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Blockade of excitatory synaptogenesis with proximal dendrites of dentate granule cells following rapamycin treatment in a mouse model of temporal lobe epilepsy

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Abstract

Inhibiting the mTOR signaling pathway with rapamycin blocks granule cell axon (mossy fiber) sprouting after epileptogenic injuries, including pilocarpine-induced status epilepticus. However, it remains unclear whether axons from other types of neurons sprout into the inner molecular layer and synapse with granule cell dendrites despite rapamycin treatment. If so, other aberrant positive-feedback networks might develop. To test this possibility stereological electron microscopy was used to estimate numbers of excitatory synapses in the inner molecular layer per hippocampus in pilocarpine-treated control mice, in mice 5 d after pilocarpine-induced status epilepticus, and after status epilepticus and daily treatment beginning 24 h later with rapamycin or vehicle for 2 months. The optical fractionator method was used to estimate numbers of granule cells in Nissl-stained sections so that numbers of excitatory synapses in the inner molecular layer per granule cell could be calculated. Control mice had an average of 2280 asymmetric synapses in the inner molecular layer per granule cell, which was reduced to 63% of controls 5 d after status epilepticus, recovered to 93% of controls in vehicle-treated mice 2 months after status epilepticus, but remained at only 63% of controls in rapamycin-treated mice. These findings reveal that rapamycin prevented excitatory axons from synapsing with proximal dendrites of granule cells and raise questions about the recurrent excitation hypothesis of temporal lobe epilepsy.

Keywords

mossy fiber sprouting; pilocarpine; mossy cell; electron microscopy; stereology; hippocampus

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Conflict of interest statement

The authors have no conflicts of interest.

Role of authors

All authors had full access to all the data in the study and take responsibility for the integrity of the data analysis. Acquisition of data: RY, KT, PSB. Study concept and design, analysis and interpretation of data, drafting of manuscript, statistical analysis, obtained funding, and study supervision: PSB.

Introduction

The recurrent excitation hypothesis of temporal lobe epilepsy proposes that after epileptogenic injuries granule cell axons (mossy fibers) sprout into the inner molecular layer and synapse with the proximal dendrites of neighboring granule cells, establishing an aberrant positive-feedback circuit that generates seizure activity (Tauck and Nadler, 1985). Abundant anatomical (Cronin and Dudek, 1988; de Lanerolle et al., 1989; Sutula et al., 1988, 1989, 1998; Houser et al., 1990; Babb et al., 1991; Isokawa et al., 1993; Mello et al., 1993; Represa et al., 1993; Franck et al., 1995; Okazaki et al., 1995; Buckmaster and Dudek, 1997, 1999; Zhang and Houser, 1999; Wenzel et al., 2000; Nissinen et al., 2001; Buckmaster et al., 2002; Cavazos et al., 2003; Pierce et al., 2005) and electrophysiological data (Cronin et al., 1992; Golarai and Sutula, 1996; Masukawa et al., 1996; Wuarin and Dudek, 1996, 2001; Simmons et al., 1997; Patrylo and Dudek, 1998; Molnár and Nadler, 1999; Okazaki et al., 1999; Lynch and Sutula, 2000; Scharfman et al., 2003) show that sprouted mossy fibers synapse with and excite granule cells. To test whether mossy fiber sprouting is epileptogenic we suppressed it by inhibiting the mTOR (mammalian or mechanistic target of rapamycin) signaling pathway with rapamycin and then measured seizure frequency in a mouse model of temporal lobe epilepsy (Buckmaster and Lew, 2011; Heng et al., 2013). Although mossy fiber sprouting was reduced substantially, seizure frequency was not affected. However, it remains unclear whether axons from other neuronal types sprout into the inner molecular layer and synapse with granule cell dendrites despite rapamycin treatment.

CA3 pyramidal cells and mossy cells could aberrantly develop positive-feedback circuits with granule cells. Normally mossy cells receive convergent synaptic input from nearby granule cells (Scharfman et al., 1990; Sík et al., 2006). In turn, they extend axon projections to the inner molecular layer of the dentate gyrus far along the septotemporal axis of the hippocampus where the vast majority of synapses they form are with spines on proximal dendrites of granule cells (Buckmaster et al., 1996). Mossy cells have been proposed to sprout axons and form new synapses with the proximal dendrites of granule cells as a mechanism of learning and memory (Namgung et al., 1997). After lesions of the entorhinal cortex, mossy cell axons sprout and form new synapses with granule cell dendrites (Del Turco et al., 2003). Many mossy cells die in patients with temporal lobe epilepsy (Margerison and Corsellis, 1966; Babb et al., 1984; Blümcke et al., 1999; Swartz et al., 2006) and in rodent models (Nadler et al., 1980; Cavazos and Sutula, 1990; Buckmaster and Dudek, 1997; Jiao and Nadler, 2007), and mossy cell axon projections in the inner molecular layer degenerate (Sloviter, 1991; Suzuki et al., 1997; Boulland et al., 2007). Nevertheless, some mossy cells survive in patients with temporal lobe epilepsy (Blümcke et al., 2000; Seress et al., 2009) and in rodent models (Buckmaster and Jongen-Rêlo, 1999; Scharfman et al., 2001; Jiao and Nadler, 2007). The irritable mossy cell hypothesis contends that surviving mossy cells amplify hyperexcitable activity patterns of granule cells (Santhakumar et al., 2000; Ratzliff et al., 2002). It has been proposed that surviving mossy cells might serve as a powerful trigger for the seizure-generating dentate gyrus network (Scharfman et al., 2001). Together, these findings suggest that after epileptogenic injuries, surviving mossy cells

might develop into aberrant network hubs, establish an excessive positive-feedback circuit with granule cells, and contribute to the generation of spontaneous seizures.

