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Impact of rapamycin on status epilepticus induced hippocampal pathology and weight gain

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Abstract

Growing evidence implicates the dentate gyrus in temporal lobe epilepsy (TLE). Dentate granule cells limit the amount of excitatory signaling through the hippocampus and exhibit striking neuroplastic changes that may impair this function during epileptogenesis. Furthermore, aberrant integration of newly-generated granule cells underlies the majority of dentate restructuring. Recently, attention has focused on the mammalian target of rapamycin (mTOR) signaling pathway as a potential mediator of epileptogenic change. Systemic administration of the mTOR inhibitor rapamycin has promising therapeutic potential, as it has been shown to reduce seizure frequency and seizure severity in rodent models. Here, we tested whether mTOR signaling facilitates abnormal development of granule cells during epileptogenesis. We also examined dentate inflammation and mossy cell death in the dentate hilus. To determine if mTOR activation is necessary for abnormal granule cell development, transgenic mice that harbored fluorescently-labeled adult-born granule cells were treated with rapamycin following pilocarpine-induced status epilepticus. Systemic rapamycin effectively blocked phosphorylation of S6 protein (a readout of mTOR activity) and reduced granule cell mossy fiber axon sprouting. However, the accumulation of ectopic granule cells and granule cells with aberrant basal dendrites was not significantly reduced. Mossy cell death and reactive astrogliosis were also unaffected. These data suggest that anti-epileptogenic effects of mTOR inhibition may be mediated by mechanisms other than inhibition of these common dentate pathologies. Consistent with this conclusion, rapamycin

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prevented pathological weight gain in epileptic mice, suggesting that rapamycin might act on central circuits or even peripheral tissues controlling weight gain in epilepsy.

Introduction

Status epilepticus (SE) selectively disrupts the integration of adult-generated hippocampal granule cells (Parent et al., 2006; Jessberger et al., 2007; Walter et al., 2007; Kron et al., 2010; Santos et al., 2011). Changes include mossy fiber axon sprouting, formation of hilar basal dendrites and ectopic migration of newly-generated cells. These phenomena have been implicated in promoting hyperexcitability in the temporal lobe and may facilitate epileptogenesis (Jung et al., 2004; 2006; Cho et al., 2015). Hilar ectopic granule cells, for example, are more excitable in rodent epilepsy models (Zhan et al., 2010; Althaus et al., 2015) and can exhibit both spontaneous and evoked bursting (Scharfman et al., 2000; Cameron et al., 2011), distinguishing them from normal granule cells. In addition to ectopically-located cells, granule cells with mossy fiber axon sprouting to the dentate inner molecular layer and hilar-projecting basal dendrites mediate the formation functional granule cell to granule cell synapses (Okazaki et al., 1999; Thind et al., 2008). These synaptic connections may mediate increased excitatory flow through the dentate gyrus. Despite the evidence that these abnormalities may contribute to epileptogenesis and associated comorbidities, no current FDA-approved treatments can prevent the aberrant integration of adult-generated neurons in epilepsy.

Recently, the mTOR pathway has emerged as a promising molecular target that may mediate aberrant granule cell integration. mTOR signaling in the dentate is enhanced in chemical, injury-induced and genetic models of epilepsy (Wong, 2013; LaSarge and Danzer, 2014), suggesting the pathway could be involved in many different forms of the disease. Treatment with rapamycin, an inhibitor of mTOR complex 1, has been shown to reduce seizures (Zeng et al., 2009; Huang et al., 2010; van Vliet et al., 2012), prevent mossy fiber sprouting (Zeng et al., 2009; Huang et al., 2010; Buckmaster et al., 2009; Buckmaster and Lew, 2011; Tang et al., 2012; van Vliet et al., 2012; Heng et al., 2013; Shima et al., 2015), mitigate cell loss (Zeng et al., 2009; van Vliet et al., 2012; Guo et al., 2013; Butler et al., 2015) and reduce reactive astrogliosis (Shima et al., 2015) in rodent modes of acquired epilepsy. Conversely, recent work from our lab demonstrates that genetically enhancing mTOR signaling by PTEN deletion in adult-generated hippocampal granule cells causes mossy fiber axon sprouting, formation of hilar basal dendrites and ectopic cell migration (Pun et al., 2012). We hypothesized, therefore, that mTOR hyperactivation underlies the cellular abnormalities evident in the hippocampus following status epilepticus.

To test our hypothesis we utilized rapamycin to directly inhibit mTOR activity in mice following pilocarpine-induced status epilepticus. A genetic fate-mapping strategy was used to determine the effectiveness of rapamycin at mitigating cellular abnormalities among adult-generated granule cells. In addition, mossy cell survival and reactive astrogliosis were examined in the animals, since these changes have also been implicated in epileptogenesis. These experiments provide new insights into the role of mTOR signaling in the formation of epilepsy-induced anatomical changes within the dentate gyrus.

Methods

Animals

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the Cincinnati Children's Hospital Research Foundation and conform to NIH guidelines for the care and use of animals. Two animal breeding schemes were followed: (1) In order to generate Gli1-CreER^{T2}::GFP mice on a pure C57BL/6NCrl background for the study, hemizygous Gli1-CreER^{T2} mice (Ahn and Joyner, 2004, 2005) on a C57BL/6NCrl background were crossed to homozygous CAG-CAT-EGFP (GFP) mice (Nakamura et al., 2006) on a C57BL/6NCrl background (B6-GFP, n=77). (2) In order to generate Gli1-CreER^{T2}::tdTomato positive mice on a 50:50 mix C57BL/6NCrl::FVB N background for the study, hemizygous Gli1-CreER^{T2} mice on an FVB N background were crossed to mice heterozygous for a CAG promoter-driven red fluorescent protein (tdTomato) reporter construct (Jax; stock 007914) on a C57BL/6NCrl background (B6/FVB-Tomato, n=9). Gli1-CreER^{T2}::tdTomato negative mice produced from this cross were also used (B6/FVB, n=5). All animals were analyzed for phosphorylated S6 protein (pS6), hilar ectopic dentate granule cell density, mossy fiber sprouting, mossy cell density, reactive astrogliosis and microgliosis. Basal dendrite frequency and granule cell hypertrophy were analyzed only in B6-GFP and B6/FVB-Tomato mice, because GFP or tdTomato expression is needed to visualize granule cell somas and dendrites. All animals were maintained as a colony in the vivarium at CCHMC. Animals were provided with food and water *ad libitum* with a 14/10 hour light/dark cycle to optimize breeding (Fox et al., 2006).

