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Disrupted hippocampal network physiology following *PTEN* deletion from newborn dentate granule cells

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Abstract

Abnormal hippocampal granule cells are present in patients with temporal lobe epilepsy, and are a prominent feature of most animal models of the disease. These abnormal cells are hypothesized to contribute to epileptogenesis. Isolating the specific effects of abnormal granule cells on hippocampal physiology, however, has been difficult in traditional temporal lobe epilepsy models. While epilepsy induction in these models consistently produces abnormal granule cells, the causative insults also induce widespread cell death among hippocampal, cortical and subcortical structures. Recently, we demonstrated that introducing morphologically abnormal granule cells into an otherwise normal mouse brain – by selectively deleting the mTOR pathway inhibitor *PTEN* from postnatally-generated granule cells – produced hippocampal and cortical seizures. Here, we conducted acute slice field potential recordings to assess the impact of these cells on hippocampal function. *PTEN* deletion from a subset of granule cells reproduced aberrant responses present in traditional epilepsy models, including enhanced excitatory post-synaptic potentials (fEPSPs) and multiple, rather than single, population spikes in response to perforant path stimulation. These findings provide new evidence that abnormal granule cells initiate a process of epileptogenesis – in the absence of widespread cell death – which culminates in an abnormal dentate network similar to other models of temporal lobe epilepsy. Findings are consistent with the hypothesis that accumulation of abnormal granule cells is a common mechanism of temporal lobe epileptogenesis.

Keywords

PTEN; dentate granule cells; epilepsy; perforant path; hippocampus

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Introduction

Dentate granule cells have long been suspected of playing a pivotal role in the development of temporal lobe epilepsy (Nadler, 2003; Sutula et al., 1989). Granule cells develop *de novo* recurrent excitatory circuits during epileptogenesis, and exhibit physiological changes indicative of increased network excitability. Ascertaining whether abnormal granule cells are cause, effect or epiphenomena, however, has been challenging due to the panoply of changes occurring throughout the brain during epileptogenesis.

We recently presented new evidence supporting a prominent role for granule cells in epileptogenesis by demonstrating that abnormal granule cells are sufficient to cause the disease (Pun et al., 2012). Specifically, we utilized a conditional, inducible transgenic mouse model system to selectively delete the mTOR pathway inhibitor Phosphatase and Tensin Homolog (*PTEN*) from granule cells born after postnatal day 14 (P14). Granule cell neurogenesis peaks around P7, and tapers off to adult levels thereafter. Inducing *PTEN* deletion in granule cell progenitors at P14, therefore, impacts about 10-20% of the total granule cell population. *PTEN* deletion leads to excess activation of the mTOR pathway, and was targeted because *PTEN* knockout granule cells develop morphological abnormalities reminiscent of temporal lobe epilepsy (e.g. basal dendrites, Pun et al., 2012). Moreover, mTOR signaling is increased during epileptogenesis (Hester et al., 2016; Zeng et al., 2009), and blocking mTOR has anti-epileptogenic properties (Ljungberg et al., 2009; Wong, 2013). *PTEN* knockout mice developed a severe epilepsy syndrome, exhibiting both hippocampal and cortical seizures in 24/7 EEG recordings -- supporting the hypothesis that abnormal granule cells can initiate temporal lobe epileptogenesis.

The extent to which *PTEN* deletion recapitulates features of traditional, status epilepticus, models of temporal lobe epilepsy, however, was not fully resolved by this previous work. Status epilepticus models cause rapid and widespread cell death throughout many brain regions, while these regions are spared in the *PTEN* model, at least initially. There are many different forms of epilepsy, so it is a formal possibility that the *PTEN* model produces a phenotype with little relevance to temporal lobe epilepsy. To further validate this model, for the present study we queried whether *PTEN* deletion from granule cells would reproduce the well-characterized abnormal responses to perforant path stimulation evident in other temporal lobe epilepsy models (for example, Patrylo et al., 1999; Shao and Dudek, 2011).

Stimulation of the perforant path in hippocampal slices elicits epileptiform bursts and negative field-potential shifts in tissue from rodents rendered epileptic using the kainic acid and pilocarpine models of epilepsy (Buckmaster and Dudek, 1997; Patrylo et al., 1999; Scharfman et al., 2002; Shao and Dudek, 2011). Similar abnormalities are evident in tissue resected from epileptic patients (Masukawa et al., 1989; for review see Williamson and Patrylo, 2007). The occurrence of multiple population spikes is believed to reflect the activation of recurrent circuits in epileptic tissue, which mediate “reverberatory” activity through the dentate. If abnormal granule cells are responsible for these abnormalities – either primarily, or by inducing secondary changes – then they should be evident in tissue from *PTEN* KO mice. Alternatively, if epileptogenesis occurs by entirely different

mechanisms in this model, abnormal field responses may be absent, or exhibit entirely unique features.

Materials and Methods

Animals

All procedures were approved by the CCHMC Animal Board (IACUC) and followed NIH guidelines. *Gli1-CreER^{T2}::PTEN^{fllox/fllox}* mice [*PTEN* KO, n=9; 7 males and 2 females] and *Gli1-CreER^{T2}::PTEN^{wt/wt}* mice [Cre control, n=10; 8 males and 2 females], aged between 2-5 months (*PTEN* KO, mean age = 95 ± 7.6 days; Cre control, mean age = 107 ± 8.8 days; $t = 1.028$, $p=0.318$) were used for histology and field recordings experiments. *Gli1-CreER^{T2}*-expressing mice (Ahn and Joyner, 2004; Ahn and Joyner, 2005) have a cDNA encoding *CreER^{T2}* inserted into the 5'UTR of the first coding exon of the *Gli1* locus. The *Gli1-CreER^{T2}* mice were crossed with *PTEN^{tm1Hwu}/J* mice (Jackson Laboratory), which contains loxP sites on either side of exon 5 of the *PTEN* gene (*PTEN* “floxed” mice). Study animals were generated by crossing *Gli1-CreER^{T2}* hemizygous, *PTEN^{fllox/wt}* male mice with *PTEN^{fllox/wt}* female mice. Animals used in this study that expressed *Gli1-CreER^{T2}* were hemizygous for the gene. All mice were maintained on a C57BL/6 background, and whenever possible, littermate controls were used. All mice used for studies (including controls) were injected with tamoxifen (250 mg/kg dissolved in corn oil) subcutaneously on postnatal day 14 (P14). The combination of *Gli1*-driven *CreER^{T2}* and postnatal induction with tamoxifen restricts *PTEN* deletion to a subset of astrocytes (<3%), postnatally-generated olfactory neurons and granule cells (Garcia et al., 2010; Pun et al., 2012).

