% of the molecular layer (West and Andersen, 1980; Deller et al., 1999; van Groen et al., 2003; Santos et al., 2011), and the middle and outer being an equal split of the remainder.

2.3. Histology and immunohistochemistry

For the brainbow control and KO mice, sections at the same medial-lateral coordinates (Lateral 1.56; (Paxinos and Franklin, 2004)) were co-immunostained for PTEN/NeuN in order to quantify the percentage of PTEN KO cells. The following antibodies were used: mouse anti-NeuN (Neuronal nuclei protein, 1:400, Millipore Cat# MAB377 RRID:AB_2298772) and rabbit anti-PTEN antibodies (Phosphatase and tensin homolog, 1:250, Cell Signaling Technology Cat# 9559 RRID:AB_390810). Secondary antibodies included goat anti-rabbit Alex Fluor 594 (Thermo Fisher Scientific Cat# A11012 RRID: AB_10562717) and goat anti-mouse Alexa Fluor 647 (Thermo Fisher Scientific Cat# A-21242 RRID: AB_2535811), all at 1:750 dilution. Sections were dehydrated in serial alcohol washes, xylene cleared for 15 min, and hard mounted with Krystalon mounting medium (EMD Millipore, Cat# 64969).

2.4. PTEN KO cell counts

PTEN/NeuN immunostained sections were imaged with a Leica SP5 inverted microscope (software RRID: SCR_013673) equipped with 63× oil objective (NA = 1.4, field size 248 × 248 μm). Confocal z-series image stacks were collected through 7 μm of tissue at a 1 μm step, excluding the top 1–2 μm of tissue to avoid sectioning artifacts. A tile scan was used to capture the entire dentate gyrus in brain hemi sections, and images were imported to Neurolucida software (Microbrightfield Inc., RRID: SCR_001775) to stitch tiles together for dentate gyrus reconstruction. Two 100 × 100 μm counting frames were placed over the midpoints of the upper and lower blades of the dentate gyrus, respectively. The total number of NeuN positive cells, and the number of NeuN positive, PTEN negative (PTEN KO) cells within these frames was