Postnatal tamoxifen treatment of Gli1-CreER^{T2} mice activates CreER^{T2} in granule cell progenitors (Ahn and Joyner, 2005), resulting in continuous expression of GFP or tdTomato in the progenitor cells and subsequent daughter cells in B6-GFP and B6/FVB-Tomato mice. All mice were given injections of tamoxifen (250 mg/kg, s.c.) at 3, 4, 5, 6 and 7 weeks of age, limiting labeling to granule cells born after three weeks of age. At eight weeks of age, mice received methyl scopolamine nitrate in sterile saline (1 mg/kg, s.c.) followed by pilocarpine (420 mg/kg, s.c.) ten minutes later. Animals were monitored for behavioral seizures and the onset of status epilepticus (SE). Following three hours of SE mice were given two injections of diazepam ten minutes apart (10 mg/kg, s.c.) to alleviate seizure activity. Mice were given sterile Ringers as needed to maintain pretreatment body weight. Animals not designated to undergo SE received saline instead of pilocarpine, but were given scopolamine and diazepam. Rapamycin (LC Laboratories) was dissolved in 5% Tween 80, 5% polyethyleneglycol and 4% ethanol in PBS and was administered daily (6 mg/kg, i.p; or vehicle control) for six weeks beginning one day after pilocarpine SE. The final treatment groups were: (1) SE+rapamycin, (2) SE+vehicle, (3) rapamycin only and (4) vehicle only. At 14 weeks of age mice were anesthetized with pentobarbital (100 mg/kg, i.p.) and perfused with 1U/ml heparin, 2.5% paraformaldehyde and 4% sucrose in PBS (pH 7.4). Brains were extracted, stored overnight at 4°C in the same fixative, cryoprotected in ascending sucrose series (10, 20, 30%) in PBS and snap-frozen in isopentane at -25°C. Brains were sectioned coronally at 60 µm, and sections were affixed to gelatin coated slides and stored at -80°C.

Immunohistochemistry

Slide mounted brain sections (two per slide) from dorsal hippocampus were processed for histological studies. Sections were immunostained with the following antibodies: rabbit anti-pS6 (1:500, Cell Signaling), chicken anti-GFP (1:500, Abcam, Boston, MA), rabbit anti-zinc transporter 3 (ZnT3) (1:3000, Synaptic Systems, Gottingen, Germany), rabbit anti-Glutamate receptor 2/3 (1:100, Millipore, Temecula, CA), rabbit anti-prox1 (1:1000, Sigma-Aldrich, St. Louis, MO), rabbit anti-GFAP (1:500, Millipore) and rabbit anti-Iba1 (1:1000, Synaptic Systems). AlexaFluor488 goat anti-chicken, AlexaFluor488 goat anti-rabbit, AlexaFluor594 goat anti-rabbit and AlexaFluor647 goat anti-rabbit secondary antibodies were used (Invitrogen, Eugene, OR). Sections were dehydrated in alcohols, cleared in xylenes and coverslips were attached with Krystalon mounting-media (Harleco, Darmstadt, Germany).

Confocal imaging and quantification of histological measures

Imaging for pS6, hilar basal dendrites, mossy fiber sprouting, dentate granule cell soma area and mossy cell density analyses was conducted using a Leica SP5 confocal system set up on a DMI 6000 inverted microscope equipped with a 63× oil immersion objective (NA 1.4; image size 240 × 240 μm in the XY plane). Images for hilar ectopic dentate granule cell density were generated using a 3024 Nikon A1Rsi inverted microscope with a 40× water objective (NA 1.15, image size 317 × 317 μm in the XY plane). Images for analyses of reactive astrocytosis and microgliosis were generated using the 3024 Nikon A1Rsi microscope with a 60× water objective (NA 1.27, image size 210 × 210 μm in the XY plane). For measures analyzing immunohistochemical staining intensity, including dentate and cortical pS6 expression, images were all collected with identical parameters. For soma areas, cell density measures and measures of cellular and neuronal process, image collection parameters were set to optimize cellular detail. For these latter measures, accurately identifying the boundaries of cellular structures is critical, so image setting were adjusted to avoid “over or underexposure”; conditions which can artifactually increase or decrease structure size, respectively. Images were collected and analyzed by an investigator blind to treatment group to avoid bias. For each parameter, dentate gyri were analyzed from dorsal hippocampus (1.6–2.0 mm posterior to bregma; Paxinos and Franklin, 2001). All cell counts were conducted using a variation of the optical dissector method (Howell et al., 2002; Hofacer et al., 2013).

1) Phosphorylated S6 protein (pS6)—To quantify pS6 immunostaining, single confocal optical sections were collected from the midpoint of the upper blade of the dentate gyrus and parietal association cortex. Images were taken 3 μm below the surface of the tissue section for all samples to control for antibody penetration. Tissue sections were imaged using 633 nm light with 10.0% laser intensity with the argon lamp set to 25.0%. For all images the PMT gain was set to 1200, PMT offset was set to −18.0, and pinhole was set to 95.2 μm. Images were all collected with 2048 × 2048 voxels at 200 Hz. Optical sections were analyzed for average pixel intensity within the upper blade of the granule cell body layer of the hippocampus using LAS AF software (Leica Microsystems). Average pixel intensity was measured on a scale of 0–255, with 0 indicating no pS6 staining, and 255 indicating complete saturation.

