RESEARCH ARTICLE

# Rapamycin prevents acute dendritic injury following seizures

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**Abstract**

**Objective**: Seizures cause acute structural changes in dendrites, which may contribute to cognitive deficits that occur in epilepsy patients. Disruption of the actin cytoskeleton of dendrites likely mediates the structural changes following seizures, but the upstream signaling mechanisms activated by synchronized physiological activity to cause seizure-induced dendritic injury are not known. In this study, we test the hypothesis that the mechanistic target of rapamycin complex 1 (mTORC1) pathway triggers structural changes in dendrites in response to seizures. **Methods**: In vivo multiphoton imaging was performed in transgenic mice expressing green fluorescent protein in cortical neurons. The effect of rapamycin pre- and posttreatment was tested on kainate-induced dendritic injury and cofilin-mediated actin depolymerization. **Results**: Kainate-induced seizures caused acute activation of mTORC1 activity, which was prevented by the mTORC1 inhibitor, rapamycin. Rapamycin pretreatment, and to a lesser degree, posttreatment attenuated acute seizure-induced dendritic injury and correspondingly decreased LIM kinase and cofilin-mediated depolymerization of actin. **Interpretation**: The mTORC1 pathway mediates seizure-induced dendritic injury via depolymerization of actin. These findings have important mechanistic and translational applications for management of seizure-induced brain injury.

## Introduction

Seizures can directly lead to brain injury, which may contribute to cognitive deficits that occur in patients with epilepsy. Although seizures cause neuronal death under certain conditions, seizures may also disrupt normal physiological mechanisms specifically involved in learning. Structural changes in dendrites represent a potential mechanism of synaptic plasticity and learning. New dendritic spine formation or other dendritic changes occurs with long-term potentiation (LTP) and behavioral learning.[1–3] Changes in spine morphology during LTP result from modulation of the filamentous actin cytoskeleton of dendrites.[4–7] Further-

more, actin polymerization can be regulated by the mechanistic/mammalian target of rapamycin (mTOR) signaling pathway, which is also necessary for LTP.[8–10]

Morphological changes in dendrites also occur under pathological conditions and in a variety of neurological disorders involving cognitive deficits.[11,12] In particular, seizures may have very acute effects on dendrites as detected by in vivo time-lapse imaging, including beading of dendrites and a loss of spines.[13–15] This acute dendritic injury may evolve over hours to days, with partial recovery of spines, but some spine loss persists for at least several weeks and may account for chronic cognitive deficits observed in epilepsy patients. Breakdown of the actin

cytoskeleton has been implicated in mediating seizure-induced dendritic injury,[15,16] but the upstream signaling mechanisms triggering actin depolymerization are unknown. In this study, we tested the hypothesis that mTOR pathway mediates acute seizure-induced dendritic injury and that the mTOR inhibitor, rapamycin, prevents this injury.

## Materials and Methods

### Animals

Two-to-three-month-old transgenic mice with a C57BL/6 background expressing enhanced green fluorescent protein (GFP) under a thy1 promoter (line GFP-M)[17] were used for all in vivo imaging experiments. In neocortex, the GFP-M mice exhibit expression of GFP in a subpopulation of pyramidal neurons, primarily in cortical layer 5 and, to a lesser extent, layer 2/3. The C57BL/6 genetic background was advantageous for these studies, as this strain of mice is resistant to kainate-induced neuronal death and epileptogenesis,[15,18,19] which otherwise could represent confounding factors in interpretation of our experiments. Two-to-three-month-old C57BL/6 wild-type mice were used for separate experiments for western blot analysis. Care and use of animals conformed to a protocol approved by the Washington University School of Medicine Animal Studies Committee.

### Surgery

For imaging and EEG recording, animal surgery was performed by similar methods as previously reported.[13,15] Briefly, mice were anesthetized with isoflurane anesthesia and held in a custom-made stereotaxic device, which could be mounted to the microscope stage. A heating pad was used to maintain body temperature while under anesthesia. A round cranial window (~2.5 mm in diameter) was first drilled in the skull with the center of the window ~3 mm posterior to bregma and 2 mm lateral to midline. Three screw electrodes were placed adjacent to the cranial window to record electroencephalography (EEG). A glass coverslip (#1.5, 8 mm) was centered over the cranial window and attached to the skull with dental acrylic, which also stabilized the EEG electrodes.

### Seizure induction and electroencephalogram recording

After obtaining baseline images, the mice were allowed to recover from anesthesia, and video and EEG data were acquired simultaneously. EEG signals were amplified and
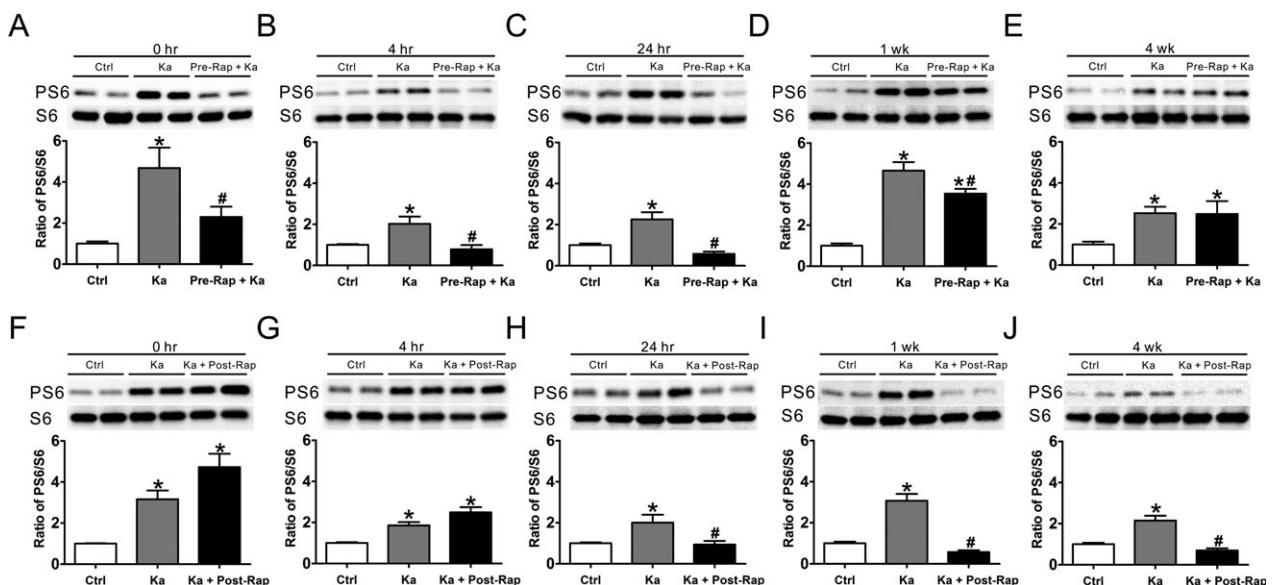