Acute slice preparation

Mice were anesthetized with pentobarbital, decapitated and the brains rapidly removed. Brains were immersed in ice-cold (<2°C) oxygenated ACSF for three minutes (95% O₂:5% CO₂). The ACSF solution had the following composition (in mM): NaCl (124), KCl (3), MgCl₂ (1.3), CaCl₂ (1.3), NaH₂PO₄ (1.4), NaHCO₃ (26), glucose (11), pH 7.4. Osmolality of ACSF was 290-305 mOs. The olfactory bulbs and the cerebella were removed, and the brain was split down the midline. The right hemisphere was prepared for immunohistochemistry (see histological analysis below) while the left hemisphere was glued onto a chuck and mounted in a bath filled with ice-cold oxygenated ACSF. This approach allowed us to conduct physiological and histological studies in the same animals. Transverse slices from the temporal 2/3 of the left hippocampal formation were cut on a tissue slicer (Campden Instruments) at 350 μ m. The hippocampus was isolated and an additional knife cut was used to transect the mossy fiber connections to CA3. Cuts were made immediately in front of the superior and inferior tips of the dentate blades, and extended through all CA3 lamina and just into CA1 stratum lacunosum/moleculare. These cuts effectively isolate the dentate from CA1 and the majority of CA3 (connections to CA3c pyramidal cells located between the dentate blades are potentially left intact). Slices were transferred to a holder perfused with oxygenated ACSF for 120 minutes before electrophysiological recordings were started.

Perforant path stimulation

Hippocampal slices were transferred to an interface chamber and maintained at 32–33°C with a 1.5 ml/min flow of oxygenated ACSF. A bipolar stimulating electrode, connected to a constant current source, was placed in either the outer or middle dentate molecular layer to activate the lateral perforant path (LPP) or medial perforant path (MPP), respectively. Evoked potentials were recorded with a ~1 mOhm ACSF-filled glass electrode placed in the dentate granule cell layer. Glass pipettes were fabricated from capillary tubing (external diameter, 1.5 mm) by a Mecnex-BB-CH puller. Evoked potentials were digitized with a Digidata 1400A A/D-D/A converter controlled by Clampex software (version 10.3, Molecular Devices, Sunnyvale, CA). Dentate recordings were conducted in both normal ACSF (3.5mM K⁺) or ACSF elevated to 6 mM [K⁺]. Field potential response characteristics were obtained for each slice using increasing current stimulation (20 to 100 μ A in 20 μ A increments, followed by 100 to 600 μ A in 100 μ A increments). A minimum of five stimuli at each current intensity, separated by five seconds, was used to generate response averages. Responses at each intensity had minimal variability at this stimulation interval, with the difference between the fEPSP slope of the first and last response of LPP path stimulation with 100 – 600 μ A of current averaging 5.1% (SD = 2.3; n = 15 mice with 6 response pairs each). The minimum intensity to evoke an fEPSP was considered threshold. fEPSP slope was calculated from the 20–80% of the signal between the onset of the response and the peak amplitude of the fEPSP. Population spikes were defined as negative deflections of at least 200 μ V, followed by a positive going signal. Population spike threshold was defined as the lowest amount of current necessary to evoke a population spike. Paired-pulse stimulation was given at inter-pulse intervals of 30, 50, 70, 100, 150 and 200 ms using a stimulation intensity 3x the threshold current to elicit an fEPSP, allowing for facilitation or suppression to be detected. Paired-pulses were repeated five times for each interval, with five seconds between each stimulation pair. The paired-pulse index (PPI; 2nd fEPSP slope/1st fEPSP slope) was calculated and compared between control and *PTEN*KO slices for the progressive intervals. A similar ratio was calculated for population spike amplitudes. These paired pulse protocols were used for both the LPP and MPP.

Histological Analysis

Acute slices were prepared from the left hemisphere, while the right hemisphere was immersed in 2.5% paraformaldehyde and 4% sucrose in phosphate-buffered saline (PBS, pH 7.4). Brains were post-fixed overnight and cryoprotected in 20% sucrose in PBS followed by 30% sucrose in PBS, each for 24 hours before flash-freezing. Frozen tissue was sectioned sagittally on a cryostat at 60 μ m, mounted to gelatin-coated slides, and stored at –80°C until use.

Immunohistochemistry

Double-immunostaining of brain sections was conducted using rabbit anti-PTEN (1:250, Cell Signaling Technology, Inc., Danvers, MA) and mouse anti-NeuN (1:400, Millipore, Temecula, CA) antibodies to confirm *PTEN* deletion. Adjacent sections were immunostained with ZnT-3 (1:500, Synaptic Systems, Gottingen, Germany) to assess mossy fiber sprouting in the dentate inner molecular layer. Alexafluor 594 goat anti-rabbit and

Alexafluor 647 goat anti-mouse secondary antibodies (all at 1:750, Invitrogen, Grand Island, NY) were used for PTEN, NeuN, and ZnT-3 immunostaining. For all immunostaining procedures, between two and four brain sections from each animal were examined. Brain sections corresponded to medial-lateral coordinates 1.3 – 1.7 mm (Paxinos and Franklin, 2001). PTEN, NeuN and ZnT-3 images were collected using a Leica SP5 confocal system equipped with 10X air (NA 0.3) and 63X oil (NA 1.4) objectives (Leica Microsystems Inc., Buffalo Grove, IL). All analyses and counts were conducted by an investigator unaware of treatment group.