2) Dentate granule cell mossy fiber axon sprouting—Granule cell mossy fiber sprouting was evaluated using ZnT3-labeling. Single confocal optical sections were collected at the midpoint of the upper and lower blades of the dentate gyrus to capture portions of the dentate inner molecular layer. Optical sections were taken 3 μm below the surface of the tissue for all samples. The area occupied by ZnT3-immunoreactive puncta within the dentate inner molecular layer was quantified using Neurolucida automated detection software (version 3.4.2). Puncta were defined as ZnT3-immunoreactive regions with a diameter greater than 0.5 μm . The degree of mossy fiber sprouting was defined as [(total area occupied by ZnT3 puncta/total inner molecular layer area) $\times$ 100].

3) Hilar ectopic dentate granule cells—The density of ectopic granule cells in the hilus was assessed from confocal z-series image stacks of sections immunostained with the granule cell marker Prox1 (1 μm step through the top 10 μm of tissue). Prox1-immunolabeled cells were considered ectopic if they were in the hilus and at least two cell body diameters [$\sim 20\mu\text{m}$] from the granule cell layer/hilar border. One hippocampal section for each animal was analyzed to determine hilar ectopic granule cell density [Prox1-expressing hilar granule cells/ mm^3 of hilus].

4) Dentate granule cells with basal dendrites—To assess basal dendrite frequency, confocal z-series image stacks of GFP or tdTomato labeled granule cells were collected (1 μm step through the top 10 μm of the tissue). One dentate gyrus/animal from dorsal hippocampus was imaged. Image stacks were imported into Neurolucida software, and cells were scored for the presence of basal dendrites projecting into the dentate hilus. Inclusion criteria for granule cells with basal dendrites were as follows: 1; Granule cells were morphologically mature, defined as cells with an axon extending into the hilus and spiny apical dendrites extending to the hippocampal fissure, 2; Cell bodies were fully contained within the tissue section, 3; Cell bodies were located within the granule cell layer and 4; Cells were well-labeled with GFP or tdTomato, such that processes could be followed to their natural endings. Data are presented as percentages (number of granule cells with basal dendrites/total number of granule cells scored).

5) Reactive astrogliosis and microgliosis—Changes in astrocytes and microglia were assessed by measuring the soma areas and total volume of GFAP- and Iba1-expressing cells located in the hilus. Image stacks were collected from within the dentate hilus (0.25 μm step through the top 5 μm of tissue). To measure soma areas, stacks were imported into Neurolucida software for measuring the maximum profile area of each respective cell type. Only cells whose somas were completely contained within the image stack were used. A total of five astrocytes and five microglia per animal were chosen at random for analysis. Soma areas presented for each animal are averages. To estimate total hilar volume occupied by astrocytes and microglia, 2 μm and 4 μm stacks (for GFAP- and Iba1-stained tissue, respectively) were imported into Imaris software. Stack size was determined so that the greatest number of sections with optimal antibody penetration for all brain preparations was used. Surfaces with a staining intensity of at least 4 $\times$ local background with a minimum detail level of 0.414 μm were automatically identified. Surfaces were added and removed manually following the automatic detection. Total volume occupied by the surfaces was

determined and data is represented as [(total volume GFAP- or Iba1-stained regions/total volume of hilus) $\times$ 100].

6) Dentate granule cell soma area—Changes in granule cell soma area were assessed by measuring the maximum profile area of GFP- or tdTomato-expressing adult-born granule cells. Image stacks used to analyses the presence of basal dendrites on GFP or tdTomato expressing cells were also used to determine dentate granule cell soma area. Measurements were completed using Neurolucida software. Granule cells with partially visible cell bodies were excluded (i.e. cut at the tissue surface or edge). Five granule cells per animal were chosen at random for analysis. For each animal, soma areas were averaged for statistical analysis.

7) Loss of hilar mossy cells—The density of mossy cells in the hilus was determined from confocal image stacks using GluR2/3-labeling (1 μ m step through the top 10 μ m of the tissue). Images were collected from the center of the hilus. Small, hilar GluR2/3-immunoreactive cells (8–12 μ m diameter) were considered to be ectopic granule cells and were excluded, while larger GluR2/3-expressing neurons (30–40 μ m diameter) localized to the hilus were counted as mossy cells (Fujise and Kosaka, 1999; Jiao and Nadler, 2007; Scharfman and Myers, 2013). Mossy cell hilar density is presented as [GluR2/3-expressing hilar mossy cells/mm³ of hilus].

Statistics

Statistical analyses were performed using Sigma Stat software (version 13.0). No differences were found between male and female animals, so data were pooled for further analysis (pilocarpine/rapamycin (n=4M, 2F), pilocarpine/vehicle (n=3M, 4F), saline/rapamycin (n=3M, 5F), saline/vehicle (n=3M, 4F). To account for differences between mouse strains, a two-way ANOVA was employed for each measure, controlling for strain (pure C57BL/6NCrl background vs. 50:50 mix C57BL/6NCrl::FVB N background) and treatment. Data that failed tests of normality or equal variance were normalized using either square root or rank transformations. Pairwise multiple comparison procedures were conducted using the Holm-Sidak method. Values are presented as least square means of raw (non-transformed) data $\pm$ standard error of the mean (SEM).

Figure preparation

Figures were prepared using Microsoft Excel and Adobe Photoshop (CS5-Extended). Brightness and contrast were adjusted to optimize cellular detail. Identical adjustments were made to all images meant for comparison. All error bars in graphs are SEM.