**Figure 1.** Rapamycin inhibits mTOR activation from kainate-induced seizures at different time points. (A–E) Kainate-induced seizures cause mTOR activation in neocortex, as reflected by increased PS6/S6 expression, immediately and for up to 4 weeks following the seizures. Rapamycin pretreatment (48 and 24 h prior to kainate seizures) inhibits the mTOR activation at 0 h, 4 h, 24 h, and 1 week, but not 4 weeks, after kainate seizures. (F–J) Rapamycin posttreatment (daily, starting at 0 h immediately after kainate seizures) has no effect on the elevated mTOR activation immediately after the kainate seizures, but inhibits mTOR activity at 24 h, 1 week and 4 weeks following kainate seizures. *$P < 0.05$ versus control mice, #$P < 0.05$ versus kainate-injected mice; $n = 6$ mice for each time point and group. mTOR, mechanistic/mammalian target of rapamycin.
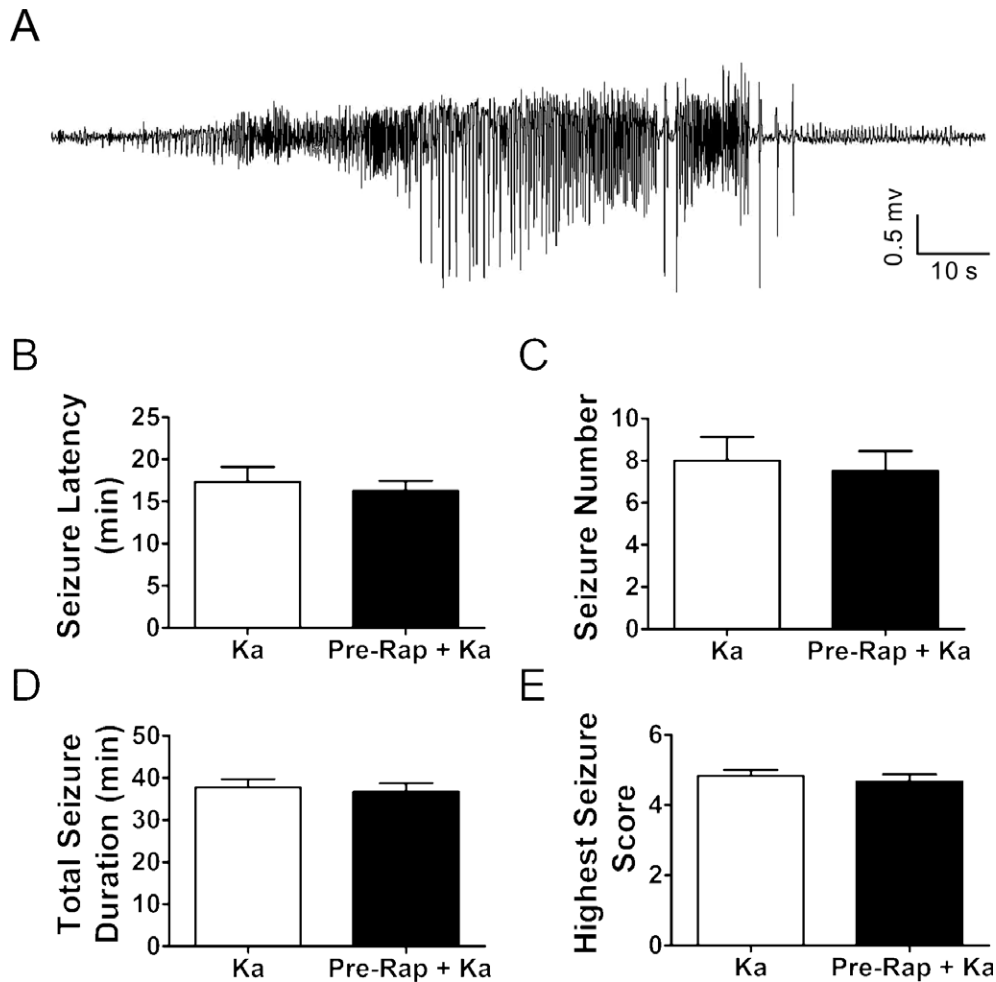
**Figure 2.** Rapamycin pretreatment has no effect on acute kainate-induced seizures. (A) Representative electrographic seizure following kainate injection. (B–E) Rapamycin pretreatment (6 mg/kg, i.p., 48 and 24 h prior to kainate) has no effect on the properties of seizure latency, duration, number, and severity during the acute episode of kainate-induced status epilepticus (defined as >30 min of cumulative seizures).

filtered (1–100 Hz) using Powerlab PL3508 amplifiers (AD Instruments, Colorado Springs, CO) and digitized (200 Hz) with LabChart (AD Instruments, Colorado Springs, CO). To induce seizures, mice were then injected with kainate (Ka, Sigma, St. Louis, MO) (20 mg/kg, i.p.). Control mice received saline injection instead of kainate. Electrographic seizures were recorded by EEG and the cumulative duration of individual seizures was monitored. An individual seizure was defined as a discrete epoch of repetitive spikes or spike-and-wave discharges lasting at least 10 sec (Fig. 2A). Seizure latency, seizure number, and total seizure duration during the acute episode of status epilepticus (defined as >30 min of cumulative seizures) were calculated and analyzed. The behavioral correlate of seizures was noted using a modified Racine scale[13]: stage 1 – behavioral arrest with mouth/facial movements, stage 2 – head nodding, stage 3 – forelimb clonus, stage 4 – rearing, stage 5 – rearing and falling, stage 6 – loss of posture, and generalized convulsive activity. After a cumulative 30–45 min duration of electrographic seizures, seizures were terminated by isoflurane anesthesia induction for subsequent postseizure imaging at 0 and 4 h. The mice were then housed and followed with postseizure time-lapse imaging for 6 weeks.