Image analysis

PTEN and NeuN immunostaining in the dentate gyrus was imaged using a 0.5 μm step through the z-axis (63X, image size 0.24 $\mu\text{m}/\text{px}$, 1024 $\times$ 1024 format), beginning 3 μm below the surface to avoid areas damaged by tissue sectioning. Images were used to estimate the percentage of knockout cells (PTEN negative / NeuN expressing cells) in each mouse. Image stacks were imported into Neurolucida software (MBF Bioscience, Williston, VT) and a modification of the optical dissector method was used for cell counts (Hofacer et al., 2013; Howell et al., 2002; Pun et al., 2012). Mossy fiber sprouting was scored from two-channel confocal optical sections of the dentate inner molecular layer (63X, 1024 $\times$ 1024 format) collected from the midpoint of the upper blade at a level 3 μm below the tissue surface. Mossy fiber sprouting was scored by determining the percentage of the total inner molecular layer area occupied by ZnT-3 immunoreactive puncta using automatic object detection (Neurolucida, MBF Bioscience, Williston, VT). Sensitivity for automatic detection was set to capture all puncta more than 2X background and larger than 0.5 μm^2 . All automated counts were reviewed manually to insure accuracy.

Statistics and data analysis

Data was exported from pClamp (version 10.3) for analysis of physiological recordings. For all analyses, statistical significance was determined using Sigma Plot software (version 12.5, Systat Software, Inc., San Jose, CA). Normality and equal variance were assessed using Shapiro-Wilk and Brown-Forsythe tests. Parametric tests were used for data that met assumptions of normality and equal variance, and non-parametric alternatives were used for data that violated either assumption. Means $\pm$ SEM are reported for parametric tests, and median [range] are reported for non-parametric tests. Specific tests were used as noted in the results. Sample sizes for statistical analysis (n) are animal number, although the number of slices {X slices} used is reported throughout the text as well. In cases in which two slices or cells from the same animal were examined, data were averaged to generate an animal mean for statistical analysis. For some measures (n) is less than the number of animals in the study. Slices were not used if fEPSP responses were less than 1 mV following 200 μA LPP stimulation. Slices were also excluded for technical problems during recording sessions (e.g. electrode breakage, perfusion system instability). All slices meeting threshold criteria and conforming to technical parameters were used for data analysis. Male and female animals were statistically equivalent for all parameters examined (data not shown), so males and females were binned for further statistical analysis. Due to the low number of females, however, the absence of differences between sexes should be interpreted cautiously.

Figure preparation

All images are either single confocal optical sections or confocal maximum projections exported as TIFF files and imported into Adobe Photoshop (version 12.0.1, Adobe Systems, San Jose, CA). Some images were adjusted using Leica Application Suite with a morphological erosion filter (radius=3; iterations=1) or Nikon Elements with a median filter (radius = 3) to reduce background artifact. Brightness and contrast of digital images were adjusted to optimize cellular detail. Identical adjustments were made to all images meant for comparison.

Results

PTEN deletion from dentate granule cells

PTEN deletion from a subset of dentate granule cells was confirmed by double immunostaining for PTEN and NeuN in brain sections from *PTEN*KO mice (Figure 1). In the *PTEN*KO animals used for the present study, *PTEN* deletion ranged from 16 to 25.5% of dentate granule cells; the same range was found to produce animals with spontaneous seizures in prior work (Pun et al., 2012).

PTEN KO slices exhibit increased field excitatory post-synaptic potential (fEPSP) slopes

To determine whether the hippocampal circuit in slices from PTEN KO mice was hyperexcitable, we electrically stimulated the lateral (LPP) and medial (MPP) perforant path; the major input pathways to the dentate gyrus from lateral and medial entorhinal cortex, respectively. As expected, increasing lateral perforant path stimulation intensity enhanced the number of axon fibers activated, in turn increasing the slope of the resulting fEPSP in field recordings from the granule cell layer from both control and *PTEN*KO slices (LPP; Control n=10 mice {13 slices}, *PTEN*KO n=9 {9}; F=68.396, p<0.001, RM ANOVA). In addition, a significant interaction was detected between LPP stimulation intensity and genotype (F= 7.059, p<0.001, RM ANOVA; Figure 2A). At stimulation intensities below 200 μ A, control and *PTEN*KO slices were similar, while *PTEN*KO slices exhibited significantly steeper slopes at intensities above 200 μ A (F=8.683, p=0.008 main effect of control vs. *PTEN*KO; LSD post-hoc tests p's<0.01). The enhanced slopes indicate that a given stimulus produces larger synaptic responses in KO slices relative to controls.

Similar to LPP stimulation, both control and PTEN KO slices showed the expected enhancement of fEPSP slopes in response to increasing intensities of stimulation (Control n=8 {8}, *PTEN*KO n=8 {8}; F=22.993, p<0.001, RM ANOVA; Figure 2B). However, although a trend was evident, the response to a given stimulation intensity was statistically equivalent between the two groups (F=2.273, p=0.15). These findings suggest that the increased excitability evident in PTEN KO slices is more pronounced for lateral over medial perforant path.

PTEN knockout slices exhibit larger evoked population spikes

Increasing perforant path stimulation intensity will eventually produce fEPSPs in the innervated granule cells of sufficient intensity to cause these neurons to fire an action potential, evident as a negative-going population spike (Figure 3). Population spike

amplitudes grew with increasing intensity of LPP stimulation for both control and *PTEN*KO slices (Figure 2C; Control n=10 mice {13 slices}, *PTEN*KO n=9 {9}; $F=22.136$, $p<0.001$, RM ANOVA); however, there was significant interaction between mouse genotype and stimulation current ($F=3.325$, $p<0.001$, RM ANOVA). Specifically, population spike amplitudes were larger in *PTEN*KOs relative to control slices at or above 100 μ A of current stimulation (μ A 100, p 's 0.01, LSD post-hoc tests). Many control slices did not generate population spikes at stimulation intensities below 100 μ A, limiting the comparisons (mean population spike threshold was 256.36 ± 56.75 μ A in controls and 66.67 ± 8.17 μ A in *PTEN*KO slices, $t=2.988$, $p=0.008$). These findings demonstrate that granule cells from *PTEN*KO slices are more likely to fire an action potential in response to a given stimulus.