Results

To induce the development of epilepsy, mice were treated with the cholinergic agonist pilocarpine to provoke status epilepticus (SE), which was allowed to proceed for three hours before seizures were mitigated by treatment with diazepam. Control animals received saline instead of pilocarpine. Studies were begun using C57BL/6NCrl mice. Because of high mortality during the 24 hour period following SE, however, mice on a 50:50 background of

C57BL/6NCrl::FVB N (B6/FVB) were also used. B6/FVB mice showed significantly improved survival one day after pilocarpine treatment relative to C57BL/6NCrl mice (C57BL/6NCrl survival, 27.7% [18/65]; B6/FVB survival, 100% [10/10]; $p < 0.001$, Mann-Whitney rank sum test), consistent with prior work showing significant strain differences in seizure susceptibility (Schauwecker, 2012). 24 hours after SE, mice were randomly assigned to receive daily treatments with either six mg/kg rapamycin (Rap) or vehicle (Veh), producing four treatment groups (SE+Rap; SE+Veh; Rap only; Veh only). Mice were killed six weeks later; a time point when spontaneous seizures are typically occurring in this model (Castro et al., 2012; Hester and Danzer, 2013). Mortality was not altered by rapamycin treatment ($p = 1$, z test, SE+Rap vs. SE+Veh). Specifically, 66.7% of SE+Rap (6 of 9 mice; 4 of 7 B6, 2 of 2 B6/FVB), 50.0% of SE+Veh (7 of 14 mice; 2 of 6 B6, 5 of 8 B6/FVB), 100% of rapamycin only (8 of 8; 6 of 6 B6, 2 of 2 B6/FVB) and 87.5% of vehicle only (7 of 8 mice; 5 of 6 B6, 2 of 2 B6/FVB) mice survived the period from one day to six weeks after pilocarpine (or saline) treatment. Not included in this count are five SE+Rap mice implanted with cortical EEG electrodes for 24/7 seizure monitoring either one or three weeks after SE (data not shown). 100% of these mice died between four and seven days after surgery.

Rapamycin reduces pS6 in the granule cell layer and the cortex

To confirm that systemic rapamycin was able to reduce mTOR activity we immunostained brain sections with antibodies against pS6, a downstream product of mTOR activation. SE + Veh-treated mice displayed increased pS6 immunoreactivity in the granule cell layer and cortex (Figure 1), confirming previous reports (Zeng et al., 2009). Systemic administration of rapamycin significantly reduced pS6 immunoreactivity in the granule cell layer of status epilepticus [SE+Rap] and control [Rap only] mice. Two-way ANOVA revealed an interaction ($p < 0.001$; square root-transformed data) between strain [B6 vs. B6/FVB mice] and treatment [Veh, Rap, SE+Veh, and SE+Rap]. Specifically, rapamycin reduced pS6 more efficiently in the dentate of SE-exposed B6/FVB mice than SE-exposed B6 mice ($p < 0.001$, Holm-Sidak). However, pS6 was significantly reduced in the dentate of both strains following rapamycin treatment (B6: SE+Veh vs. SE+Rap, $p < 0.001$; B6/FVB: SE+Veh vs. SE+Rap, $p < 0.001$, Holm-Sidak). In cortex, rapamycin decreased the amount of pS6 expression (SE+Veh vs. SE+Rap, $p = 0.003$; Veh only vs. Rap only, $p = 0.014$). A significant strain effect was observed as well, with less pS6 expression observed in the cortex of B6/FVB mice compared to B6 mice ($p < 0.001$, two-way ANOVA). These results demonstrate the ability of systemic rapamycin to effectively block phosphorylation of S6.

Rapamycin reduces status epilepticus-induced weight gain

Pilocarpine epileptogenesis leads to obesity in rodents (Scharfman et al., 2008; Ruiz et al., 2011) and a similar phenomenon has been described in patients with epilepsy (Daniels et al., 2009). In the present study, SE+Veh mice exhibited a significant 35% increase in body weight over the course of the experiment ($p < 0.001$, one-way RM ANOVA). Both male and female mice exhibited increases (Figure 2, top). Vehicle-treated mice also grew, weighing 12% more at the end of the experiment ($p < 0.034$, one-way RM ANOVA). By contrast, neither SE+Rap mice ($p < 0.066$, one-way RM ANOVA) nor Rap only mice ($p < 0.456$, one-way RM ANOVA) exhibited significant weight gain (Figure 2). When normalized to percent change in body weight, SE+Veh mice gained significantly more weight relative to all other

groups (Figure 2, bottom; $p < 0.001$ vs SE+Rap and Rap only, $p = 0.003$ vs. Vehicle; Holm-Sidak). No sex or strain differences were found among the treatment groups (data not shown). Furthermore, rapamycin-treated and vehicle-treated mice that did not undergo SE exhibited identical outcomes for all additional measures in the study. Therefore, these animals were pooled into a common control group for the remainder of the manuscript.

Mossy fiber sprouting is reduced in rapamycin treated mice

Mossy fiber sprouting in the dentate inner molecular layer was assessed in each animal by ZnT3-immunohistochemistry. ZnT3 is localized to the axon terminals of granule cells, providing a reliable measure of mossy fiber sprouting (Wenzel et al., 1997; McAuliffe et al., 2011). Two-way ANOVA revealed a significant strain effect between B6 and B6/FVB mice ($p = 0.016$). B6 mice in the SE+Veh group exhibited robust mossy fiber sprouting compared to controls ($p < 0.001$, square root-transformed data) and rapamycin treatment significantly reduced this sprouting ($p = 0.042$, Figure 3). Pilocarpine treatment also significantly increased mossy fiber sprouting in B6/FVB strain relative to controls ($p = 0.003$, Holm-Sidak), however, the effect was not as dramatic as that observed in B6 mice, producing a significant difference between strains for within SE+Veh ($p = 0.003$, Holm-Sidak) and within SE+Rap comparisons ($p = 0.008$, Holm-Sidak). In addition, rapamycin did not significantly reduce mossy fiber sprouting in the B6/FVB line (SE+Veh vs. SE+Rap, $p = 0.120$, Holm-Sidak), perhaps because the reduced sprouting overall in this line limited statistical power. Notably, however, rapamycin-treated B6/FVB mice were also statistically indistinguishable from controls ($p = 0.307$, Holm-Sidak), suggesting that the drug has some efficacy.