## Rapamycin treatment

Rapamycin (LC Labs, Woburn, MA, USA) was initially dissolved in 100% ethanol (30 mg/mL), stored at −20°C, and diluted (1:10) in a vehicle solution containing 5% Tween 80, 5% PEG 400 (low-molecular-weight grade of polyethylene glycol), and 4% ethanol immediately before injection. All other chemicals were obtained from Sigma unless indicated otherwise. Two different rapamycin
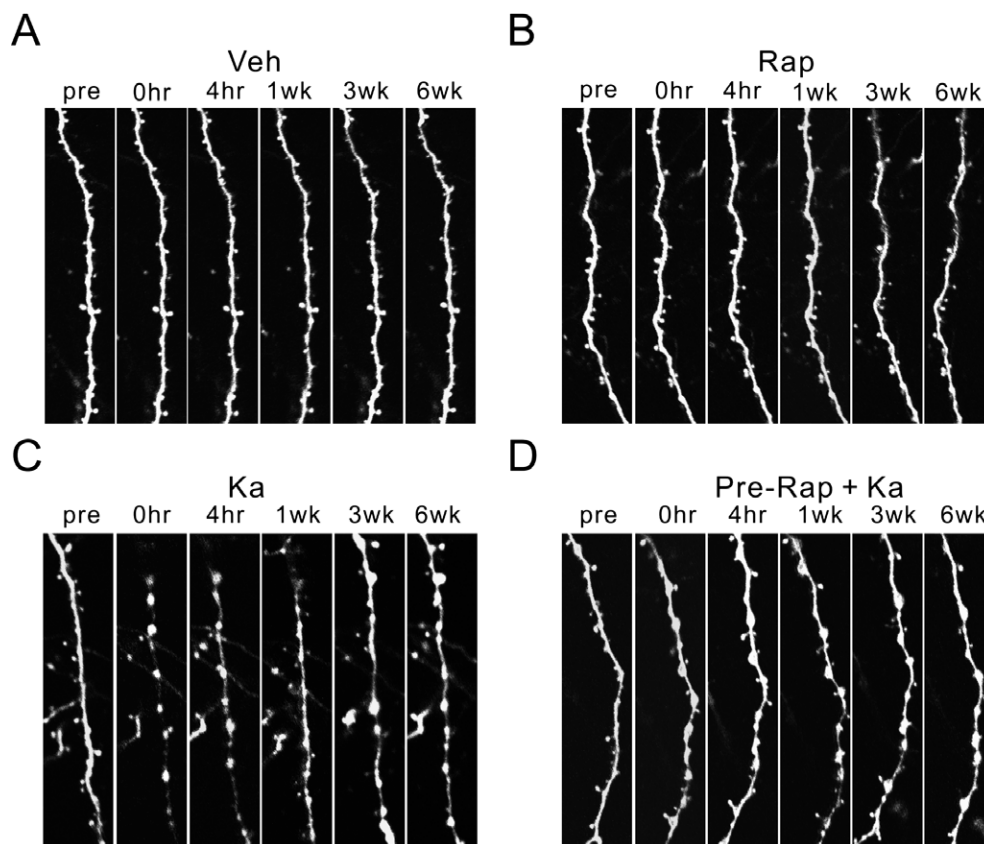
**Figure 3.** Representative images of the effect of rapamycin pretreatment of kainate seizure-induced dendritic injury. (A and B) There are no obvious changes in dendrites or spines in vehicle-treated (Veh) or rapamycin-treated (Rap) control mice. (C) Kainate-induced seizures cause severe dendritic beading with extensive spine loss which partially recovers over 6 weeks (Ka). (D) In contrast there is minor dendritic beading with less severe spine loss in mice receiving rapamycin pretreatment at 0 h which persists through 6 weeks without further recovery (Rap + Ka).

treatment paradigms were performed in this study, based on previous studies examining inhibitory effects of rapamycin on kainate seizure-induced mTOR activation. For a pretreatment study, vehicle or rapamycin (6 mg/kg, i.p.) was administered 48 and 24 h before Ka injection (Ka or Pre-Rap + Ka groups). For a posttreatment study, vehicle or rapamycin (6 mg/kg, i.p.) was administrated daily for up to 6 weeks, starting immediately after Ka-induced status epilepticus was terminated (Ka or Ka + Post-Rap groups) and mice were followed up for imaging or harvested for western blotting at various times points (0 h defined as immediately after seizure termination and rapamycin administration). Both studies also included two control groups of mice without Ka-induced seizures injected with vehicle (Veh) or rapamycin (Rap). Each group included at least six mice.

## Multiphoton imaging

Baseline images of dendrites and dendritic spines of neocortical neurons expressing GFP were obtained through the cranial window with a multiphoton microscope (LSM 510; Zeiss, Thornwood, NY) and a water immersion objective (Zeiss, 40×, 0.8 numerical aperture, IR-adjusted, Zeiss). A Titanium–Sapphire pulsed infrared laser (Coherent, Santa Clara, CA) was used to stimulate GFP at 900 nm. Low-magnification images ~50–100 $\mu$m below the neocortical surface were first obtained to identify regions with GFP-expressing dendrites. At higher magnification (3× digital zoom; 280 × 280 $\mu$m), z-stacks of 6 to 10 images separated by 1 $\mu$m steps were taken of dendrites and accompanying spines. Individual images were acquired at 12 bits with frame averaging (2–4 times). Following seizures, surface blood vessels were used to identify the same dendrites for post-seizure time-lapse imaging at various times (0 h, 4 h, 24 h, 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, and 6 weeks).

## Post hoc image analysis

Post hoc image analysis was performed using LSM 5 Image Examiner software (Zeiss) in a blinded fashion to evaluate changes in dendrites over time, as described
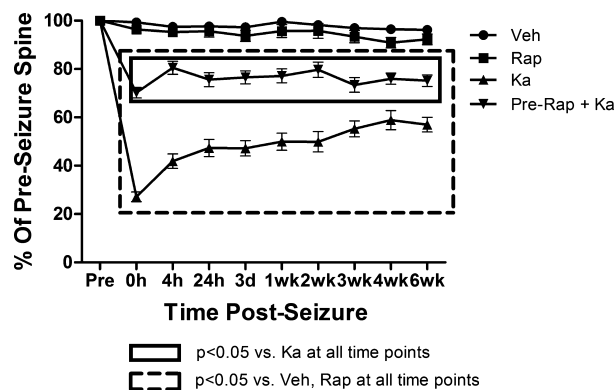
**Figure 4.** Quantitative analysis of effect of rapamycin pretreatment on kainate seizure-induced dendritic spine loss. Control mice (Veh, Rap) had no significant change in spine number over 6 weeks. In contrast, kainate-induced seizures led to an acute spine loss immediately after termination of the seizures (0 h), that only partially recovered over 6 weeks of observation. Rapamycin pretreatment had partial protective effects at 0 h, which persisted through 6 weeks without further recovery. $n = 21$–24 dendrites and $n = 6$–7 animals per group. Statistical comparisons by two-way repeated measures analysis of variance.

**Table 1.** Effect of rapamycin pretreatment on kainate seizure-induced dendritic beading.

| Group/time after seizures | Total dendrites | No beading | Mild beading | Severe beading |
|---|---|---|---|---|
| **Veh** | | | | |
| Preseizures | 24 | 24 (100%) | 0 (0%) | 0 (0%) |
| 0 h | 24 | 24 (100%) | 0 (0%) | 0 (0%) |
| 4 h | 24 | 24 (100%) | 0 (0%) | 0 (0%) |
| 24 h | 24 | 24 (100%) | 0 (0%) | 0 (0%) |
| 1 week | 24 | 24 (100%) | 0 (0%) | 0 (0%) |
| 4 weeks | 24 | 24 (100%) | 0 (0%) | 0 (0%) |
| 6 weeks | 24 | 24 (100%) | 0 (0%) | 0 (0%) |
| **Rap** | | | | |
| Preseizures | 23 | 23 (100%) | 0 (0%) | 0 (0%) |
| 0 h | 23 | 23 (100%) | 0 (0%) | 0 (0%) |
| 4 h | 23 | 23 (100%) | 0 (0%) | 0 (0%) |
| 24 h | 23 | 23 (100%) | 0 (0%) | 0 (0%) |
| 1 week | 23 | 23 (100%) | 0 (0%) | 0 (0%) |
| 4 weeks | 23 | 23 (100%) | 0 (0%) | 0 (0%) |
| 6 weeks | 23 | 23 (100%) | 0 (0%) | 0 (0%) |
| **Ka** | | | | |
| Preseizures | 21 | 21 (100%) | 0 (0%) | 0 (0%) |
| 0 h | 21 | 0 (0%) | 4 (19%) | 17 (81%) |
| 4 h | 21 | 0 (0%) | 13 (62%) | 8 (38%) |
| 24 h | 21 | 0 (0%) | 13 (62%) | 8 (38%) |
| 1 week | 21 | 1 (5%) | 18 (85%) | 2 (10%) |
| 4 weeks | 21 | 3 (14%) | 17 (81%) | 1 (5%) |
| 6 weeks | 21 | 5 (24%) | 16 (76%) | 0 (0%) |
| **Pre-Rap + Ka** | | | | |
| Preseizures | 23 | 23 (100%) | 0 (0%) | 0 (0%) |
| 0 h[1] | 23 | 2 (9%) | 17 (74%) | 4 (17%) |
| 4 h | 23 | 2 (9%) | 18 (78%) | 3 (13%) |
| 24 h | 23 | 2 (9%) | 18 (78%) | 3 (13%) |
| 1 week | 23 | 3 (13%) | 20 (87%) | 0 (0%) |
| 4 weeks | 23 | 6 (26%) | 17 (74%) | 0 (0%) |
| 6 weeks | 23 | 6 (26%) | 17 (74%) | 0 (0%) |