Similar to results with LPP stimulation, stimulation of the MPP also produced population spikes of greater amplitude from the granule cell layer with increasing stimulation intensity in both groups (Control n=8 {8}, *PTEN*KO n=8 {8}; $F=14.973$, $p<0.001$, RM ANOVA). MPP stimulation produced larger amplitude population spikes in KOs relative to controls (Figure 2D; $F=6.413$, $p=0.024$, RM ANOVA). Differences were evident at all current intensities tested. In combination with fEPSP data, increases in population spike amplitude suggest that *PTEN*KO cells receive more excitatory input than control cells, require less stimulation to fire an action potential, or some combination of both.

PTEN KO slices exhibit multiple population spikes following perforant path stimulation

Perforant path stimulation typically evokes only a single population spike in the granule cell layer. In the kainic acid and pilocarpine models of epilepsy multiple population spikes can be induced (Dudek et al., 1994; Haas et al., 1996; Lynch and Sutula, 2000; Patrylo et al., 1999; Scharfman et al., 2002). Multiple population spikes are hypothesized to reflect the *de novo* creation of "epileptogenic" circuitry in the dentate. In *PTEN*KO slices, perforant path stimulation consistently produced multiple population spikes (normal ACSF; Figure 3B). Moreover, following stimulation, the signals from KO slices overshot baseline with an elongated negative phase (Figure 3B').

To quantify repetitive firing, population spikes were counted following LPP and MPP stimulation at 80 and 200 μ A; currents below and at the intensity, respectively, at which fEPSP slope differences were detected between groups. As seen in Figure 3C, *PTEN*KO slices had significantly more population spikes with LPP stimulation at both 80 μ A (Control: n=10 mice, {12 slices}, 0.00 events [range: 0.00 – 1.00]; *PTEN*KO: n=9 {9}, 2.0 [range: 0.00 – 5.00]; $U=16.00$, $p=0.003$ RST) and 200 μ A (Control: n=10 {12} slices, 1.00 [range: 0.00 – 1.00]; *PTEN*KO: n=9 {9}, 3.00 [range: 2.00 – 6.00]; $U=0.00$, $p<0.001$ RST). Similarly, MPP stimulation produced more population spikes in slices from *PTEN*KOs relative to controls at both 80 μ A (Control: n=8 {8}, 0.00 events [range: 0.00 – 1.00]; *PTEN*KO: n=8 {8}, 2.50 [range: 0.00 – 8.00]; $U=7.00$, $p=0.007$ RST) and 200 μ A (Control: n=8 {8}, 1.00 [range: 0.00 – 2.00]; *PTEN*KO: n=8 {8}, 4.0 [range: 1.00 – 8.00]; $U=4.00$, $p=0.002$ RST). Indeed, current intensities that produced multiple population spikes from *PTEN*KO slices were often subthreshold for producing even a single population spike in controls. Notably, the difference in the number of evoked spikes still persisted for 200 μ A even after excluding stimuli from control slices that failed to evoke a single spike (LPP:

Control n= 6 {7} slices, 1.0 events [range: 1.00 – 1.00]; *PTEN*KO: n=9 {9}, 3.0 [range: 2.00 – 6.00]; U= 0.00, p<0.001 RST; MPP: Control n= 4 {5} slices, 1.00 events [range: 1.00 – 2.00]; *PTEN*KO: n=8 {8}, 4.00 [range: 1.00 – 8.00]; U= 4.00, p=0.019 RST).

The generation of multiple granule cell layer spikes from *PTEN*KO mice was remarkably robust. For the present study, slices from all 9 *PTEN*KO mice exhibited at least two population spikes 100% of the time with a 200 μ A LPP stimulation, whereas control slices never exhibited more than one population spike. Similarly, MPP stimulation with the same current yielded similar results, with slices from seven of eight *PTEN*KO mice showing two or more population spikes (one of the eight had a single population spike). MPP stimulation of control slices, on the other hand, yielded no spikes in slices from 3 of 8 mice, single spikes from 4 of 8, and two spikes from 1 of 8. These data emphasize the finding that *PTEN*KO mice consistently and reliably generate multiple population spikes, providing evidence of a recurrent, hyperexcitable network.

Normal ACSF versus High K⁺ solution

Inducing multiple population spikes in tissue from pilocarpine-treated, kainic acid-treated and kindled animals typically requires “unmasking”, Specifically, either excitation is increased or inhibition is reduced; usually by elevating extracellular K⁺ or Ca²⁺, or by adding the GABA antagonist bicuculline (Haas et al., 1996; Lynch and Sutula, 2000; Sutula and Dudek, 2007). Notably, unmasking is not required to generate multiple spikes in tissue from *PTEN*KO mice (Fig.3). Nonetheless, we queried whether a standard unmasking procedure – high K⁺ – would alter the threshold current needed to elicit population spikes.

High K⁺ treatment significantly reduced the LPP stimulation current required to elicit a population spike in control tissue (Figure 4A and B), but not in *PTEN*KO tissue (Figure 4D-F; Control in ACSF: n=10 mice {12}, 256.36 $\pm$ 56.75 μ A; Control with High K⁺: n=6 {6}, 100.00 $\pm$ 21.29 μ A; *PTEN*KO in ACSF: n=9 {9}, 66.67 $\pm$ 8.17 μ A; *PTEN*KO with High K⁺: n=7 {7}, 51.43 $\pm$ 8.57 μ A; group F= 8.566, p=0.007; treatment F= 4.443, p=0.044; two-way ANOVA). Moreover, population spike thresholds were statistically equivalent between *PTEN*KO slices and control slices in high K⁺ (p = 0.449, Fisher LSD), meaning that high K⁺ treatment caused control slices to behave more like *PTEN*KO slices. In contrast to population spike thresholds, fEPSP thresholds were not altered by high K⁺ treatment, and did not differ between genotypes (Control in ACSF: 50.91 $\pm$ 5.44 μ A; Control with High K⁺: 46.67 $\pm$ 7.37 μ A; *PTEN*KO in ACSF: 35.56 $\pm$ 6.02 μ A; *PTEN*KO with High K⁺: 40.00 $\pm$ 6.82 μ A; group F= 2.910, p=0.099.; treatment F= 0.000, p=0.988; two-way ANOVA; Figure 4C).