Other evidence suggests that after epileptogenic injuries surviving CA3 pyramidal cells sprout axon collaterals that synapse with the proximal dendrites of granule cells forming an aberrantly over-developed positive-feedback circuit. Tracer injections reveal enhanced retrograde CA3 pyramidal cell axon projections to the inner molecular layer of the dentate gyrus after kainate-induced status epilepticus in rats (Siddiqui and Joseph, 2005). Similarly, transplanted CA3 pyramidal cells develop retrograde axon projections to the inner molecular layer of the dentate gyrus in kainate-treated rats (Shetty et al., 2005). In hippocampal slices from epileptic pilocarpine-treated rats, excitation of proximal CA3 pyramidal cells (and hilar neurons, which probably include surviving mossy cells) evokes more excitatory monosynaptic input to granule cells compared to controls (Zhang et al., 2012). It is reasonable to hypothesize, therefore, that surviving CA3 pyramidal cells and mossy cells develop aberrant positive-feedback networks with granule cells after epileptogenic injuries. Only the axons of granule cells are darkly labeled by Timm staining (Haug, 1967). Therefore, Timm staining cannot reveal whether rapamycin inhibits the development of other positive-feedback projections to granule cells. The present study used a stereological electron microscopic approach to test whether rapamycin nonspecifically blocks excitatory synaptogenesis with the proximal dendrites of granule cells after an epileptogenic injury.

Materials and Methods

Animals

All animal procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by an institutional animal care and use committee at Stanford University. Male GIN mice (FVB-Tg(GadGFP)4570Swn/J, The Jackson Laboratory, Bar Harbor, ME) at 56 ± 6 d old (mean $\pm$ sem, range, 28–108 d) were treated with pilocarpine. Pilocarpine hydrochloride (300 mg/kg, i.p.) was administered 40 ± 3 min (range, 19–53 min) after atropine methyl bromide (5 mg/kg, i.p.). Diazepam (10 mg/kg, i.p.) was administered 2 h after the onset of motor seizures and repeated as needed to suppress convulsions. Control mice were treated identically but did not develop status epilepticus. The present study did not evaluate naïve controls, but previous studies found no differences between pilocarpine-treated and naïve control groups of rats (Kobayashi and Buckmaster, 2003; Thind et al., 2010; Zhang et al., 2012) or mice (Zhang et al., 2009). After status epilepticus, mice were kept warm and received lactated Ringer's solution with dextrose. Beginning 24 h after status epilepticus, mice in the 2 month survival groups began receiving daily treatment with 3 mg/kg i.p. rapamycin (LC Laboratories, Woburn, MA) in vehicle (5% Tween 80, 5% polyethylene glycol 400, and 4% ethanol) or vehicle alone.

Tissue processing

Mice were killed with urethane (2 gm/kg i.p.) and perfused through the ascending aorta at 15 ml/min for 1 min with 0.9% sodium chloride and 30 min with 2.5% formaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4) at 4°C. Brains postfixed overnight at

4°C. Right hippocampi were isolated, equilibrated in 30% sucrose in PB at 4°C, gently straightened, frozen, and sectioned perpendicular to the septotemporal axis with a sliding microtome set at 40 µm. Beginning at random starting points near the septal pole and extending through the entire septotemporal length, 1-in-12 series of sections were Nissl-stained with 0.25% thionin. Other 1-in-12 series of sections from the same hippocampi were prepared for electron microscopy. Sections postfixed with potassium ferricyanide-reduced 1% OsO₄ in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h, dehydrated in a series of ethanols, were placed in propylene oxide, and then embedded in Epon and propylene oxide mix (1:1 and 3:1) before being gradually transferred to pure Araldite/Eponate-12 (Ted Pella, Redding, CA), and flat-embedded between sheets of ACLAR at 60°C for 24 h. Sample areas on flat-embedded tissue sections were isolated and mounted on blank epoxyresin capsules. Block faces were trimmed and ultrathin sections of silver interference color were cut from re-embedded blocks with an ultramicrotome (Reichert Ultracut S, Leica, Vienna, Austria). Serial sections were collected on coated, nickel single-slot grids. In an attempt to label GABAergic axons, post-embedding GABA-immunocytochemistry was performed on ultrathin sections by blocking nonspecific labeling with 0.8% ovalbumin and 5% fetal calf serum in 0.05 M TBS (pH 7.6) for 1 h followed by incubation overnight in GABA antiserum (Sigma, St. Louis, MO, #A2052, Table 1) in blocking solution. Grids were gently rinsed and then incubated in anti-rabbit colloidal gold (10 nm diameter, 1:80, Ted Pella) in 0.1% Triton X-100 and 0.05 M tris buffer (pH 8.2) for 90 min. However, immunostaining was not sufficiently sensitive or specific to be used for data analysis. Sections were post-stained with 2% aqueous uranyl acetate for 6 min and Sato's lead stain for 4–6 min.