[1]$P < 0.05$ by chi-square test of independence for distribution of beading categories compared to Ka at 0 h. $n = 6$–7 mice per group.

previously.[13,15] Briefly, spines were defined as perpendicular projections out of the main axis of the dendrite that were narrower than the dendrite from which they arose and could progressively taper, maintain their width, or form "caps." The numbers of spines at different time points after the seizures were normalized to those at baseline before the seizures in each group. In addition to spine counting, a qualitative scoring system was also used to grade the degree of beading that frequently occurred after seizures: no beading; mild beading (visible beads with diameter of beads <3 times the diameter of the original dendrite with normal intervening segments of dendrite); severe beading (visible beading with diameter of beads >3 times the diameter of the original dendrite without normal intervening segments of dendrite) (see Fig. S1 for examples). Both summed projections of z-stacks and individual images (typically 6–10) of the z-stack were analyzed, allowing more accurate identification of spines, mild beading, and artifacts (such as turns in dendrites, mimicking beading). Mice that developed obvious technical complications (e.g., bleeding or severe clouding over of the cranial window) at any point during the period were excluded from analysis. All remaining mice included for analysis were successfully followed up for the 6 week duration without any loss or "drop-out" of imaged dendrites.

## Western blotting

Western blot analysis of phospho-S6, total S6, P-LIM kinase, LIM kinase, P-cofilin, cofilin, actin, P-Akt, Akt, and P-PKCα was performed as described previously (Zeng et al.[15]). Briefly, brains were removed from C57BL/6 wild-type mice at various times following saline or kainate (20 mg/kg, i.p.) injection. After 30–45 min Ka-induced status epilepticus, the seizures were stopped by diazepam (Sigma, St. Louis, MO) (10 mg/kg, i.m.). The neocortex was dissected out and sonicated individually in lysis buffer. Protein extracts from neocortex were homogenized in sodiumdodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 3% SDS, 2% L-mercaptoethanol, and 5% glycerol in 60 mmol/L Tris buffer, pH 6.7. Samples were boiled for 5 min and stored at −20°C. Protein concentration was determined with the Lowry method. Thirty micrograms of protein were separated by 15% SDS-PAGE and transferred to polyvinylidene difluoride membranes. After
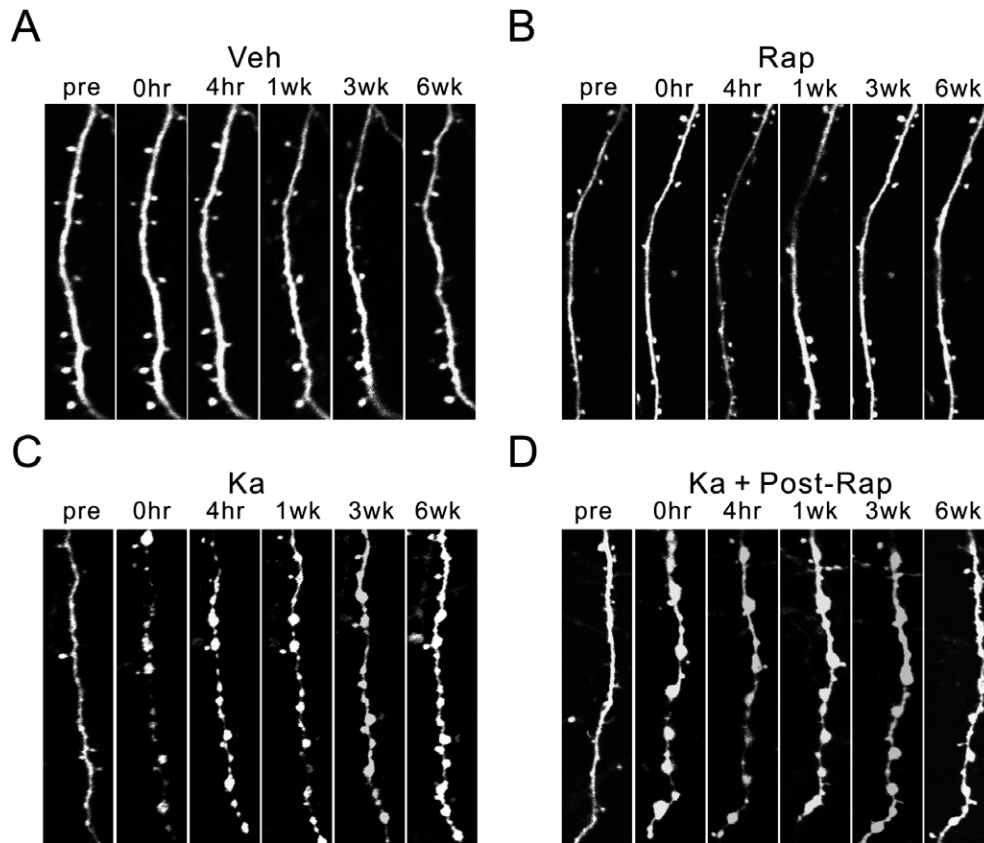
**Figure 5.** Representative images of the effect of rapamycin posttreatment of kainate seizure-induced dendritic injury. (A and B) There are no obvious changes in dendrites or spines in vehicle-treated (Veh) or rapamycin-treated (Rap) control mice. (C) Kainate-induced seizures cause severe dendritic beading with extensive spine loss which partially recovers over 6 weeks (Ka). (D) Rapamycin posttreatment showed mild protection only at 6 weeks against Ka-induced dendrite injury.