Rapamycin does not affect dentate granule cell soma size

mTOR is a known regulator of cell growth (Fingar and Blenis, 2004). Hyperactivation of the mTOR pathway has been shown to increase soma size (Kwon et al., 2001), while suppression of mTOR reduces soma size (Thomanetz et al., 2013). Status epilepticus also results in increased granule cell soma size (Suzuki et al., 1995; Young et al., 2009; Murphy et al., 2011; 2012). To see if rapamycin reduces the soma size of adult-born granule cells following SE we measured maximum profile area of GFP or tdTomato positive cell bodies. We found increased soma size in vehicle-treated mice following pilocarpine induced SE ($51.7 \pm 2.6 \mu\text{m}^2$) compared to control mice ($34.5 \pm 2.7 \mu\text{m}^2$; $p < 0.001$; two-way ANOVA, Holm-Sidak, Figure 4). However, rapamycin treatment did not reduce soma size following pilocarpine treatment ($50.6 \pm 2.6 \mu\text{m}^2$; $p = 0.773$). Mean values between mouse backgrounds were not different ($p = 0.705$, two-way ANOVA). These findings suggest that increased soma size following SE may be mediated by mTORC2, which is rapamycin insensitive. mTORC2 has been shown to control actin cytoskeleton (Jacinto et al., 2004) and soma size (Thomanetz et al., 2013) in other models.

Rapamycin does not alter the number of hilar ectopic granule cells

Granule cells born after an epileptic insult commonly migrate to ectopic locations in the dentate hilus (Parent et al., 2006; Jessberger et al., 2007; Walter et al., 2007; Kron et al., 2010). Ectopic granule cells are rare in non-epileptic animals. Here, we used the granule cell-specific marker *prox1* to label hilar ectopic granule cells for quantification (Figure 5). Hilar ectopic granule cell density was significantly greater in vehicle-treated epileptic mice

($17.2 \pm 2.6 \times 10^3$ cells/mm³) relative to controls ($2.3 \pm 1.8 \times 10^3$ cells/mm³; $p < 0.001$; two-way ANOVA on rank-transformed data, Holm-Sidak). Rapamycin treatment following SE had no effect on ectopic granule cell accumulation ($17.8 \pm 2.6 \times 10^3$ cells/mm³; $p = 0.566$) and treated animals continued to exhibit significantly more ectopic granule cells than controls ($p < 0.001$), confirming previously reported data (Buckmaster and Lew, 2011). No differences were found between mouse strains ($p = 0.092$, two-way ANOVA on rank-transformed data).

Rapamycin may reduce the number of granule cells with basal dendrites

GFP and tdTomato expression was used to score granule cells for the presence of basal dendrites, which form recurrent excitatory currents and are implicated in the development of epilepsy (Ribak et al., 2000; Austin and Buckmaster, 2004; Thind et al., 2008; Hester and Danzer, 2013). The total numbers of granule cells scored in each treatment group were as follows: SE+Veh (180 cells, 15–49 cells/animal); SE+Rap (300, 8–105) and control (321, 8–71). The percentage of adult-born granule cells possessing basal dendrites was significantly increased in vehicle-treated epileptic mice ($33.3 \pm 4.3\%$) relative to controls ($3.5 \pm 4.4\%$; $p < 0.001$; two-way ANOVA on square root transformed data, Holm-Sidak; Figure 6). Rapamycin-treatment of epileptic mice produced a trend towards fewer granule cells with basal dendrites; however, this reduction did not reach significance ($15.9 \pm 4.3\%$; $p = 0.066$). This suggests that basal dendrite formation may be partially mediated through mTOR signaling, but other pathways are likely involved. No differences in basal dendrite frequency were found between mouse strains ($p = 0.963$, two-way ANOVA on square root transformed data).

Rapamycin treatment does not affect reactive astrocytosis or microgliosis

Reactive astrocytosis may facilitate the progression of epileptogenesis (Gibbons et al., 2013). Rapamycin has been shown to inhibit immune responses in normal animals (Lu et al., 2015) and can attenuate epilepsy-associated inflammation (Brewster et al., 2013; Nguyen et al., 2015; Shima et al., 2015), leading us to ask whether rapamycin affected the activation state of astrocytes following SE. Astrocytic activation was first assessed by measuring the soma area of astrocytes in the dentate hilus. As astrocytes become reactive, they lose their fine processes and their cell bodies become enlarged. Consistent with prior work (Shapiro et al., 2008), we observed an increase in astrocytic soma size in vehicle-treated epileptic mice ($47.8 \pm 2.5 \mu\text{m}^2$) compared to control mice ($26.1 \pm 1.7 \mu\text{m}^2$; $p < 0.001$; two-way ANOVA on square root transformed data, Holm-Sidak, Figure 7). Rapamycin treatment of epileptic mice did not reduce soma size ($43.6 \pm 2.5 \mu\text{m}^2$; $p = 0.240$), indicating that the drug had no effect on this measure of inflammation. No differences in astrocyte soma area were found between mouse backgrounds ($p = 0.089$, two-way ANOVA on square root transformed data). As a second test of astrocyte activation, we determined the total volume of tissue occupied by astrocytic somata and processes in a sample of the dentate hilus. SE+Veh and SE+Rap mice exhibited greater hilar volume occupied by astrocytes than control mice ($p < 0.001$ and $p = 0.010$, respectively, two-way ANOVA, Holm-Sidak, Figure 7). Rapamycin treatment, however, did not significantly reduce the volume of tissue occupied by astrocytes following status epilepticus (SE+Veh vs. SE+Rap, $p = 0.097$). Two-way ANOVA revealed a strain difference between B6 and B6/FVB mice, with B6/FVB exhibiting less hilar volume occupied by astrocytes than B6 mice ($p = 0.031$).

