Microglial mTOR is Neuronal Protective and Antiepileptogenic in the Pilocarpine Model of Temporal Lobe Epilepsy

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Excessive activation of mammalian target of rapamycin (mTOR) signaling is epileptogenic in genetic epilepsy. However, the exact role of microglial mTOR in acquired epilepsy remains to be clarified. In the present study, we found that mTOR is strongly activated in microglia following excitatory injury elicited by status epilepticus. To determine the role of microglial mTOR signaling in excitatory injury and epileptogenesis, we generated mice with restrictive deletion of mTOR in microglia. Both male and female mice were used in the present study. We found that mTOR-deficient microglia lost their typical proliferative and inflammatory responses to excitatory injury, whereas the proliferation of astrocytes was preserved. In addition, mTOR-deficient microglia did not effectively engulf injured/dying neurons. More importantly, microglial mTOR-deficient mice displayed increased neuronal loss and developed more severe spontaneous seizures. These findings suggest that microglial mTOR plays a protective role in mitigating neuronal loss and attenuating epileptogenesis in the excitatory injury model of epilepsy.

Key words: epilepsy; microglia; mTOR; neuronal loss; phagocytosis; seizure

Significance Statement

The mammalian target of rapamycin (mTOR) pathway is strongly implicated in epilepsy. However, the effect of mTOR inhibitors in preclinical models of acquired epilepsy is inconsistent. The broad presence of mTOR signaling in various brain cells could prevent mTOR inhibitors from achieving a net therapeutic effect. This conundrum has spurred further investigation of the cell type-specific effects of mTOR signaling in the CNS. We found that activation of microglial mTOR is antiepileptogenic. Thus, microglial mTOR activation represents a novel antiepileptogenic route that appears to parallel the proepileptogenic route of neuronal mTOR activation. This may explain why the net effect of mTOR inhibitors is paradoxical in the acquired models of epilepsy. Our findings could better guide the use of mTOR inhibitors in preventing acquired epilepsy.

Introduction

Epileptogenesis is a pathologic process that transforms a normal brain into an epileptic brain, typically initiated by genetic mutations and neurologic insults such as status epilepticus (SE). Excessive activation of mammalian target of rapamycin (mTOR)

signaling is recognized as a pathomechanism underlying both genetic and acquired epilepsy (Wong, 2013; D’Gama et al., 2015; Crino, 2016; Talos et al., 2018). Modeling of mTOR hyperactivation in rodents demonstrates a very robust epileptogenic effect (Uhlmann et al., 2002; Meikle et al., 2007; Zeng et al., 2009; Orlova et al., 2010; Feliciano et al., 2011; Sunnen et al., 2011; Carson et al., 2012; McMahon et al., 2012; Zhang et al., 2016; Nguyen et al., 2019). However, over the past decade, mTOR inhibitors have been found to have very minimal or inconsistent effects in preventing acquired epilepsy (Buckmaster et al., 2009; Zeng et al., 2009; Gericke et al., 2020). As mTOR is expressed ubiquitously in neuronal and non-neuronal cells and its mode of action is very wide ranging, it is perhaps not surprising that the outcomes from broad mTOR inhibition are less than desirable. Therefore, it is of paramount importance to further dissect the cell-specific roles of mTOR signaling in epileptogenesis so as to

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develop better antiepilepsy strategies. Previous studies mainly focused on modeling hyperactivation of mTOR signaling in neurons and astrocytes, which nicely recapitulates the epileptogenic activity (Uhlmann et al., 2002; Meikle et al., 2007). The role of microglia and microglial mTOR signaling in epileptogenesis has only recently been increasingly explored (Boer et al., 2008; Sierra et al., 2010; Abraham et al., 2012; Schartz et al., 2018; Zhao et al., 2018; Mo et al., 2019).