blocking with 5% skim milk, the membranes were incubated with rabbit anti-P-S6 antibody (Ser 240/244; 1:1000; Cell Signaling Technology, Danvers, MA, USA), P-cofilin (Ser3; 1:1000; Cell Signaling Technology) or P-LIMK1 (Thr508)/LIMK2 (Thr505) (1:1000; Cell Signaling Technology), P-Akt (S473) (1:1000; Cell Signaling Technology), P-PKCα (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA) followed by peroxidase-conjugated secondary antibody, and visualized with ECL reagent (Pierce/Thermo Fisher Scientific, Waltham, MA, USA). In some cases, the membranes were reprobed and incubated with the rabbit antibody for S6 (1:1000; Cell Signaling Technology), cofilin (1:1000), LIMK1 (1:1000), Akt (1:1000) $\beta$-actin (1:4000), or glyceraldehyde-3-phosphate dehydrogenase (GADPH) (1:1000). Signals were quantitatively analyzed using NIH ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Measurements of the F-actin to G-actin ratio were also made by western blotting, as previously described.[15] Cortex was isolated and homogenized in cold lysis buffer (10 mmol/L $K_2HPO_4$, 100 mmol/L NaF, 50 mmol/L KCl, 2 mmol/L $MgCl_2$, 1 mmol/L ethylene glycol tetraacetic acid (EGTA), 0.2 mmol/L dithiothreitol, 0.5% Triton X-100, 1 mol/L sucrose, pH 7.0) and then centrifuged at 15,000$g$ for 30 min. The supernatant was used for measurement of soluble actin (G-actin). To measure F-actin, the pellets were resuspended in lysis buffer plus an equal volume of 1.5 mol/L guanidine hydrochloride, 1 mol/L sodium acetate, 1 mmol/L $CaCl_2$, 1 mmol/L ATP, and 20 mmol/L Tris–HCl (pH 7.5) and incubated on ice for 1 h to depolymerize F-actin, with gentle mixing every 15 min. The samples were centrifuged at 15,000$g$ for 30 min and this supernatant was also used to measure actin (as a reflection of insoluble F-actin). Samples from the supernatant (G-actin) and pellet (F-actin) fractions were proportionally loaded and analyzed by western blotting.

## Statistics

Repeated measures two-way analysis of variance (ANOVA) with Bonferroni posttests for multiple comparisons

was used to compare changes in dendritic spine number and protein expression between different groups. One-way ANOVA with Tukey's multiple comparison test was used to compare PS6/S6 ratio among three groups. Student's *t*-test was used to compare seizure parameters between the KA and Pre-Rap + KA groups. Chi-square test of independence was used to compare the distribution of dendritic beading severity between different groups. All data are expressed as mean ± SEM. Statistical significance was defined as $P < 0.05$.

## Results

### Rapamycin pretreatment significantly attenuates acute seizure-induced dendritic injury

Previous studies with in vivo imaging show that kainate seizures cause an immediate beading of dendrites and loss of dendritic spines.[15] Kainate seizures also cause activation of the mTOR pathway, and the mTOR inhibitor, rapamycin, administered prior to kainate, prevents this mTOR activation and reduces mossy fiber sprouting and subsequent development of seizures in rats,[20] but the effect of mTOR inhibition on dendritic morphology was not examined. As our previous studies of rapamycin in the kainate model were performed in rats, we first examined mTOR activation and the effects of rapamycin in the kainate model in mice. mTOR activity, as reflected by increased phospho-S6 expression, was increased immediately following status epilepticus and persisted for a few weeks. Rapamycin pretreatment at 48 and 24 h prior to injection of kainate inhibited this mTOR activation at 0 h, 4 h, 24 h, and 1 week, but not at 4 weeks, following kainate seizures (Fig. 1A–E). In evaluating the possibility that rapamycin pretreatment affected the duration or severity of status epilepticus, there was no difference in seizure latency, duration, number, and severity between mice with vehicle and rapamycin pretreatment prior to kainate (Fig. 2B–E). This indicates that rapamycin directly inhibited kainate seizure-induced mTOR activation without affecting the properties of the kainate seizures themselves.

To test whether rapamycin prevents seizure-induced dendritic injury, rapamycin or vehicle was administered 48 and 24 h prior to kainate. There was minimal change in gross dendritic morphology and spine number in vehicle or rapamycin-injected control mice over 6 weeks (Fig. 3A and B). Consistent with our previous work, kainate seizures for 30 min caused acute dendritic beading and loss of spines immediately after the seizures were terminated (Fig. 3C). The spines partially recovered, but ~50% loss of spines persisted for at least 6 weeks. Rapamycin pretreatment significantly reduced

dendritic spine loss immediately after the seizure, which lasted up to 6 weeks (Fig. 3D). Quantitative analysis of both spine number (Fig. 4) and dendritic beading (Table 1) found a significant protective effect of rapamycin pretreatment.

### Rapamycin posttreatment only has mild late protection against seizure-induced dendrite injury

We next tested whether rapamycin treatment initiated immediately after status epilepticus would also rescue or reverse seizure-induced dendritic injury. First, rapamycin posttreatment had no significant effect on the elevated mTOR activation immediately after the kainate seizures, but inhibited mTOR activity at 24 h, 1 week, and 4 weeks following kainate seizures (Fig. 1F–J). As the dendritic injury is already apparent immediately after termination

**Table 2.** Effect of rapamycin posttreatment on kainate seizure-induced dendritic beading.

| Group/time after seizures | Total dendrites | No beading | Mild beading | Severe beading |
|---|---|---|---|---|
| Veh | | | | |
| Preseizures | 37 | 37 (100%) | 0 (0%) | 0 (0%) |
| 0 h | 37 | 37 (100%) | 0 (0%) | 0 (0%) |
| 4 h | 37 | 36 (97%) | 1 (3%) | 0 (0%) |
| 24 h | 37 | 35 (95%) | 2 (5%) | 0 (0%) |
| 1 week | 37 | 36 (97%) | 1 (3%) | 0 (0%) |
| 4 weeks | 37 | 35 (95%) | 2 (5%) | 0 (0%) |
| 6 weeks | 37 | 35 (95%) | 2 (5%) | 0 (0%) |
| Rap | | | | |
| Preseizures | 23 | 23 (100%) | 0 (0%) | 0 (0%) |
| 0 h | 23 | 23 (100%) | 0 (0%) | 0 (0%) |
| 4 h | 23 | 23 (100%) | 0 (0%) | 0 (0%) |
| 24 h | 23 | 23 (100%) | 0 (0%) | 0 (0%) |
| 1 week | 23 | 23 (100%) | 0 (0%) | 0 (0%) |
| 4 weeks | 23 | 23 (100%) | 0 (0%) | 0 (0%) |
| 6 weeks | 23 | 23 (100%) | 0 (0%) | 0 (0%) |
| Ka | | | | |
| Preseizure | 25 | 25 (100%) | 0 (0%) | 0 (0%) |
| 0 h | 25 | 0 (0%) | 7 (28%) | 18 (72%) |
| 4 h | 25 | 2 (8%) | 23 (92%) | 0 (0%) |
| 24 h | 25 | 2 (8%) | 23 (92%) | 0 (0%) |
| 1 week | 25 | 4 (16%) | 21 (84%) | 0 (0%) |
| 4 weeks | 25 | 5 (20%) | 20 (80%) | 0 (0%) |
| 6 weeks | 25 | 5 (20%) | 20 (80%) | 0 (0%) |
| Ka + Post-Rap | | | | |
| Preseizures | 32 | 32 (100%) | 0 (0%) | 0 (0%) |
| 0 h | 32 | 0 (0%) | 10 (31%) | 22 (69%) |
| 4 h | 32 | 4 (13%) | 23 (71%) | 5 (16%) |
| 24 h | 32 | 4 (13%) | 27 (84%) | 1 (3%) |
| 1 week | 32 | 5 (16%) | 27 (84%) | 0 (0%) |
| 4 weeks | 32 | 6 (19%) | 26 (81%) | 0 (0%) |
| 6 weeks | 32 | 7 (22%) | 25 (78%) | 0 (0%) |