High K⁺ ACSF also reduced MPP population spike thresholds for control, but not *PTEN*KO slices (Figure 4F; Control in ACSF: n=8 mice {8}, 225.00 $\pm$ 38.11 μ A; Control with High K⁺: n=6 {6}, 80.00 $\pm$ 44.01 μ A; *PTEN*KO in ACSF: n=8 {8}, 42.50 $\pm$ 38.11 μ A; *PTEN*KO with High K⁺: n=6 {6}, 56.67 $\pm$ 44.01 μ A; group F= 6.250, p = 0.020; treatment F= 2.525, p = 0.125.; two-way ANOVA). There was no change in the fEPSP threshold in either of the groups with the high K⁺ solution (Figure 4C; Control in ACSF: 57.50 $\pm$ 5.90 μ A; Control with High K⁺: 46.67 $\pm$ 6.67 μ A; *PTEN*KO in ACSF: 37.50 $\pm$ 7.01 μ A; *PTEN*

KO with High K⁺: $43.33 \pm 6.15 \mu\text{A}$; group $F = 3.126$, $p = 0.090$; treatment $F = 0.144$, $p = 0.708$; two-way ANOVA).

Altogether, high K⁺ bath solution made control slices more excitable, but it did not change response dynamics in *PTEN*KO slices. The control slices showed a decrease in the population spike threshold current with a high K⁺ solution, but there was no change between the ACSF or high K⁺ solution in the amount of current needed to elicit a population spike from *PTEN*KO slices. One interpretation of these data is that *PTEN*KO cells have already achieved maximal excitability, such that further depolarization with high K⁺ provides no further enhancement.

Impaired paired-pulse facilitation in *PTEN* KO slices

Paired-pulse facilitation is a measure of short term synaptic plasticity. It can be induced in dentate granule cells by application of two closely-timed stimulations. Facilitation is evident as an increase in the slope of the fEPSP, or amplitude of the population spike, evoked by the second stimulation (McNaughton, 1980; for review Zucker and Regehr, 2002). The timing of the second stimulation influences the amount of facilitation. Facilitation was previously reported with interpulse intervals of 30 ms, with the amount of facilitation decreasing with increasing interval duration (McNaughton, 1980). Paired-pulse facilitation is often impaired in tissue from epileptic animals (Klapstein et al., 1999; Scimemi et al., 2006; Zhao and Leung, 1992).

For the present study interpulse intervals of 30, 50, 70, 100, 150 and 200 ms were used to assess this form of short-term synaptic plasticity in control and *PTEN*KO slices. Responses are expressed as the paired-pulse index (PPI: 2nd response/1st response; Table 1). Paired pulse stimulation of the LPP produced robust facilitation of the fEPSP slope in control slices at interpulse intervals of 30 – 150 ms, with an increase in the slope of 46% at 50 ms (Table 1). In contrast, facilitation in *PTEN*KO slices was significantly reduced compared to controls at interpulse intervals of 30 – 150 ms (RM ANOVA and Holm-Sidak method for post-hoc comparisons), with the increase peaking at $\approx 15\%$ (Figure 5, Table 1). Decreased facilitation in *PTEN*KO slices may reflect responses that are already near maximal following the initial stimulation. Seizures can produce long-lasting synaptic potentiation (Ben-Ari and Gho, 1988; Ben-Ari and Represa, 1990) and if synapses are already potentiated, subsequent stimuli may not be able to produce much additional facilitation.

As expected, reduced fEPSP facilitation following paired stimuli in *PTEN*KO slices was accompanied by impaired population spike facilitation for interpulse intervals of 30 – 150 ms (Figure 5, Table 2). For example, while paired pulse stimulation at 50 ms produced only a modest 14% increase in the amplitude of the second spike in *PTEN*KO slices, a 135% increase was observed in control slices.

In contrast to findings with the LPP, responses to MPP stimulation were equivalent between controls and KOs. MPP stimulation typically produces paired-pulse depression at short intervals (< 25 ms), followed by modest facilitation as intervals increase up to 200 ms (Petersen et al., 2013). This is true for both fEPSP slopes and population spike amplitudes. In the present study, paired pulse stimulation of the MPP followed this pattern for both

control and *PTEN*KO slices, with facilitation evident at short intervals and neutral or depressed responses evident at long intervals (Table 1). Notably, however, these responses were statistically identical between groups (RM ANOVA; all *p*'s not significant). Taken together, these data clearly demonstrate afferent-specific effects of *PTEN* deletion on short term plasticity, with LPP responses exhibiting robust differences from controls, while MPP responses are unaffected.