Analysis

The optical fractionator method (West et al., 1991) was used to estimate numbers of Nissl-stained granule cells and hilar neurons. An average of 14 sections/mouse was analyzed. Borders of the granule cell layer and hilus were outlined using a 10X objective, and sample sites were determined randomly and systematically with Stereo Investigator (MBF Bioscience, Williston, VT). Dissector height was total section thickness. Only nuclei that were not cut at the upper surfaces of sections were counted using a 100X objective and Lucivid (MBF Bioscience). Parameters of stereological analysis are listed in Table 2. Coefficients of variation were much larger than mean coefficients of error, suggesting sufficient within-animal sampling.

Established methods were used to estimate numbers of synapses in the inner molecular layer of the dentate gyrus (Thind et al., 2010). To measure areas of the inner molecular layer, embedded sections were analyzed with NeuroLucida (MBF Bioscience), a microscope equipped with a 10X objective, and a Lucivid. Contours were drawn around the granule cell layer and molecular layer. Borders between the inner third and outer two-thirds of the molecular layer were determined by measuring total height of the molecular layer and placing a series of points one-third of the way from the granule cell layer toward the outer border of molecular layer. Six sites per hippocampus were sampled systematically and randomly for electron microscopy. Cumulative length of the granule cell layer was measured and divided into 6 uniform intervals. Placements of first sample sites were randomly located within first intervals, and subsequent samples were at equal intervals. At each sample site,

tissue was trimmed to include the entire height of the molecular layer and granule cell layer. Counting frames were determined within the inner molecular layer by the location of a randomly positioned score mark made by small imperfections in the cutting edges of diamond knives used to make ultrathin sections. Low magnification electron micrographs were used to verify score-mark locations. Counting frames were the area of electron micrographs ($3.69 \times 5.11 \mu\text{m}$) photographed using a transmission electron microscope (JEOL 100CX; JEOL, Peabody, MA) at 19,000X magnification and printed at a final magnification of 49,000X. Counting frames were aligned at one axis by the edge of the tissue block and at the other axis by a score-mark. These fiduciary marks were determined without bias and were independent of tissue-based landmarks. For each hippocampus, the fraction of area sampled was calculated by multiplying the number of sample sites (6) by counting frame area and dividing by the summed area of the inner molecular layer for all sections in the 1-in-12 series. Tissue samples were sectioned completely, and the total number of ultrathin sections was determined, so sampled fractions of tissue thickness could be calculated. Each sample site consisted of 50 serial aligned electron micrographs that represented a $3.69 \times 5.11 \times 2.75 \mu\text{m}$ block of tissue in the inner molecular layer. If 5 or more consecutive sections were unavailable for analysis in a series, an equal number was added to the end of the series to compensate.

Asymmetric synapses were identified by parallel membranes, concentration of presynaptic vesicles, and a thick postsynaptic density (Gray, 1959). All asymmetric synapses that appeared at least partially within the field of view were counted, unless they were evident in the first section of a series or touched the upper or left-hand borders. Postsynaptic structures were identified by ultrastructural characteristics, including microtubules and mitochondria for dendritic shafts, and a spine apparatus and lack of other organelles for dendritic spines (Peters et al., 1991).

Synapse sizes were measured for a randomly sampled subset of synapses whose postsynaptic densities were within or touching a $1 \times 1 \mu\text{m}$ area centered in counting frames of the first 40 electron micrographs of each sample site. Lengths of postsynaptic densities were measured in each section, summed, and multiplied by ultrathin section thickness (55 nm) to yield synapse area.

A total of 12,140 asymmetric synapses (1012 ± 82 per mouse) was counted at 72 sample sites consisting of over 3600 electron micrographs from 12 mice. A subset of 960 synapses was evaluated for size (80 ± 6 per mouse).

Study design and statistics

Investigators were blind to experimental groups during data acquisition and analysis. To reduce confounding effects of variation from sources other than experimental group differences, the following steps were taken. All mice were handled, treated, and perfused, and all hippocampi were dissected and sectioned by one investigator. Generation of ultrathin sections and electron micrographs was divided equally between two investigators. For example, one investigator was responsible for the first, third, and fifth sample point along the septotemporal axis of a hippocampus, and the other investigator was responsible for the

second, fourth, and sixth sample point. Finally, all light and electron microscopic stereological analysis was performed by one investigator.

To test for differences between experimental groups, one way ANOVA was used, which assumes that samples were drawn from normally distributed populations with the same variances (SigmaPlot 12, Systat, San Jose, CA). The Shapiro-Wilk test was used to measure normality. Equal variance was tested by checking the variability about the group means. Differences were considered significant if $p < 0.05$. Results are reported as mean $\pm$ s.e.m.

Images were prepared using Adobe Photoshop (San Jose, CA). Only brightness, contrast, and levels were adjusted.

Results

Hilar neuron loss occurs rapidly after pilocarpine-induced status epilepticus (Wang et al., 2008; do Nascimento et al., 2012), but synaptic reorganization requires weeks to months to fully develop (Mello et al., 1993). Therefore, experimental groups included pilocarpine-treated control mice, mice 5 d after status epilepticus to measure the maximal extent of acute synaptic loss before sufficient time for reactive synaptogenesis, and other mice that were evaluated after status epilepticus and daily treatment beginning 24 h later with 3 mg/kg rapamycin or vehicle for 2 months. Each group consisted of 3 mice.