$n$ = 6–7 mice per group.
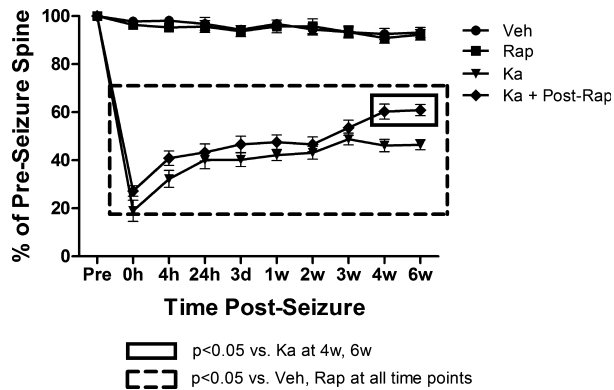
**Figure 6.** Quantitative analysis of effect of rapamycin posttreatment on kainate seizure-induced dendritic spine loss. Control mice (Veh, Rap) had no significant change in spine number over 6 weeks. In contrast, kainate-induced seizures led to an acute spine loss immediately after termination of the seizures (0 h), that only partially recovered over 6 weeks of observation. Rapamycin posttreatment had a small protective effect only at 4–6 weeks. $n = 23–37$ dendrites and $n = 6–7$ animals per group. Statistical comparisons by two-way repeated measures analysis of variance.

of the seizures, it's not surprising that dendritic beading and loss of spines was not affected by rapamycin post-treatment during the first few hours after kainate seizures (Fig. 5, Table 2). In fact, there was no significance difference between rapamycin and vehicle-treated mice for the first few weeks after kainate. However, by 4–6 weeks after kainate seizures, there was a small but significant improvement in the recovery of spines in the rapamycin posttreatment group (Fig. 6).

### Rapamycin reverses seizure-induced depolymerization of F-actin and dephosphorylation of LIM kinase and cofilin

Seizures have previously been shown to induce actin depolymerization (conversion of filamentous F-actin into soluble G-actin) via activation (dephosphorylation) of the actin-binding protein, cofilin.[15,16] We next tested whether rapamycin treatment prevents the seizure-induced regulation of the cofilin–actin axis. As demonstrated previously, kainate seizures induced a change in F-actin to G-actin. Rapamycin pretreatment prevented the seizure-induced conversion of F-actin to G-actin at all time points examined (Fig. 7A and C). In contrast, rapamycin posttreatment had no effect on actin polymerization during the first few hours following kainate seizures, but had a significant effect at 4 weeks (Fig. 7B and D), correlating with the protective effects of rapamycin posttreatment on spine injury. Kainate seizures also caused the expected dephosphorylation of cofilin, which was reversed by rapamycin pretreatment (Fig. 8A and B). In addition, LIM kinase is an upstream

regulator of cofilin. We first showed that kainate seizures regulate phosphorylation of LIM kinase. Rapamycin pre-treatment also prevented the kainate-induced modulation of LIM kinase (Fig. 8A and C). Similar to the effects on actin polymerization and spine density, rapamycin post-treatment only had a significant opposing effect on kai-nate-induced cofilin and LIM kinase phosphorylation at 4 weeks after kainate seizures (Fig. 8D–F). In contrast, there was no evidence of mTORC2 regulation by kainate-induced seizures or rapamycin alone, as assayed by P-PKCα (data not shown) and P-Akt levels (Fig. S2).

## Discussion

Regulation of dendritic structure has both important physiological roles and pathological relevance to neurological disorders. There may be overlap in the mechanisms mediating physiological and pathological modulation of dendrites. In this study, we demonstrate that mechanisms involved in LTP, a putative mechanism of learning, are involved in seizure-induced dendritic injury. In particular, the mTORC1 signaling pathway, which is necessary for LTP, mediates the acute effects of kainate seizures on neocortical dendrites. The mTOR inhibitor, rapamycin, prevented the seizure-induced dendritic beading and loss of spines. This effect appeared to be mediated by LIM kinase and cofilin regulation of actin.

mTOR is a relatively ubiquitous protein kinase involved in a number of physiological processes, including cell growth, metabolism, and survival.[21] mTOR is part of two major functional multiprotein complexes, mTORC1 and mTORC2. mTORC1 primarily regulates protein synthesis and is highly sensitive to inhibition with rapamycin. Although the molecular pathways and functions of mTORC2 are not as well delineated, mTORC2 regulates cytoskeletal organization and is relatively insensitive to rapamycin inhibition, but does respond to chronic rapamycin treatment. Somewhat surprisingly, despite the link between mTORC2 and actin, we found no evidence of mTORC2 activation in neocortex following kainate seizures. Rather, the mTORC1 downstream mediators, such as S6, were activated by kainate and reversed by rapamycin. Thus, mTORC1 signaling appears to mediate the effects of kainate seizures on dendritic structure. It is also possible that other parallel signaling pathways, such as Rho-Rock/Rac-Pak or Ras/ERK, may mediate effects of seizure-induced dendritic injury, but this is a subject of future studies.