We also assessed the impact of rapamycin on microglial activation in the hilus. Microglial soma area was increased following SE (SE+Veh vs. control, $p=0.001$, two-way ANOVA, Holm-Sidak, Figure 8), but again, rapamycin did not block this increase (SE+Veh vs. SE+Rap, $p=0.710$). Percent hilar volume occupied by microglial processes was statistically equivalent among groups (SE+Veh, SE+Rap, or control, $p=0.161$, Figure 8). No strain effects were observed for either measure (microglial soma area, $p=0.100$; microglial volume/hilar volume, $p=0.736$).

Mossy cell loss is unaffected by rapamycin treatment

Hilar mossy cell death following seizures has been reported for decades (Sloviter, 1987) and has been suggested to play a role in epileptogenesis (Sloviter et al., 2003; Jinde et al., 2013). To quantify mossy cell numbers, sections were immunostained for GluR2/3, a receptor expressed by glutamatergic mossy cells. We confirmed that hilar mossy cell density was significantly reduced in vehicle-treated epileptic mice ($10.9 \pm 6.1 \times 10^3$ cells/mm³) compared to controls ($56.4 \pm 4.2 \times 10^3$ cells/mm³; $p<0.001$; Figure 9). Rapamycin treatment after SE had no effect on mossy cell density ($13.1 \pm 6.3 \times 10^3$ cells/mm³), with SE+Rap mice exhibiting significantly lower values than controls. Mossy cell loss occurs hours to days after SE, so it was expected that rapamycin treatment would have limited effects on this measure. No differences in mossy cell density were found between mouse strains ($p=0.516$, two-way ANOVA).

Discussion

mTOR signaling is enhanced during the development of acquired temporal lobe epilepsy (Figure 1), and rapamycin treatment has been shown to reduce seizures frequency in a variety of epilepsy models, including kainate-SE (Zeng et al., 2009), pilocarpine-SE (Huang et al., 2010), angular bundle electrical stimulation (van Vliet et al., 2012), neonatal hypoxia (Talós et al., 2012), traumatic brain injury (Guo et al., 2013), models of TSC (Zeng et al., 2008; Goto et al., 2011; Zeng et al., 2011) and PTEN inactivation (Ljungberg et al., 2009; Sunnen et al., 2011; Pun et al., 2012). Negative findings have also been reported (Buckmaster and Lew, 2011; Heng et al., 2013; Sliwa et al., 2012). In addition to improving seizure outcome, animal studies have demonstrated that rapamycin improves cognitive and memory deficits following SE (Brewster et al., 2013). The mechanisms by which rapamycin produces these positive effects, however, are unclear. For the present study, we examined several hippocampal abnormalities implicated in the development of temporal lobe epilepsy (Parent and Kron, 2012; Hester and Danzer, 2014), including mossy fiber sprouting (Buckmaster, 2014), hilar ectopic granule cell accumulation (Scharfman and Pierce, 2012), basal dendrite formation (Ribak et al., 2012), reactive astrogliosis and microgliosis (Sharma et al., 2008; Gibbons et al., 2013) and mossy cell loss (Jinde et al., 2013). Consistent with previous studies (Zeng et al., 2009; Buckmaster et al., 2009), we observed a significant reduction in mossy fiber sprouting with rapamycin treatment. Rapamycin was ineffective, however, at significantly mitigating any of the other pathological changes. With the exception of mossy fiber sprouting, our findings suggest that it is unlikely that rapamycin targets these common dentate pathologies to produce its positive effects in animal models of epilepsy.

Rapamycin was highly efficient at blocking mTOR pathway signaling

The negative results presented here cannot be explained by a failure of the drug regimen to inhibit the mTOR pathway in the dentate, since staining for pS6 (a kinase that is one of the primary mediators of mTOR pathway signaling) was dramatically reduced in treated animals. Replication of the effect on mossy fiber sprouting provides additional evidence that the drug worked as expected. Unfortunately it was not possible to combine the rapamycin treatment regimen used here with chronic EEG recording, because drug treatment dramatically increased mortality following electrode implantation surgery. We suspect that the poor survival of SE+Rap mice reflects combined effects of seizure-induced stress, and reduced weight gain. The radio telemetry system used for 24/7 EEG monitoring requires implanting a transmitter (1.6 g, 1.1 cc) under the skin of the back. Animals weighing less than 18 g do not tolerate the implant, and animals greater than 20 g do best. In our past studies, both healthy control and epileptic mice weighing >18 g have tolerated the transmitters well (Castro et al., 2012; Pun et al., 2012; Hester and Danzer, 2013; Jiang et al., 2015). The combination of SE and reduced weight gain in SE+Rap mice, however, may have been too great a stressor for successful use of the radio telemetry system. While weight gain is considered to be a comorbidity of epilepsy, it likely enhances the animal's ability to tolerate the transmitters. Investigators conducting similar studies in the future should consider using larger animals, or less invasive recording systems.

Significance of reduced weight gain in epileptic mice treated with rapamycin

Pilocarpine status epilepticus leads to obesity in rodents (Scharfman et al., 2008; Ruiz et al., 2011). Obesity is also a common comorbidity of human epilepsy (Ladino et al., 2014; Janousek et al., 2013). The present findings indicate that the mTOR pathway is critical for SE-induced weight gain in rodents. Rapamycin could act as a non-specific growth retardant (van Vliet et al., 2016), or it could act more specifically to mitigate dysfunction of brain circuits regulating the neuroendocrine system, which are known to be disrupted in epilepsy (for review see Luef and Rauchenzauner, 2009). Alternatively, rapamycin could act directly on adipose tissue by inhibiting mTOR signaling in these cells. mTOR signaling plays a major role in regulating a variety of adipose tissue functions, including adipogenesis (for review see Cai et al., 2015). Notably, the pronounced ability of rapamycin to block weight gain following SE raises the possibility that the drug's anti-epileptogenic effects may be mediated by tissues outside the CNS. Changes in metabolism can alter neuronal excitability, as evidenced by the success of the ketogenic diet in treating epilepsy (Nangia et al., 2012; Neal et al., 2008). Indeed, rapamycin treatment can increase ketone body levels in mice (Fang et al., 2013). The possibility that rapamycin produces some of its positive effects in epilepsy by altering metabolism deserve further investigation.