Nissl-stained sections revealed hilar neuron loss in all mice that experienced status epilepticus but not in pilocarpine-treated controls (Figure 1). After epileptogenic injuries, ectopic granule cells are generated and they migrate into the hilus (Houser, 1990; Parent et al., 1997), which can confound measurement of hilar neuron loss. To reduce the likelihood of including ectopic granule cells, only Nissl-stained profiles $>10 \mu\text{m}$ in diameter in at least one axis of the soma were counted. Differences in mean values of hilar neurons among experimental groups were greater than would be expected by chance ($p < 0.001$, one way ANOVA, Table 3). Control mice had 8190 ± 270 large, hilar neurons per hippocampus, which was more than all other experimental groups ($p < 0.003$, one way ANOVA with the Holm-Sidak method) (Figure 2D1). In mice 5 d after status epilepticus hilar neuron numbers were reduced to 29% of controls, vehicle-treated mice had 48% of controls, and rapamycin-treated mice had 49% of controls. There was no significant difference between groups that had experienced status epilepticus. In mice that had experienced status epilepticus hilar neuron loss was evident at all septotemporal levels except the septal pole (Figure 2D2).

The volume of the inner molecular layer was $0.35 \pm 0.03 \text{ mm}^3$ (mean $\pm$ s.e.m.) in control mice (Figure 2B1). Differences in mean values of inner molecular layer volume among experimental groups were greater than would be expected by chance ($p = 0.022$, one way ANOVA, Table 3). The volume of the inner molecular layer was 106% of controls in mice 5 d after status epilepticus, 114% of controls in rapamycin-treated mice 2 months after status epilepticus, and 144% of controls in vehicle-treated mice 2 months after status epilepticus. The difference between control and vehicle-treated groups was significant ($p = 0.031$, one way ANOVA with Holm-Sidak method). Increased average area of the inner molecular layer in vehicle-treated mice 2 months after status epilepticus was evident at all

septotemporal levels except the septal pole (Figure 2B2). These findings are consistent with previous reports of dentate gyrus hypertrophy in epileptic pilocarpine-treated mice (Zhang et al., 2009) and suppression of hypertrophy by rapamycin (Buckmaster and Lew, 2011; Heng et al., 2013).

Serial electron micrographs (50/site) were evaluated at 6 random sites in the inner molecular layer per hippocampus. The average number of ultrathin sections generated from the entire thickness of a sample site was 497 ± 11 . Therefore, to estimate synapse number, a dissector height sampling adjustment factor of 10 (500/50 ultrathin sections) was used. Assuming ultrathin sections were 55 nm thick, these findings suggest initial sections shrank from 40 μ m to 28 μ m during processing. Tissue shrinkage would not have affected the accuracy of stereological estimates.

Numbers of asymmetric synapses in the inner molecular layer were counted. Asymmetric synapses were recognized by their thicker postsynaptic density compared to symmetric synapses (Figure 3). Asymmetric synapses were abundant in the inner molecular layer of control mice (Figure 4) and in vehicle-treated mice 2 months after status epilepticus. Although less abundant, many excitatory synapses were found in the inner molecular layer in rapamycin-treated mice 2 months after status epilepticus (Figure 5) and in mice 5 d after status epilepticus. Differences in mean values of synapses per sample site among experimental groups were greater than would be expected by chance ($p=0.023$, one way ANOVA, Table 3). Control mice had 240 ± 9 asymmetric synapses per sample site (Figure 2A1). All other groups had fewer. Mice that had experienced status epilepticus and were treated with vehicle had 74% of controls. Those treated with rapamycin had 64% of controls. And mice 5 d after status epilepticus had 59% of controls, which was a significant difference ($p=0.033$, one way ANOVA with Holm-Sidak method). The average number of synapses counted per sample site was roughly similar at different septotemporal levels, and control mice had more than all other groups (Figure 2A2).

Stereological methods were used to estimate the total number of asymmetric synapses in the inner molecular layer of control mice, which was 1.09 ± 0.03 billion (Figure 6A). This value for control mice in the present study is one-third of the estimated number of asymmetric synapses in the inner third of the molecular layer previously reported for control rats (Cardoso et al., 2008). Differences in mean values of synapses among experimental groups were greater than would be expected by chance ($p<0.001$, one way ANOVA, Table 3). In mice 5 d after status epilepticus, average synapse number per hippocampus was reduced to 59% of controls. In mice 2 mo after status epilepticus synapse number recovered to 101% of controls in vehicle-treated mice but was only 70% of controls in rapamycin-treated mice. Average values of control and vehicle-treated groups were similar and significantly greater than those of 5 d and rapamycin-treated groups ($p<0.008$, one way ANOVA with Holm-Sidak method). Average values of 5 d and rapamycin-treated groups were not significantly different from each other. These findings revealed substantial reductions of excitatory synapse numbers in the inner molecular layer 5 d after status epilepticus that recovered by 2 months. That recovery was blocked by rapamycin.

To reveal excitatory synapse numbers on a per-cell basis, Nissl-stained sections from the same mice (Figure 1) were evaluated with stereological methods to estimate numbers of granule cells per hippocampus. Control mice had $484,000 \pm 36,000$ granule cells/hippocampus (Figure 2C1). Average granule cell numbers were 95% of controls in mice 5 d after status epilepticus, 108% in vehicle-treated mice 2 months after status epilepticus, and 109% in rapamycin-treated mice. There were no significant differences in average numbers of granule cells between groups (Table 3). However, trends were similar to results from pilocarpine-treated rats, which show a modest reduction in granule cell numbers 5 d after status epilepticus (Thind et al., 2010). Trends also were similar to previous results from vehicle- and rapamycin-treated mice that display elevated numbers of granule cells 2 months after status epilepticus (Buckmaster and Lew, 2011). In all groups the average number of granule cells per section tended to be highest near the septal end and lowest at the temporal end of the hippocampus (Figure 2C2).