mTORC1 is necessary for some forms of LTP.[9,10] As LTP and seizures both involve highly synchronized, excessive electrical activity, it is perhaps not surprising that there could be overlap in downstream mechanisms involved in dendritic plasticity. In addition to a role of
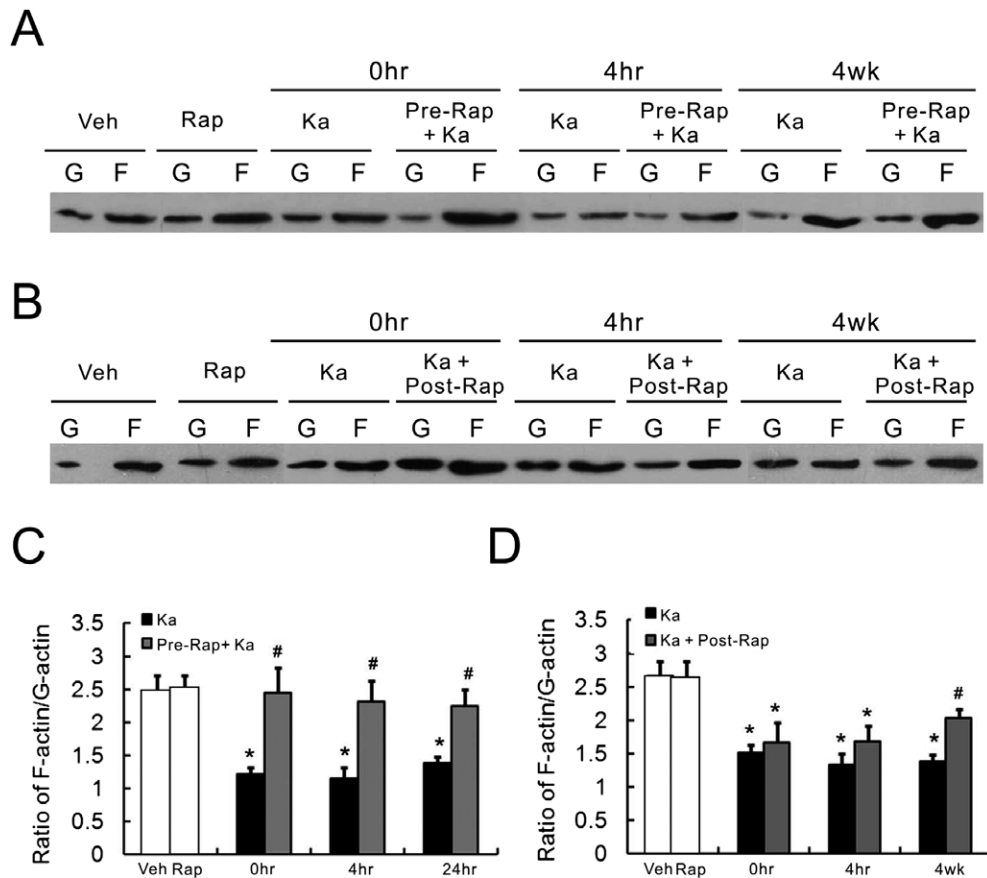
**Figure 7.** Rapamycin inhibits kainate seizure-induced actin depolymerization. Western blotting for actin was performed on fractions of soluble actin (G-actin) and insoluble actin (F-actin) separated by centrifugation from homogenized cortex. Representative western blots are shown (A,B). Kainate seizures cause a decrease F/G-actin ratio at 0 h, 4 h, and 4 weeks following the seizures, indicating F-actin depolymerization, and rapamycin pretreatment reverses this effect of kainate on F-actin depolymerization (C). In contrast, rapamycin posttreatment only had a significant effect at 4 weeks (D). *$P < 0.05$ compared with vehicle-treated control mice; #$P < 0.05$ compared with kainate-treated mice by two-way repeated measures analysis of variance ($n = 8$ mice per group).

mTOR, involvement of actin in dendritic plasticity has been strongly implicated in LTP.[4–7] As mTOR is activated under conditions of increased neuronal excitability, including seizures, mTOR may be a common signaling mechanism linking neuronal activity to actin dynamics under both physiological and pathological conditions, the main difference potentially being the degree of mTOR activation. The mTOR pathway has previously been directly linked to actin regulation of dendritic morphology via modulation of the actin depolymerization factor, cofilin, and upstream LIM kinase under genetic conditions of mTOR hyperactivity.[22] In our study, kainate seizure-induced changes in dendritic morphology were similarly associated with mTOR-dependent changes in actin, cofilin, and LIM-kinase dynamics.

The timing of mTOR inhibition was critical in exerting an effect on dendritic morphology, with rapamycin pretreatment having a much stronger effect than posttreatment. As mTOR is already activated immediately after

seizures are terminated, it is not surprising that rapamycin posttreatment did not have a significant acute effect in preventing dendritic injury. Since dendritic beading and spine loss is already maximal immediately after seizure termination, it is possible that posttreatment would have no effect. However, rapamycin posttreatment did induce a small but significant improvement in dendritic spine recovery after 4 weeks, suggesting that ongoing actin dynamics or other mTOR-dependent mechanisms contribute to chronic spine turnover and recovery. Our results with rapamycin posttreatment are similar to a recent report that showed a mild decrease in spine loss following pilocarpine status epilepticus, which was partially reversed with rapamycin initiated 2 weeks after status.[23] It is also possible that rapamycin pretreatment was more effective than posttreatment because pretreatment affected the severity of the acute kainate-induced seizures. However, consistent with our previous study,[15] no difference in seizure latency, severity, or duration was detected with rapamycin pretreatment.
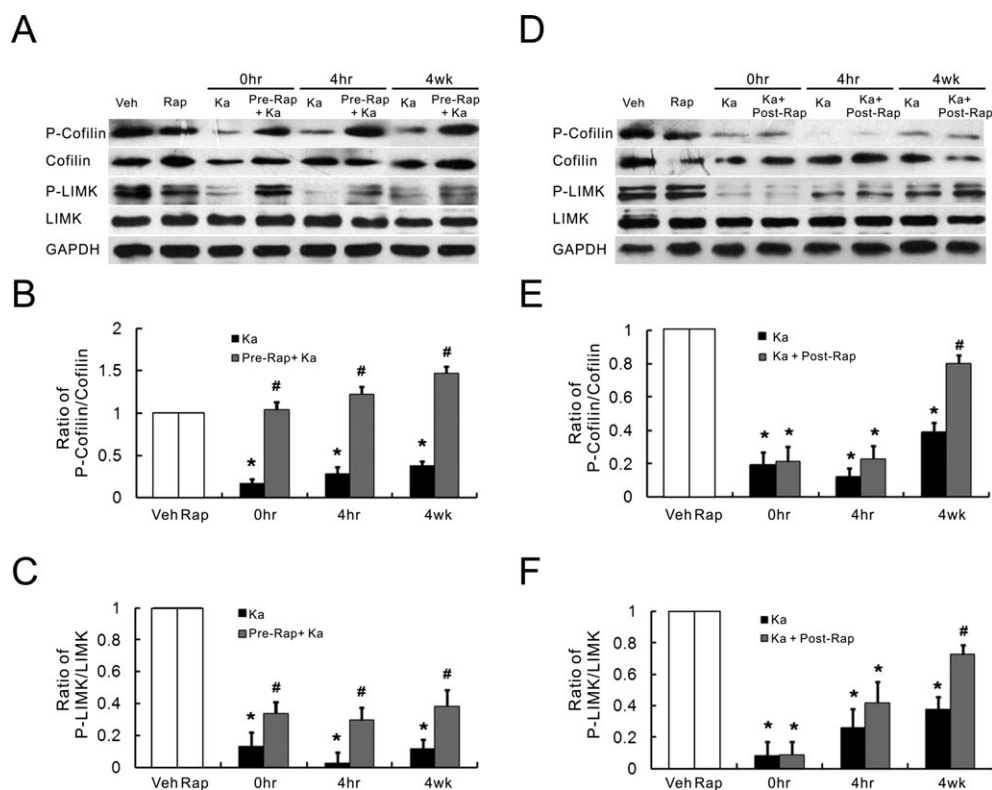
**Figure 8.** Rapamycin antagonizes the effects of kainate seizures on cofilin and LIM kinase dephosphorylation. (A) Phosphorylated cofilin (p-cofilin), total cofilin, phosphorylated LIM kinase, and total LIM kinase were assayed by western blotting of cortex, with and without rapamycin pretreatment. A representative western blot is shown. (B and C) Summarized data show a decrease in relative protein expression of P-cofilin and P-LIM kinase at 0 h, 4 h, and 4 weeks following kainate seizures. Rapamycin pretreatment (48 and 24 h prior to kainate seizures) reverses the effect of kainate on cofilin and LIM kinase dephosophorylation. The ratio of P-cofilin to total cofilin and P-LIM kinase to total LIM kinase was calculated for each condition/experiment and normalized to the preseizure control. (D–F) Rapamycin posttreatment (daily, starting at 0 h immediately after kainate seizures) has a small but significant effect in opposing the dephosphorylation of cofilin and LIM kinase only at 4 weeks following kainate seizures. All data were normalized to GADPH. *$P < 0.05$ compared with vehicle-treated control mice; #$P < 0.05$ compared with kainate-treated mice by two-way repeated measures ANOVA ($n = 10$ mice per group). ANOVA, analysis of variance.