Rapamycin reduced mossy fiber sprouting, but had no effect on other granule cell pathologies

Mossy fiber sprouting is a common hallmark of temporal lobe epilepsy. This sprouting creates recurrent excitatory circuits in the dentate gyrus, promoting local hyperexcitability (Sutula and Dudek, 2007). Mossy fiber sprouting occurs following gene mutations that enhance mTOR signaling (LaSarge and Danzer, 2014; LaSarge et al., 2015). Inhibiting the

pathway with rapamycin has consistently been demonstrated to block sprouting in acquired epilepsy models (Zeng et al., 2009; Buckmaster et al., 2009). It remains unclear, however, whether preventing mossy fiber sprouting will have any effect on epileptogenesis (Buckmaster, 2014). Numerous lines of evidence suggest that mossy fiber sprouting may not play an important role in epileptogenesis, including work with rapamycin, which has produced a dissociation between mossy fiber sprouting and seizure occurrence (Buckmaster and Lew, 2011; Heng et al., 2013). Why some groups see reductions in seizure frequency following rapamycin treatment and others do not will require additional studies, but differences among animal models, drug dosing regimens or drug metabolism among rodent species/strains could all contribute to the conflicting findings. Regardless, it is clear that mossy fiber sprouting is not necessary for the development of epilepsy, and the ability of rapamycin to block mossy fiber sprouting is unlikely to be responsible for its effects on seizure prevention.

Rapamycin did not reduce hippocampal astrogliosis or microgliosis

Given the absence of rapamycin-effects on granule cell morphology, we queried whether the drug mitigates inflammatory changes in epilepsy (Russo et al., 2013). Inflammatory changes are a prominent feature of the epileptogenic process (Vezzani et al., 2013). Moreover, rapamycin has robust anti-inflammatory properties (Soliman, 2013). It prevents reactive gliosis following a variety of injuries (Carson et al., 2012; Goldshmit et al., 2015; Li et al., 2015), including seizures (Brewster et al., 2013; Nguyen et al., 2015; Shima et al., 2015). Finally, rapamycin has long been used clinically as an immune suppressant following organ transplants (Geissler and Schlitt, 2010).

Status epilepticus leads to reactive astrogliosis and microgliosis (Garzillo and Mello, 2002; Borges et al., 2003). During this inflammatory process, reactive astrocytes and microglia change their morphology from a ramified structure, with many fine processes, to a more amoeboid morphology, with enlarged somas and shorter, thicker processes (Sofroniew, 2009; Lee and MacLean, 2015). Increased phosphorylation of S6 protein is evident in astrocytes in the kainic acid model of epilepsy (Sha et al., 2012; Macias et al., 2013), suggesting that the mTOR pathway might mediate these changes. Nevertheless, despite the compelling evidence linking mTOR signaling to reactive astrogliosis, rapamycin treatment had no effect on reactive astrogliosis or reactive microgliosis in the dentate gyrus. Enlarged astrocyte and microglia somas were evident in both vehicle and rapamycin treated epileptic animals. These findings suggest that in the pilocarpine model of epilepsy, hippocampal astrogliosis and microgliosis is likely a result of other cellular mechanisms and is not mediated by the mTOR signaling pathway. Inflammation may occur through other pathways which were not analyzed in the present study, including cytokine activation. Notably, these findings contrast with recent work by Shima and colleagues (2015), in which they did demonstrate reduced astrogliosis with rapamycin treatment in the intrahippocampal kainic acid mouse model of epilepsy. Much higher doses of rapamycin were used (40 vs. 6 mg/kg/day), however, increasing the likelihood of mTORC 2 inhibition (Sarbanov et al., 2006; Urbanska et al., 2012).

Alternate mechanisms by which rapamycin might mitigate epileptogenesis

In the present study, we tested several mechanisms by which rapamycin might act to mitigate epileptogenesis, including reducing aberrant neurogenesis, blocking hippocampal inflammatory changes and preventing mossy cell loss. Rapamycin had modest effects on mossy fiber sprouting, and was ineffective in preventing changes among the other parameters, strongly suggesting that the beneficial effects of the drug in epilepsy are not mediated by changes in any of these processes.

Notably, the specific pathological changes required to produce epilepsy have yet to be fully defined, so it is a formal possibility that the processes examined here are not integral to epileptogenesis, and the drug acts on brain regions or by mechanisms not yet assessed. In addition to hippocampus, pilocarpine SE causes neuropathological changes in a variety of other brain regions, including olfactory cortex, amygdala, thalamus, neocortex and substantia nigra (Turski et al., 1983). Systemically-administered rapamycin has the potential to impact all these brain regions, and therefore the drug might act anywhere in the CNS. It is also conceivable that rapamycin might act outside the nervous system, with direct effects on the immune system being the most likely target with the potential to modulate seizure incidence (Soliman, 2013; Vezzani and Viviani, 2015).

In addition to the effects on cell morphology and survival examined here, rapamycin might act by a variety of alternate mechanisms. One unlikely scenario, however, is that it acts like an anticonvulsant, akin to currently available anti-epileptic drugs. When evaluated through a battery of acute seizure tests, rapamycin exhibited minimal acute anticonvulsant properties (Chachua et al., 2012; Hartman et al., 2012; Siebel et al., 2015) and may exhibit some pro-convulsant effects (Huang et al., 2012; Macias et al., 2013). This is not surprising, since the majority of current anti-epileptic drugs directly inhibit or activate ion channels (excitatory or inhibitory, respectively) -- mechanisms not attributed to rapamycin (Daoud et al., 2007; Ruegg et al., 2007). The mTOR pathway is, however, a key regulator of synaptogenesis (Li et al., 2010), synaptic strength (Weston et al., 2014) and long-term potentiation (Stoica et al., 2011). The pathway also modulates the expression of a large number of genes, including voltage-gated sodium, potassium and calcium channels (Huang et al., 2013; Jiang et al., 2013; Sosanya et al., 2015). Changes in ion channel expression could significantly impact seizure occurrence (Snowball and Schorge, 2015) and changes in synaptic strength are likely important for epileptogenesis (Casillas-Espinosa et al., 2012). Recent work by Yamawaki and colleagues (2015) demonstrated that rapamycin reduces synapse density in the dentate gyrus following epileptogenesis. Such changes in synaptic strength and number are not detectable with the methodologies used in the current study, but could be fruitful avenues for investigation of mTOR signaling in epilepsy.