Microglia are generally considered to be resident macrophages. They are the main innate immune cells in the CNS and the principal producer of proinflammatory cytokines in response to brain injury (Wolf et al., 2017). In addition to their innate immune activity, microglia also play various roles in brain development and CNS homeostasis through processes such as synapse pruning, clearing of apoptotic cells, and repair of minute insults (Nimmerjahn et al., 2005; Paolicelli et al., 2011; Sierra et al., 2013). Microglia also regulate myelination (Miron, 2017; Włodarczyk et al., 2017) as well as proliferation and activation of astrocytes (Liddel et al., 2017). Microglia become activated in response to epileptic insults (Boer et al., 2006; Eyo et al., 2016; Zhang et al., 2016; Schartz et al., 2018), and their inflammatory response has long been postulated to be epileptogenic (Quan et al., 2013; Vezzani and Viviani, 2015; Aronica et al., 2017). Morphologically reactive microglia have been found in the brains of animal models of temporal lobe epilepsy (TLE; van Vliet et al., 2012; Brewster et al., 2013) and in surgical resections of epilepsy patients (Beach et al., 1995; Sosunov et al., 2012; Liu et al., 2014). Pharmacological inhibition of microglial activation appears to attenuate epileptogenesis (Abraham et al., 2012). The proinflammatory action of microglia is postulated to be an etiologic driver of epileptogenesis. We recently discovered an alternative epileptogenic process involving microglia that is independent of an inflammatory response (Zhao et al., 2018).

mTOR signaling was found to be elevated in microglia in human epileptic resections (Nonoda et al., 2009; Sosunov et al., 2012) and a preclinical model (Brewster et al., 2013). Pharmacological inhibition of mTOR by rapamycin suppresses microglial activation and/or proliferation (Brewster et al., 2013; Nguyen et al., 2015; van Vliet et al., 2016), while other studies reported that the mTOR pathway may have inconsistent effects on microglia activation (van Vliet et al., 2012) or the effect on epileptogenesis was driven by unintended activation of mTOR signaling in neurons and astrocytes (Zhang et al., 2018). mTOR signaling was reported to regulate microglial behavior via several other mechanisms (Kassai et al., 2014; Shibuya et al., 2014; Li et al., 2016; Shen et al., 2016). Our recent study revealed that mice with excessive activation of mTOR restrictively in microglia develop spontaneous recurrent seizures (SRSs), suggesting a proepileptogenic role of upregulated microglial mTOR (Zhao et al., 2018, 2019). However, the exact role of activated microglial mTOR in epileptogenesis in acquired epilepsy remains to be determined. In the present study, we generated Cx3cr1-cre; mTOR^{fl/fl} mice to probe the role of microglial mTOR signaling in epileptogenesis in response to SE.

Materials and Methods

Animals. *Tg(Cx3cr1-cre)MW126Gsat/Mmud* (Cx3cr1-cre) mice were acquired from the MMRRC (Zhao et al., 2018, 2019), *B6.129S4-Mtor^{tm1.2Koz}/J* (mTOR^{fl/fl}) mice were acquired from The Jackson Laboratory. Both sexes were used. Animals were housed in a pathogen-free, temperature- and humidity-controlled facility with a 12 h light/dark cycle (lights on at 7:00 A.M.) and given *ad libitum* access to food and water. All experiments were performed according to the guidelines

set by the Institutional Animal Care and Use Committee as well as the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Efforts were made to minimize suffering and unnecessary use of animals.

Pilocarpine treatment. SE was induced by pilocarpine as described in our previous work (Zhao et al., 2019). In brief, to minimize the peripheral cholinergic side effects of pilocarpine, 8- to 10-week-old mice of either sex were first injected intraperitoneally with 1 mg/kg methyl scopolamine (Sigma-Aldrich) in 0.9% NaCl for 10 min before injection of pilocarpine (Sigma-Aldrich). For all groups, pilocarpine administration was started at the same time of day, 9:00 A.M. to 3:00 P.M. mTOR^{Cx3cr1-creCKO} mice and their controls (mTOR^{fl/fl}) from the same litter were used for the experiments. There is a variation in seizure threshold to the convulsant pilocarpine between control and knock-out mice or even within control mice from the same litter. To induce comparable levels of SE among mice, we used a modified ramping-up pilocarpine injection protocol. We treated mice with an initial dose of pilocarpine at 200 mg/kg by intraperitoneal injection, followed by 50 mg/kg every 15 min until the onset of stage 4 or 5 seizures. Seizures were classified according to the Racine scale (Racine, 1972), with modifications made by Borges et al. (2003), as follows: stage 0, normal activity; stage 1, rigid posture or immobility; stage 2, stiffened, extended and often arched tail; stage 3, partial body clonus, including forelimb or hindlimb clonus or head bobbing; stage 4, whole-body continuous clonic seizures with rearing; stage 5, severe whole-body continuous clonic seizures with rearing and falling; and stage 6, tonic-clonic seizures with loss of posture or jumping. Animals were allowed to develop SE for 4 h. The total time of seizures at Racine scale level 3 and above was recorded. The mortality rates to pilocarpine treatment were 60.7 ± 11.4% and 53.3 ± 11.3% for control and mTOR^{Cx3cr1-creCKO} mice, respectively. The surviving pilocarpine-treated animals were assigned to each group by pairing animals based on total seizure duration to ensure that the overall severity of SE induced by pilocarpine in each group was comparable. To further ensure that the animals started with a similar intensity of SE to pilocarpine treatment, animals that developed mild seizures (defined as a total seizure duration <50% of average) were excluded from further study. SE was terminated by diazepam treatment (10 mg/kg, i.p.; Sigma-Aldrich), followed by administration of a single dose of dextrose (1.5 g/kg, i.p.). Animals were placed on a 30°C warm pad for recovery for 1 h.