Furthermore, rapamycin has been shown to have minimal or no direct anticonvulsant effects.[24,25] Thus, the effect of rapamycin is most likely independent of any acute, direct effect on neuronal excitability.

The potential clinical applications of this work for epilepsy are strong. mTOR inhibitors are already being considered as potential agents to treat seizures or prevent epileptogenesis.[26,27] While dendritic spine loss might affect epileptogenesis, seizure-induced dendritic injury more likely relates to cognitive and memory deficits in epilepsy patients.[11,28,29] Thus, mTOR inhibition may have translational applications for preventing seizure-induced brain injury.

## Acknowledgments

## Conflict of Interest

None declared.

## References

1. Kasai H, Fukuda M, Watanabe S, et al. Structural dynamics of dendritic spines in memory and cognition. Trends Neurosci 2010;33:121–129.
2. Yuste R, Bonhoeffer T. Morphological changes in dendritic spines associated with long-term synaptic plasticity. Annu Rev Neurosci 2001;24:1071–1089.

3. Sala C, Segal M. Dendritic spines: the locus of structural and functional plasticity. Physiol Rev 2014;94:141–188.

4. Fukazawa Y, Saitoh Y, Ozawa F, et al. Hippocampal LTP is accompanied by enhanced F-actin content within the dendritic spine that is essential for late LTP maintenance in vivo. Neuron 2003;38:447–460.

5. Kramar EA, Lin B, Rex CS, et al. Integrin-driven actin polymerization consolidates long-term potentiation. Proc Natl Acad Sci USA 2006;103:5579–5584.

6. Lin B, Kramar EA, Bi X, et al. Theta stimulation polymerizes actin in dendritic spines of hippocampus. J Neurosci 2005;25:2062–2069.

7. Okamoto K, Nagai T, Miyawaki A, Hayashi Y. Rapid and persistent modulation of actin dynamics regulates postsynaptic reorganization underlying bidirectional plasticity. Nat Neurosci 2004;7:1104–1112.

8. Huang W, Zhu PJ, Zhang S, et al. mTORC2 controls actin polymerization required for consolidation of long-term memory. Nat Neurosci 2013;16:441–448.

9. Stoica L, Zhu PJ, Huang W, et al. Selective pharmacogenetic inhibition of mammalian target of rapamycin complex I (mTORC1) blocks long-term synaptic plasticity and memory storage. Proc Natl Acad Sci USA 2011;108:3791–3796.

10. Tang SJ, Reis G, Kang H, et al. A rapamycin-sensitive signaling pathway contributes to long-term synaptic plasticity in the hippocampus. Proc Natl Acad Sci USA 2002;99:467–472.

11. Wong M, Guo D. Dendritic spine pathology in epilepsy: cause or consequence? Neuroscience 2013;251:141–150.

12. Penzes P, Cahill ME, Jones KA, et al. Dendritic spine pathology in neuropsychiatric disorders. Nat Neurosci 2011;14:285–293.

13. Guo D, Arnspiger S, Rensing NR, Wong M. Brief seizures cause dendritic injury. Neurobiol Dis 2012;45:348–355.

14. Rensing N, Ouyang Y, Yang XF, et al. In vivo imaging of dendritic spines during electrographic seizures. Ann Neurol 2005;58:888–898.

15. Zeng LH, Xu L, Rensing NR, et al. Kainate seizures cause acute dendritic injury and actin depolymerization in vivo. J Neurosci 2007;27:11604–11613.

16. Kurz JE, Moore BJ, Henderson SC, et al. A cellular mechanism for dendritic spine loss in the pilocarpine model of status epilepticus. Epilepsia 2008;49:1696–1710.

17. Feng G, Mellor RH, Bernstein M, et al. Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. Neuron 2000;28:41–51.

18. Schauwecker PE, Steward O. Genetic determinants of susceptibility to excitotoxic cell death: implications for gene targeting approaches. Proc Natl Acad Sci USA 1997;94:4103–4108.

19. Yang J, Houk B, Shah J, et al. Genetic background regulates semaphorin gene expression and epileptogenesis in mouse brain after kainic acid status epilepticus. Neuroscience 2005;131:853–869.

20. Zeng LH, Rensing NR, Wong M. The mammalian target of rapamycin signaling pathway mediates epileptogenesis in a model of temporal lobe epilepsy. J Neurosci 2009;29:6964–6972.

21. Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. Nat Rev Mol Cell Biol 2011;12:21–35.

22. Tavazoie SF, Alvarez VA, Ridenour DA, et al. Regulation of neuronal morphology and function by the tumor suppressors Tsc1 and Tsc2. Nat Neurosci 2005;8:1727–1734.

23. Brewster AL, Lugo JN, Patil VV, et al. Rapamycin reverses status epilepticus-induced memory deficits and dendritic damage. PLoS One 2013;8:e57808.

24. Chachua T, Poon KL, Yum MS, et al. Rapamycin has age-, treatment paradigm-, and model-specific anticonvulsant effects and modulates neuropeptide Y expression in rats. Epilepsia 2012;53:2015–2025.

25. Hartman AL, Santos P, Dolce A, Hardwick JM. The mTOR inhibitor rapamycin has limited acute anticonvulsant effects in mice. PLoS One 2012;7:e45156.

26. Wong M. A critical review of mTOR inhibitors and epilepsy: from basic science to clinical trials. Expert Rev Neurother 2013;13:657–669.

27. Wong M. Mammalian target of rapamycin (mTOR) inhibition as a potential antiepileptogenic therapy: from tuberous sclerosis to common acquired epilepsies. Epilepsia 2010;51:27–36.

28. Wong M. Modulation of dendritic spines in epilepsy: cellular mechanisms and functional implications. Epilepsy Behav 2005;7:569–577.

29. Casanova JR, Nishimura M, Owens JW, Swann JW. Impact of seizures on developing dendrites: implications for intellectual developmental disabilities. Epilepsia 2012;53 (Suppl 1):116–124.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Examples of mild (A – preseizure; B – postseizure) and severe (C – preseizure; D – postseizure) beading.

**Figure S2.** Lack of difference in P-Akt (S473) following kainate-seizures and with rapamycin treatment ($n = 6$ mice per group).