The estimated number of asymmetric synapses in the inner molecular layer for each mouse was divided by each individual's estimated number of granule cells per hippocampus to calculate an average number of excitatory synapses with proximal dendrites per granule cell. Control mice had 2280 ± 230 asymmetric synapses in the inner molecular layer per granule cell (Figure 6B), assuming all counted synapses were with granule cell dendrites, which might be a slight overestimation. Differences in mean values of synapses per granule cell among experimental groups were greater than would be expected by chance ($p=0.013$, one way ANOVA, Table 3). The number of excitatory synapses in the inner molecular layer per granule cell in control mice is slightly less than that of control rats (2730) (Thind et al., 2010). The average number of excitatory synapses in the inner molecular layer per granule cell was reduced to 63% of controls 5 d after status epilepticus ($p<0.05$, one way ANOVA with Holm-Sidak method), recovered to 93% of controls at 2 months in vehicle-treated mice, and was only 63% of controls in rapamycin-treated mice ($p<0.05$ versus controls). These findings revealed that rapamycin blocked development of excitatory synapses in the inner molecular layer after pilocarpine-induced status epilepticus.

To test whether ultrastructural features were affected by status epilepticus and rapamycin-treatment, synapses in the inner molecular layer were evaluated with respect to size, target structure, and whether they were perforated or not. In all groups the vast majority of asymmetric synapses in the inner molecular layer were with dendritic spines (Figures 4 and 5). In pilocarpine-treated control mice, for example, $99.3 \pm 0.4\%$ of all synapses were with dendritic spines, and only 15 ± 7 excitatory synapses in the inner molecular layer per granule cell were with dendritic shafts. Differences in mean values of shaft synapses per granule cells among experimental groups were greater than would be expected by chance ($p=0.031$, one way ANOVA, Table 3). The average number of ax shaft synapses per granule cell was almost 6-times higher (89 ± 24 , $p=0.039$, one way ANOVA with Holm-Sidak method) in mice 5 d after status epilepticus (Figure 7A). An example of an ax shaft synapse in a mouse 5 d after status epilepticus is shown in Figure 3. Similarly, ax shaft synapses with granule cell dendrites are more numerous 5 d after status epilepticus in rats (Thind et al., 2010). Compared to controls there were over twice as many ax shaft synapses per granule cell in mice 2 months after status epilepticus (vehicle-treated, 33 ± 11 ; rapamycin-treated, 33 ± 8), but the differences were not statistically significant.

Nevertheless, these findings are consistent with reports that granule cells have fewer spines on their proximal dendrites after pilocarpine-induced status epilepticus (Santos et al., 2011).

Most asymmetric synapses in the inner molecular layer had a postsynaptic density consisting of a simple, complete disc. In pilocarpine-treated control mice, for example, $96 \pm 1\%$ of asymmetric synapses in the inner molecular layer were simple, complete discs. Some larger synapses, however, had perforated postsynaptic densities. After pilocarpine-induced status epilepticus, granule cell mossy fiber boutons form more perforated synapses in stratum lucidum of CA3 (Upreti et al., 2012), suggesting sprouted mossy fibers in the inner molecular layer might make more perforated synapses. Control mice had 109 ± 36 perforated synapses per granule cell (Figure 7B). The average number of perforated synapses in the inner molecular layer per granule cell was almost twice as high in mice 5 d after status epilepticus (216 ± 67) and higher than controls but to a smaller degree in vehicle-treated (147 ± 3) and rapamycin-treated mice (122 ± 11). However, none of the differences was significant (Table 3). The average size of asymmetric synapses in the inner molecular layer of pilocarpine-treated control mice was $0.032 \pm 0.002 \mu\text{m}^2$ (Figure 7C), which is slightly smaller than asymmetric synapses in the inner molecular layer of control rats ($0.045 \mu\text{m}^2$) (Thind et al., 2010). Differences in mean values of synapse size were not greater than would be expected by chance (Table 3).

Discussion

The major finding of the present study is that rapamycin treatment following an epileptogenic injury prevented excitatory synaptogenesis with the proximal dendrites of granule cells.

Rapamycin nonspecifically blocked epilepsy-related excitatory synaptogenesis

Activation of the mTOR signaling pathway in granule cells results in excitatory synapse formation with granule cell dendrites (including proximal dendrites) and increases the frequency of spontaneous and miniature excitatory postsynaptic currents (Luikart et al., 2011). Mossy fiber sprouting develops in transgenic animals with excessive mTOR activity, even without a preceding epileptogenic injury (Kwon et al., 2006). The mTOR pathway is activated by brain injuries that result in mossy fiber sprouting (Chen et al., 2007; Shacka et al., 2007). Rapamycin inhibits mTOR, suppresses mossy fiber sprouting (Buckmaster et al., 2009; Zeng et al., 2009; Huang et al., 2010; Sunnen et al., 2011; van Vliet et al., 2012; Guo et al., 2013), inhibits axon sprouting from the dentate gyrus in organotypic hippocampal cultures (Berdichevsky et al., 2013), and inhibits excitatory synaptic output of cultured dentate neurons probably by reducing numbers of synapses (Weston et al., 2012). However, it has remained unclear whether rapamycin specifically blocks mossy fiber sprouting but spares other types of reactive synaptogenesis. If so, without competition from sprouting mossy fibers that are inhibited by rapamycin, axons from surviving mossy cells and CA3 pyramidal cells might have been more likely to hyper-innervate granule cell proximal dendrites. If that had occurred, stereological electron microscopic analysis would have revealed recovery of excitatory synapses in inner molecular layer of rapamycin-treated mice 2 months after status epilepticus. That was not the case. Instead, numbers of excitatory