Mossy fiber sprouting does not correlate with physiological measures

In epileptic patients and animals, granule cell mossy fibers axons form *de novo* projections into the dentate inner molecular layer, where they can form connections with granule cell apical dendrites (for review Buckmaster, 2014; Sutula et al., 1989; Tauck and Nadler, 1985). These recurrent circuits have been hypothesized to underlie the multiple population spikes evident following stimulation of tissue from epileptic animals. To investigate this possibility, zinc transporter 3 (ZnT3) labelling was used to analyze the location of the zinc-rich mossy fiber terminals (McAuliffe et al., 2011). *PTEN*KO slices exhibited significantly more ZnT3 labeled puncta in the inner molecular layer compared to controls (percent of molecular layer occupied by ZnT3 labelling; Control: *n*=7 mice, 0.00 % [range: 0.00 – 0.12; *PTEN*KO: *n*=7, 4.02% [range: 0.11 – 9.38]; *U* = 2.000, *p*<0.001 RST; Figure 6A), confirming the presence of mossy fiber sprouting in the *PTEN*KO animals. The degree of sprouting, however, did not correlate with fEPSP threshold (LPP, *R* = -0.254, *p* = 0.583; MPP, *R* = -0.497, *p* = 0.316, Pearson Correlation), population spike threshold (LPP, *R* = -0.428, *p* = 0.338; MPP, *R* = -0.170, *p* = 0.747, Pearson Correlation), or the number of evoked spikes (with 200 μ A of stimulation: LPP, *R* = -0.069, *p* = 0.884; MPP, *R* = 0.210, *p* = 0.690, Pearson Correlation). In fact, *PTEN*KO mice with little mossy fiber sprouting (<1%) still exhibited multiple population spikes following perforant path stimulation (Figure 6B), indicating that robust mossy fiber sprouting is not required for this particular pathological response.

Severity of physiological abnormalities are largely stable with increasing age in *PTEN* KO mice

*PTEN*KO mice between 9 and 20-weeks-old were used for the present study, and as a group displayed reduced fEPSP and population spike thresholds and increased numbers of population spikes following stimulation. To determine whether pathology increases with age, correlation analyses were conducted. Age did not correlate with the threshold current for a population spike from the LPP (*R* = 0.082, *p* = 0.834, Pearson) or with the number of population spikes following 200 μ A of LPP stimulation (*R* = -0.301, *p* = 0.432), although there was a trend for fEPSP threshold current to decrease with age (*R* = -0.659, *p* = 0.054). Data from the MPP largely matched the LPP data, with no correlation between age and population spike threshold current (*R* = -0.684, *p* = 0.062, Pearson) or population spike number following a 200 μ A stimulus (*R* = -0.598, *p* = 0.117). MPP fEPSP threshold current, however, was negatively correlated with age (*R* = -0.708, *p* = 0.049); although this was largely dependent on a single animal, and therefore should be interpreted cautiously. Overall, these data indicate that physiological abnormalities are present by nine weeks of age in the *PTEN*KO mice, and that abnormalities remain relatively stable, at least out till 20 weeks.

Discussion

Abnormal hippocampal dentate granule cells have been suspected of playing a causal role in temporal lobe epileptogenesis. While abnormal granule cells are a common feature of most temporal lobe epilepsy models, epilepsy in these models is typically initiated by brain insults, such as status epilepticus, that cause rapid cell loss and widespread brain changes. Whether abnormal granule cells are key participants in epileptogenic process, therefore, or are mere epiphenomena, is difficult to establish. We recently demonstrated that deletion of the *PTEN* gene from a small number of adult-born dentate granule cells causes a severe epilepsy syndrome (Pun et al., 2012). Here, we queried whether brain slices from these animals would exhibit physiological abnormalities hypothesized to be mediated by abnormal granule cells. Similar to other models of temporal lobe epilepsy, hippocampal slices from *PTEN*KO mice were hyperexcitable, exhibiting multiphasic responses in the granule cell layer following perforant path stimulation. This demonstrates that very distinct brain injuries (e.g. status epilepticus vs. focal *PTEN* deletion) can converge to produce common circuit abnormalities in the hippocampus, and supports a hypothesized role for abnormal granule cells in temporal lobe epileptogenesis.

Circuit abnormalities are more robust in the PTEN model

The epilepsy field has produced an extensive literature showing abnormal responses to perforant path stimulation in a variety of extremely well-characterized temporal lobe epilepsy models. Multiple population spikes in the granule cell layer following perforant path stimulation have been described following systemic injection of pilocarpine to induce status epilepticus (Scharfman et al., 2002), systemic injection of kainic acid to induce status epilepticus (Haas et al., 1996; Patrylo et al., 1999) and kindling epileptogenesis (Lynch and Sutula, 2000; Naylor and Wasterlain, 2005). There is substantial variability, however, in the incidence of multiple population spikes in normal ACSF in these models, with response rates ranging from 0 – 93% (Cronin et al., 1992; Dudek et al., 1994; Patrylo et al., 1999; Scharfman et al., 2002; Shao and Dudek, 2011; Sloviter, 1992). Treatments to enhance excitability or reduce inhibition increase the percentage of slices with multiple spikes, although some still fail to show the effect (Lynch and Sutula, 2000; Sutula and Dudek, 2007). This variability in slice physiology likely reflects, at least in part, wide differences in histopathology and epilepsy severity within these models (Danzer et al., 2010; Hester and Danzer, 2013). In this regard, the consistency among *PTEN* KO slices demonstrates one of the strengths of the model. Further, this consistency supports the hypothesis that abnormal granule cells drive hippocampal pathophysiology in temporal lobe epilepsy. Specifically, the dominant pathology of *PTEN* KO mice is abnormal granule cells. They are present in all animals in large numbers. By contrast, abnormal granule cells appear at widely variable rates in the pilocarpine model, and are not always present in significant numbers (Hester and Danzer, 2013; Walter et al., 2007). Although correlative, the robust expression of hallmark physiological abnormalities in slices from *PTEN* KO mice may reflect the dominant presence of abnormal granule cells in these animals.

PTEN KO cell mediated changes in hippocampal circuitry

The addition of *PTEN*KO cells to the hippocampus in this model fundamentally changes hippocampal circuitry. *PTEN*KO cells contribute to mossy fiber sprouting (Pun et al., 2012), expand their axonal projections in the mossy fiber pathway (LaSarge et al., 2015), develop hilar-projecting basal dendrites (Pun et al., 2012), and occasionally migrate to ectopic locations in the dentate hilus. All of these changes have been hypothesized to contribute to recurrent circuitry in the epileptic temporal lobe.