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Highlights

- Rapamycin prevents pathological weight gain in epileptic rodents
- Rapamycin reduces mossy fiber sprouting in epilepsy
- Rapamycin does not block ectopic granule cell accumulation or somatic hypertrophy
- Rapamycin does not block hippocampal inflammatory changes
- Rapamycin does not prevent hilar mossy cell death

Figure 1.

Rapamycin decreases pS6 levels in epileptic and non-epileptic mice. Confocal maximum projections of pS6 immunolabeling in the hippocampus of mice receiving the following treatments: vehicle (Veh), vehicle following status epilepticus (SE+Veh), rapamycin (Rap) and rapamycin following status epilepticus (SE+Rap). The graph shows optical density quantification of pS6 intensity in the granule cell layer (left) and cortex (right). Scale bar, 100 μ m.

Figure 2.

Rapamycin blocks weight gain following SE. Bar graphs show animal weights at the beginning of the experiment (top) and prior to sacrifice (middle). The lower graph shows the percent change in body weight for animals in each group. **, $p < 0.01$ vs. all other groups.

***, $p < 0.001$ vs all other groups.

Figure 3.

Rapamycin reduces mossy fiber sprouting. Confocal maximum projections of ZnT3 immunolabeling (a marker of granule cell axon terminals) in the hippocampi of C57BL6 SE +Veh, SE+Rap and control (Veh only) mice. Graphs show quantification of ZnT3-expressing puncta density in the inner molecular layer (IML) of C57BL6 and C57BL6/FVB mice. DGC-L, dentate granule cell layer. *, p<0.05; **, p<0.01; ***, p<0.001. Scale bar = 10 μ m.

Figure 4.

Rapamycin does not prevent SE-induced granule cell enlargement. Confocal maximum projections of GFP-expressing granule cells. Mice that underwent SE and received daily vehicle injections contained granule cells with enlarged somas. Mice receiving rapamycin treatment following SE also possessed large granule cell somas. Non-epileptic mice (control) contained granule cells with normal cell bodies ($\sim 10 \mu\text{m}$ diameter). Micrographs on the right are enlargements of the boxed regions to the left. The graph shows quantification of granule cell soma size. ***, $p < 0.001$. Scale bars: Left panels = $25 \mu\text{m}$; Right panels = $10 \mu\text{m}$.

Figure 5.

Rapamycin has no effect on ectopic granule cell migration. Confocal maximum projections of Prox1 immunolabeling (a mature granule cell marker) in the hippocampus of SE+Veh, SE+Rap and control (Rap only) brains. Right panels are enlargements of the boxed regions on the left. Arrowheads denote hilar ectopic granule cells. The graph shows quantification of ectopic granule cell densities in the hilus. ***, $p < 0.001$. Scale bars: Left panels = 100 μm ; Right panels = 50 μm .

Figure 6.

Rapamycin does not significantly affect basal dendrite formation. Confocal maximum projections of GFP-expressing granule cells. SE+Veh mice contained granule cells that possessed basal dendrites (arrowheads) extending across the granule cell layer-hilar border (white dashed lines). SE+Rap treatment produced a non-significant trend towards reduced basal dendrite frequency. Mice that did not undergo SE rarely contained basal dendrites (control). The graph shows the percentage of GFP-expressing granule cells that contained basal dendrites. *, $p < 0.05$; ***, $p < 0.001$. Scale bar, 20 μm .

Figure 7.

Rapamycin does not reduce reactive astrogliosis. Confocal maximum projections of GFAP immunolabeling of astrocytes in the hippocampus. Mice that underwent SE and received daily vehicle injections contained astrocytes with enlarged somas, indicative of astrocyte activation. Mice receiving rapamycin treatment following SE also possessed activated astrocytes. Non-epileptic mice (control) contained astrocytes with smaller cell bodies that did not display an activated morphology. Lower panels are enlargements of the boxed regions in the top panels. The top graph depicts astrocyte soma area, while the lower graph depicts the percentage of the hilar region occupied by astrocytic somata and processes. Strains were graphed separately in the lower panel to show differences. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Scale bars: Upper panels = 25 μm; Lower panels = 5 μm.

Figure 8.

Rapamycin does not affect reactive microgliosis. Confocal maximum projections of Iba1 immunolabeling of microglia in the hippocampus. SE+Rap and SE+Veh mice exhibited enlarged soma areas relative to non-epileptic (Control) mice, suggestive of increased microglial activation. Lower panels are enlargements of the boxed regions in the top panels. The top graph depicts microglial soma area, while the lower graph depicts the percentage of the hilar region occupied by Iba1 positive microglia. **, $p < 0.01$ vs. control. Scale bars: Upper panels = 25 μm; Lower panels = 10 μm.

Figure 9.

Mossy cell loss is not affected by rapamycin. Confocal maximum projections of GluR2/3 expressing cells. SE leads to hilar mossy cell death, as shown by the lack of large cell bodies within the hilus of SE+Veh mice. Rapamycin treatment did not reduce mossy cell loss (SE+Rap). Mossy cells were prominent in control mice that did not undergo SE (arrowheads). Mossy cells are distinguished from ectopic dentate granule cells (arrows) based on their larger soma size. The graph shows quantification of mossy cell density. Scale bar, 50 μ m.