synapses in the inner molecular layer failed to recover in rapamycin-treated mice and remained at the same level as in mice 5 d after status epilepticus. These findings suggest rapamycin nonspecifically blocked all forms of reactive excitatory synaptogenesis in the inner molecular layer after pilocarpine-induced status epilepticus in mice. Other neuronal types also depend on mTOR activation for axon sprouting and synaptogenesis (Campbell and Holt, 2001; Verma et al., 2005; Park et al., 2008; Li et al., 2010; Buckmaster and Wen, 2011). Together these findings suggest rapamycin might nonspecifically block epilepsy-related synaptic reorganization in other brain regions.

How does rapamycin block synaptic reorganization? mTOR is a serine-threonine protein kinase that regulates homeostasis by coordinating nutrient availability, extracellular signals, and cell growth (Swiech et al., 2008; Sengupta et al., 2010). mTORC1 activation promotes protein synthesis, and rapamycin might block synaptogenesis by inhibiting BDNF-induced local protein synthesis in dendrites (Takei et al., 2004). In contrast however, BDNF, together with FGF-2, has been reported to reduce mossy fiber sprouting after pilocarpine-induced status epilepticus (Paradiso et al., 2011). Because rapamycin treatment was prolonged in the present study (2 months), mTORC2 might have been inhibited in addition to mTORC1 (Sarbasov et al., 2006). mTORC2 controls the actin cytoskeleton (Jacinto et al., 2004), which might be involved in axon sprouting. Thus, a number of possibilities exist, and the specific molecular mechanisms underlying rapamycin's effect on epilepsy-related excitatory synaptogenesis remain unclear.

Homeostasis of excitatory synaptic input to granule cells

Numbers of excitatory synapses in the inner molecular layer per hippocampus and per granule cell were substantially reduced 5 d after status epilepticus but recovered to control levels by 2 months in vehicle-treated mice. However, control mice and vehicle-treated mice that had experienced status epilepticus obtained similar numbers of synapses through different mechanisms. Control mice had higher synapse density but lower inner molecular layer volume; whereas, vehicle-treated mice had higher inner molecular layer volume but lower synapse density. The difference cancelled out, yielding similar average synapse numbers in both groups. These findings support the hypothesized existence of a homeostatic mechanism to control numbers of excitatory synapses received by granule cells (Buckmaster, 2014). If such a mechanism exists, reduced synapse numbers in rapamycin-treated mice suggests mTOR signaling might be necessary for excitatory synaptic homeostasis. Consistent with that possibility, mossy fiber sprouting develops after cessation of rapamycin treatment (Lew and Buckmaster, 2011).

Synaptic input to the proximal dendrites of granule cells

Normally, mossy cells are a major source of excitatory synaptic input to the proximal dendrites of granule cells, and when mossy cells are killed, their axons degenerate (see Introduction). Loss of excitatory synapses in the inner molecular layer after pilocarpine-induced status epilepticus was substantial (down to 63% of controls). However, synapse loss in mice was less severe than that found in pilocarpine-treated rats (down to 38% of controls) (Thind et al., 2010). Less severe hilar neuron loss would be expected to result in less severe synapse loss, but the extent of hilar neuron loss in the present study was similar to that of

pilocarpine-treated rats. In rats, some asymmetric synapses in the inner molecular layer are formed by axons projecting from cells in the medial septum (Clark, 1985) and supramammillary nucleus (Maglóczy et al., 1994; Soussi et al., 2010). Mice have similar projections to granule cells (Vivar et al., 2012), and species differences in numbers of subcortical afferents might account for differences in the extent of synapse loss in the inner molecular layer after status epilepticus, but the relative numbers of subcortical afferents versus those from mossy cells in rats versus mice is not known. After epileptogenic treatments, axons from cells in the septum and supramammillary nucleus sprout in the inner molecular layer (Nadler et al., 1980; Soussi et al., 2014). Assuming sprouted axons develop new synapses, results of the present study suggest rapamycin also might have blocked synaptic reorganization by subcortical afferents. The proximal dendrites of granule cells also receive GABAergic input from HICAP cells (Halasy and Somogyi, 1993) and CCK-immunoreactive axons (Hefft and Jonas, 2005). GABAergic axons were not evaluated in the present study. However, axon sprouting by GFP-positive somatostatin-immunoreactive neurons in GIN mice (like the mice used in the present study) is inhibited by rapamycin (Buckmaster and Wen, 2011), suggesting GABAergic synaptogenesis depends on mTOR activation.