Mossy fiber sprouting into the dentate molecular layer creates recurrent circuitry within the dentate, and such sprouting is evident in a subset *PTEN*KO animals (Figure 6A). In the present study, both *PTEN*KO slices with and without mossy fiber sprouting showed multiple population spikes, and the degree of mossy fiber sprouting in *PTEN*KO mice did not correlate with any physiological measure analyzed. These findings are consistent with prior work, in which seizure frequency was not found to be correlated with mossy fiber sprouting (Pun et al., 2012). Taken together, the results support the conclusion that mossy fiber sprouting is not critical for physiological abnormalities in this model. Indeed, the exact role of this phenomenon in epilepsy remains controversial (Buckmaster, 2014).

The majority of *PTEN*KO cells exhibit large, spiny basal dendrites which appear to receive recurrent, mossy fiber input (Pun et al., 2012). Recurrent activation of granule cells through these dendrites could produce secondary spikes, and the large number of cells with these abnormal processes makes them an appealing candidate for mediating this recurrent activity. Moreover, among granule cells with basal dendrites in status epilepticus models of epilepsy, the axon often originates from the basal dendrite (Dashtipour et al., 2002; Walter et al., 2007). Recent work from Thome and colleagues (2014) indicates that basal dendrite-originating axons in CA1 can elicit action potential more efficiently than somatic axons. Whether this is also true for granule cells remains to be determined; nevertheless, basal dendrites might have a disproportionate effect on circuit function.

A subset of *PTEN*KO cells (~3%) migrate ectopically to the dentate hilus (Pun et al., 2012). Studies of ectopic granule cells in status epilepticus models of epilepsy demonstrate that these neurons are hyperexcitable and prone to burst firing (Cameron et al., 2011; Scharfman et al., 2000; Zhan and Nadler, 2009), which might lead to additional tertiary and quaternary spikes. Hilar mossy cells - which directly excite granule cells in addition to mediating feedback inhibition through granule cells - might also provoke abnormal activity (Scharfman et al., 2001; Zhang et al., 2015).

Possible mechanisms of circuit hyperexcitability: Cell intrinsic changes among PTEN KO cells

Intrinsic changes in *PTEN*KO cells have been investigated with several *PTEN*KO models using different Cre-driver lines as well as viral deletion strategies (Amiri et al., 2012; Fraser et al., 2004; Haws et al., 2014; Kwon et al., 2006; Kwon et al., 2003; Luikart et al., 2011; Sperow et al., 2012; Takeuchi et al., 2013; Williams et al., 2015; Zhou et al., 2003). *PTEN* deletion is targeted to distinct cell populations in these models, producing a range of phenotypes. Studies that specifically targeted dentate granule cells using a viral-deletion

strategy, which is perhaps most similar to the model used here, reveal that changes in *PTEN* KO cell intrinsic properties contribute to dentate excitability. *PTEN* KO cells also fire at lower intensities of afferent stimulation and exhibit increases in spine density and synaptic input (Luikart et al., 2011; Weston et al., 2014; Williams et al., 2015). Together, the findings suggest that network excitability likely reflects the combined effects of cell intrinsic changes in excitability, and changes in wiring and connectivity.

Secondary epileptogenic changes initiated by *PTEN* KO cells

An alternate possibility is that – rather than directly driving circuit abnormalities – *PTEN* KO cells induce a cascade of secondary changes which culminate in the abnormalities described here. Indeed, since *PTEN* KO mice develop spontaneous seizures as early as four weeks after tamoxifen treatment (Pun et al., 2012), secondary, seizure-induced changes are likely. In prior work, we demonstrated that the axons of *PTEN*-expressing granule cells contribute to mossy fiber sprouting, confirming that secondary changes do occur. It is conceivable that *PTEN* KO cells produce low-grade epileptiform activity which eventually disrupts the hippocampal circuit – reminiscent of repeated kindling stimulations of the brain – producing changes similar to those described here (Sayin et al., 2003). Indeed, disruption of the hippocampal circuit may contribute to the observed impairment in paired-pulse facilitation. Altered paired-pulse facilitation is thought to reflect a change in presynaptic release probability (McNaughton, 1980). In this case, layer II neurons in lateral entorhinal cortex are implicated, providing evidence that changes have spread beyond *PTEN* KO dentate granule cells.

Conclusions

The present findings demonstrate that selective deletion of *PTEN* from a subset of granule cells reproduces hippocampal pathophysiologies evident in traditional models of temporal lobe epilepsy. Cell-intrinsic increases in *PTEN* KO cell excitability and circuit level changes mediated by *PTEN* KO cell axonal and dendritic sprouting are consistent with the interpretation that KO cells underlie these pathologies. However, the possibility that secondary changes among other neuronal populations drives the phenotype cannot be excluded. In either case, the present findings reveal that *PTEN* deletion from a subset of granule cells can *initiate* the development of hippocampal abnormalities common across multiple temporal lobe epilepsy models, supporting the overall hypothesis that aberrant granule cell integration is epileptogenic.

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Highlights

- ⊃ *PTEN* removal from newborn granule cells causes a hyperexcitable dentate network.
- ⊃ Granule cells in *PTEN* KO mice have a lowered population spike current threshold.
- ⊃ Perforant path stimulation elicits multiple population spikes in *PTEN* KO slices.
- ⊃ Paire-pulse facilitation is impaired in the dentate network of *PTEN* KO mice.

Figure 1.

Dentate gyrus sections from control and *PTEN*KO mice showing NeuN (blue) and PTEN (red) immunostaining. *PTEN*KO (NeuN positive/PTEN negative; yellow arrows) and control dentate granule cells (NeuN/PTEN positive; white arrows) are shown. Scale bars = 50 μ m (A-F) and 10 μ m (C' and F').

Figure 2.

Increasing lateral (A), but not medial (B), perforant path stimulation intensity caused a steeper fEPSP slope in the *PTEN* KO slices compared to controls. Population spike amplitudes, however, were larger following both lateral and medial perforant path area stimulation in *PTEN* KO slices (C and D).

Figure 3.