Functional implications

The present study confirmed previous reports that rapamycin blocks mossy fiber sprouting (Buckmaster et al., 2009; Zeng et al., 2009; Huang et al., 2010; Sunnen et al., 2011; van Vliet et al., 2012; Guo et al., 2013) and extended those findings by revealing that no other excitatory axons replaced sprouted mossy fibers. These results suggest rapamycin might block all types of epilepsy-related excitatory synaptogenesis with proximal dendrites of granule cells. However, rapamycin does not reduce the development or frequency of spontaneous seizures after pilocarpine-induced status epilepticus in mice (Buckmaster and Lew, 2011), even at high doses (Heng et al., 2013). Together these findings suggest that contrary to recurrent excitation hypothesis, development of aberrant positive-feedback networks with proximal dendrites of granule cells might not be necessary for temporal lobe epileptogenesis, however there are caveats.

Rapamycin suppresses seizure frequency in some other models of temporal lobe epilepsy (Zeng et al., 2009; Huang et al., 2010; van Vliet et al., 2012; Guo et al., 2013) but not all (Sliwa et al., 2012). Possible reasons why rapamycin reduces mossy fiber sprouting and seizures in other models include an acute anti-seizure effect in rats (Huang et al., 2010) and not mice (Hartman et al., 2012), but that seems unlikely because rapamycin is a poor anticonvulsant in rats (Chachua et al., 2012). Another possibility is that other forms of aberrant positive-feedback might develop despite rapamycin treatment. For example, after epileptogenic treatments, granule cells develop basal dendrites that extend far into the hilus and receive mossy fiber input (Ribak et al., 2000; Thind et al., 2008). In addition, ectopic granule cells are generated in rapamycin-treated mice after pilocarpine-induced status epilepticus (Buckmaster and Lew, 2011). However, rapamycin is reported to block the development of all functional, aberrant, recurrent excitation in the dentate gyrus of pilocarpine-treated mice (Tang et al., 2012). Yet another possibility is that in some previous studies rapamycin was administered so soon after an epileptogenic treatment that it might

have had a protective effect, thereby reducing the severity of the initial injury, which could have indirectly reduced epileptogenesis (van Vliet et al., 2012; Guo et al., 2013). Furthermore, rapamycin has many side-effects (Swiech et al., 2008) and some, like inhibition of GABAergic axon sprouting (Buckmaster and Wen, 2011), might be pro-epileptogenic and counteract possible anti-epileptogenic effects on excitatory synaptogenesis.

Mossy fiber sprouting is a common neuropathological feature in patients with temporal lobe epilepsy and in animal models, but its functional consequences are unclear (Buckmaster, 2012). It has been proposed to increase feedback inhibition by exciting basket cells (Sloviter, 1992; Sloviter et al., 2006), but ~95% of synapses formed by sprouted mossy fibers are with granule cells (Buckmaster et al., 2002). Abnormal positive-feedback among granule cells is found in epileptic tissue with mossy fiber sprouting (Wuarin and Dudek, 1996; Molnár and Nadler, 1999; Lynch and Sutula, 2000; Scharfman et al., 2003), but largely remains functionally masked until tissue is bathed in proconvulsive media (Cronin et al., 1992; Gabriel et al., 2004). Mossy fiber sprouting is not necessary for development of epilepsy after hyperactivation of the mTOR pathway in granule cells (Pun et al., 2012). Similarly, rapamycin's inhibitory effect on mossy fiber sprouting does not correlate with inhibition of epileptogenesis after traumatic brain injury in mice (Guo et al., 2013). Together, these findings suggest that if rapamycin has anti-epileptogenic effects in some models of acquired epilepsy, it probably is through mechanisms other than reduced mossy fiber sprouting or any other type of excitatory synaptic reorganization with the proximal dendrites of granule cells.

Caveats

Stereological electron microscopic approaches, like the one used in the present study, provide estimates of the total number of synapses in a neuroanatomically defined region. Such estimates are useful for comparing experimental groups, in part because they avoid confounding effects of changes in tissue volume (Geinisman et al., 1996). However, stereological electron microscopic approaches pose logistical challenges. As in previous studies (Geinisman et al., 2000), six sites per hippocampus were sampled for electron microscopy. Instead of 100 serial ultrathin sections per sample site used in our previous study that focused on GABAergic synapses, in the present study only 50 serial sections per site were sampled, because glutamatergic synapses are more abundant. Nevertheless, the total number of electron micrographs generated and analyzed was substantial and limiting, and only three mice each were evaluated to compare the four experimental groups. Steps were taken to minimize variation other than that from group effects (see Materials and Methods), and tests of equal variance and normality were applied when groups were compared by ANOVA. However, although the Shapiro-Wilk test used is the most powerful normality test, its power is low for small sample sizes (Razali and Wah, 2011). Even doubling the sample sizes in the present study would not solve this problem. Therefore, the normality assumption of ANOVA may have been violated, and results should be interpreted with caution. Despite these caveats, the descriptive statistics of inner molecular layer synapses per hippocampus and per granule cell shown in Figure 6 suggest many excitatory

synapses were lost 5 d after status epilepticus and recovered to control values by 2 months, but recovery was blocked by rapamycin.

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Figure 1.

Nissl stained dentate gyrus of a pilocarpine-treated control mouse (**A**), a mouse 5 d after status epilepticus (**B**), a mouse that was treated with vehicle for 2 months after status epilepticus (**C**), and a mouse that was treated with rapamycin for 2 months after status epilepticus (**D**). m=molecular layer, g=granule cell layer, h=hilus, CA3=CA3 pyramidal cell layer. Scale bar (in **D**)=250 μ m.

Figure 2.

Group averages and septotemporal distributions of synapses, inner molecular layer volume and area, number of granule cells, and number of hilar neurons in pilocarpine-treated control mice, mice 5 d after status epilepticus, and mice 2 months after status epilepticus and daily treatment with vehicle or rapamycin. **A1** Number of asymmetric synapses counted per sample site. Values represent mean $\pm$ s.e.m. Average number of synapses was larger in control mice compared to mice 5 d after status epilepticus ($p=0.033$, one way ANOVA with Holm-Sidak method). **A2** Septotemporal distribution. **B1** Volume of the inner molecular per

hippocampus. Average volume of the inner molecular layer was larger in vehicle-treated mice compared to controls ($p=0.031$). **B2** Inner molecular layer area per section. **C1** Number of Nissl-stained granule cells per hippocampus. No significant differences between groups. **C2** Number of granule cells per section. **D1** Number of Nissl-stained large ($>10\text{ }\mu\text{m}$ soma diameter) hilar neurons per hippocampus. Control mice had more hilar neurons than all other groups ($p<0.003$). **D2** Large hilar neurons per section.

Figure 3.
An asymmetric (arrow) and symmetric synapse (arrowhead) with a dendritic shaft in the inner molecular layer of the dentate gyrus in a mouse 5 d after status epilepticus. Scale bar=0.25 μ m.

Figure 4.

Asymmetric synapses in serial electron micrographs of the inner third of the dentate gyrus molecular layer in a pilocarpine-treated control mouse. An axospinous synapse (arrow) with a spine neck connected to a dendritic shaft in the plane of section. Other axospinous synapses are indicated by arrowheads. Scale bar (in **E**)=0.5 μ m.

Figure 5. Asymmetric synapses in serial electron micrographs of the inner third of the dentate gyrus molecular layer in a mouse that experienced pilocarpine-induced status epilepticus and then was treated for 2 months with 3 mg/kg/d rapamycin. An axospinous synapse (arrow) with a spine neck connected to a dendritic shaft in the plane of section. Other axospinous synapses are indicated by arrowheads. Scale bar (in **E**)=0.5 μ m.

Figure 6.

Number of asymmetric synapses in the inner molecular layer of pilocarpine-treated control mice, mice 5 d after pilocarpine-induced status epilepticus, and mice 2 months after status epilepticus and daily treatment with vehicle or rapamycin. Values represent mean $\pm$ s.e.m. **A** Average numbers of asymmetric synapses in the inner molecular per mouse of control group and vehicle-treated mice 2 months after status epilepticus were larger than those of 5 d and rapamycin-treated groups ($p < 0.008$, one way ANOVA with Holm-Sidak method). **B** The

average number of asymmetric synapses per granule cell was larger in the control group than in the 5 d and rapamycin-treated group ($p < 0.05$).

Figure 7.

Ultrastructural characteristics of asymmetric synapses in the inner molecular layer in pilocarpine-treated control mice, mice 5 d after status epilepticus, and mice that experienced status epilepticus and then were treated with vehicle or rapamycin for 2 months. **A** Number of shaft synapses per granule cell. Values represent mean $\pm$ s.e.m. The average value for mice 5 d after status epilepticus is larger than that of controls ($p=0.039$, ANOVA with Holm-Sidak

method). **B** Number of perforated synapses per granule cell. No significant differences. **C** Synapse size. No significant differences.

Table 1

Primary antibody used.

<u>Antigen</u>	<u>Immunogen</u>	<u>Manufacturer</u>	<u>Dilution</u>
GABA	GABA conjugated to bovine serum albumin	Sigma (St. Louis, MO), rabbit polyclonal, #A2052	1:70

Table 2

Parameters of light microscopic stereological analysis.

	<u>hilar neurons</u> [*]	<u>granule cells</u>
counting grid	75 × 75 μm	175 × 175 μm
counting frame	50 × 50 μm	10 × 10 μm
cells counted / mouse ^{**}	170 ± 25	131 ± 9
coefficient of variation	0.51	0.23
mean coefficient of error ^{***}	0.08	0.05

* somata >10 μm diameter

** mean ± s.e.m.

*** calculated according to West et al. (1991)

Table 3
Results of normality tests (Shapiro-Wilk), equal variance tests, one way ANOVA between groups, and power analysis ($\alpha=0.05$).

Parameter	Normality test (p [*])	Equal variance test (p [*])	ANOVA (F)	ANOVA (p)	power
hilar neurons per hippocampus ^{**}	0.956	0.488	25.176	<0.001	1.000
inner molecular layer (mm ³)	0.995	0.440	5.660	0.022	0.688
synapses per sample site ^{***}	0.991	0.314	5.579	0.023	0.680
synapses per hippocampus ^{***}	0.858	0.492	20.738	<0.001	1.000
granule cells per hippocampus	0.324	0.390	0.841	0.509	0.050
synapses per granule cell ^{***}	0.464	0.697	6.955	0.013	0.801
shaft synapses per granule cell	0.416	0.562	5.001	0.031	0.615
perforated synapses per granule cell	0.286	0.158	1.521	0.282	0.115
synapse size (μm ²)	0.408	0.869	1.335	0.329	0.090

* p values represent the probability of being incorrect that the data are not normally distributed or do not have equal variance

** somata >10 μm diameter

*** asymmetric synapses in the inner molecular layer of the dentate gyrus