Responses to lateral perforant path (LPP) stimulation from control and *PTEN* KO mice. In slices from control mice (A) the field excitatory post-synaptic potential (fEPSP) increased in amplitude with greater stimulation current and was followed by the appearance of a single population spike (negative going event) once threshold was reached. The signal returned to baseline within 25 ms (A'). Slices from *PTEN* KO mice (B) also showed increasing fEPSP slope with increasing current, however, multiple population spikes were evoked and responses featured an elongated negative phase (B'). *PTEN* KO slices had significantly more population spikes than controls (C).

Figure 4.

High K⁺ solution reduced population spike threshold in control but not *PTEN*KO slices. The lateral perforant path was stimulated in slices immersed in normal ACSF (A and D) or High K⁺ (B and E) bath solution. In normal ACSF, substantial current (≈ 250 μ A, pink line in A) was required to evoke a modest population spike from control slices, while *PTEN*KO slices exhibited multiple population spikes at all stimulation intensities shown (D). When slices were exposed to a High K⁺ bath solution, population spike threshold dropped significantly in controls (B), while the threshold among KO slices remained unchanged (E). Panel F shows a graphical representation of mean population spike responses. In contrast to population spikes thresholds, fEPSP thresholds did not differ by treatment or bath solution (C). LPP = lateral perforant path; MPP = medial perforant path.

Figure 5.

Lateral perforant path paired-pulse facilitation is impaired in slices from *PTEN* KO mice. (A) Paired-pulse responses to interpulse intervals of 30, 70, and 100 ms from control (blue) and *PTEN* KO (purple) slices. (B) Superimposed traces from the first (black line) and second (red line) stimulations with a 70 ms interpulse interval. The control slice exhibited increased slope of the fEPSP and amplitude of the population spike, while facilitation was largely absent from the *PTEN* KO slice. Quantification of fEPSP slope (C) and population spike facilitation (D) are shown in the graphs. The degree of facilitation was significantly reduced in *PTEN* KO slices relative to controls at interpulse intervals between 30 and 150 ms.

Figure 6.

ZnT-3 immunostaining was used to analyze the distribution of mossy fiber axons in control and *PTEN* KO slices. (A) In control mice, ZnT3 staining was concentrated in the hilus and stratum lucidum. *PTEN* KO mice showed variability in their ZnT3 expression. Some *PTEN* KO mice exhibited clear mossy fiber sprouting, evident as an immunoreactive band in the inner molecular layer (middle panels, white arrow), whereas other mice had expression patterns similar to controls (lower panels). Higher resolution images (expansions of the boxed regions) highlight immunoreactive puncta (middle, yellow arrow) in the inner molecular layer (IML) of one *PTEN* KO mouse, but not the other (lower). (B) There was no correlation between abnormal physiology and mossy fiber sprouting. *PTEN* KO mice with and without mossy fiber sprouting responded with multiple population spikes following lateral perforant path stimulation, whereas control slices exhibited only single population spikes (5 stimulations of 200 μ A shown with 5 seconds between stimulations). Scale bars = 250 (low magnification) and 25 μ m (high magnification).

Table 1

Paired-pulse Index of fEPSP slope

		Interpulse Interval (ms)					
	group	30	50	70	100	150	200
LPP	Control n=10 {11}	1.425 ± 0.05	1.466 ± 0.06	1.427 ± 0.03	1.377 ± 0.05	1.249 ± 0.05	1.169 ± 0.04
	<i>PTEN</i> KO n=9 {9}	1.120 ± 0.02	1.146 ± 0.03	1.155 ± 0.02	1.119 ± 0.02	1.038 ± 0.03	1.051 ± 0.04
	RM ANOVA: F(group) = 32.099, p<0.001; F(IPI) = 13.920, p<0.001; F(inx) = 2.875, p=0.019						
	post-hoc t / p- value	5.170 / p<0.001	5.433 / p<0.001	4.386 / p<0.001	4.329 / p<0.001	3.539 / p<0.001	2.000 / p=0.051
MPP	Control n=8 {8}	1.122 ± 0.06	1.165 ± 0.07	1.170 ± 0.10	1.073 ± 0.06	1.097 ± 0.09	1.024 ± 0.07
	<i>PTEN</i> KO n=8 {8}	1.100 ± 0.08	1.067 ± 0.09	1.088 ± 0.06	1.080 ± 0.03	1.034 ± 0.03	0.963 ± 0.03
	RM ANOVA: F(group) = 0.715, n.s.; F(IPI) = 1.759, n.s.; F(inx) = 0.247, n.s.						

LPP, lateral perforant path; MPP, medial perforant path; n = mice {slices}, Mean ± S.E.

Table 2

Paired-pulse Index of population spike amplitude

		Interpulse Interval (ms)					
	group	30	50	70	100	150	200
LPP	Control n=10 {11}	2.437 ± 0.30	2.353 ± 0.37	2.338 ± 0.29	2.138 ± 0.27	1.679 ± 0.09	1.373 ± 0.07
	<i>PTEN</i> KO n=9 {9}	1.113 ± 0.10	1.137 ± 0.05	1.116 ± 0.05	1.089 ± 0.06	1.043 ± 0.05	1.004 ± 0.06
	RM ANOVA: F(group) = 42.197, p<0.001; F(IPI) = 13.195, p<0.001; F(inx) = 8.651, p<0.001						
	Post-hoc t / p- value	6.854 / p<0.001	6.154 / p<0.001	6.190 / p<0.001	5.312 / p<0.001	3.126 / p=0.004	1.867 / p=0.072
MPP	Control n=8 {8}	1.783 ± 0.27	1.572 ± 0.26	1.492 ± 0.25	1.186 ± 0.16	1.132 ± 0.13	1.164 ± 0.15
	<i>PTEN</i> KO n=8 {8}	1.437 ± 0.18	1.297 ± 0.12	1.759 ± 0.53	1.408 ± 0.19	1.262 ± 0.13	1.042 ± 0.09
	RM ANOVA: F(group) = 0.144, n.s.; F(IPI) = 2.053, n.s.; F(inx) = 0.593, n.s.						

LPP, lateral perforant path; MPP, medial perforant path; n = mice {slices}, Mean ± S.E.