Rapamycin treatment. Rapamycin treatment was performed as previously described (Huang et al., 2010). In brief, wild-type mice (mixed background, C57BL/6N) were first treated with pilocarpine to allow SE for 4 h, immediately followed by intraperitoneal injection of either vehicle (5% Tween-20 and 4% ethanol) or rapamycin (6 mg/kg/d; LC Laboratories). Mice then received two additional doses of rapamycin 24 h apart. Mouse brain samples were harvested and processed for further analysis.

Immunohistochemistry and acquisition of images. Animals were anesthetized with pentobarbital (100 mg/kg, i.p.; Sigma-Aldrich) and transcardially perfused with PBS followed by 4% paraformaldehyde (PFA) in PBS, pH 7.4. Brains were postfixed overnight in 4% PFA buffer, followed by cryoprotection in 30% sucrose in PBS for at least 48 h. Mouse brains were then embedded in Neg-50 frozen section medium (Thermo Fisher Scientific) and sectioned on a cryostat at 35 μm thickness for all histologic analyses unless otherwise described. For most immunohistological experiments, unless otherwise specified, brain sections were washed with PBS, blocked and permeabilized in 10% BSA (Sigma-Aldrich) and 0.3% Triton X-100 (Sigma-Aldrich) in PBS at room temperature for 2 h. Sections were incubated with primary antibodies in the blocking buffer overnight at 4°C, and then washed with PBS for 5 min and repeated three times, followed by incubation with appropriate fluorescent conjugated secondary antibodies for 2 h at room temperature. For staining of activated caspase-3, brain sections were permeabilized with 0.5% Tween 20. For staining with goat anti-Iba1 (1:250; catalog #NB100-1028, Novus Biologicals), free-floating sections were permeabilized and blocked with blocking buffer containing 0.3% Triton X-100 and 5% normal donkey serum in PBS at room temperature for 1 h. Sections were then incubated with goat anti-Iba1 antibody diluted in blocking buffer overnight at 4°C. Nuclei were counterstained with DAPI (Sigma-Aldrich), and coverslips

were coated with Fluoromount G (SouthernBiotech) and sealed with nail polish. Images were acquired using a Zeiss LSM 880 confocal microscope with Airyscan and processed with Zen Black 2.1 or Zen Blue lite 2.3 Software (Carl Zeiss). Immunofluorescence signals were quantified using the National Institutes of Health (NIH) image analysis software ImageJ.

Brain coronal sections with similar anatomic locations (position –2 mm from bregma based on the mouse brain atlas) from all groups of control and *mTOR^{Cx3cr1-cre}*CKO mice were selected for all histologic analyses. To show the staining of microglia, astrocytes, and neurons in the entire cortex and hippocampus, images were acquired using the tiles and positions module and a 25× water-objective lens. For the quantification of microglia density, brain sections were stained with anti-Iba1 (Wako), anti-CD68 (Bio-Rad), and DAPI (Sigma-Aldrich). For evaluating the proliferation of microglia, microglia were colabeled with anti-Iba1 (Wako) and anti-Ki67 (Abcam). For the quantification of astrocyte density, brain sections were stained with anti-Iba1 (Wako), anti-GFAP (Millipore Sigma), and DAPI. For the quantification of cleaved Caspase-3⁺ cell density, brain sections were stained with anti-cleaved Caspase-3 (Cell Signaling Technology) and DAPI (Sigma-Aldrich). Confocal image stacks were collected using a 25× water-objective lens with a 1 μm interval through a 10 μm z-depth of the tissue under the tiles and stitching mode covering an area of 1656 × 3250 μm. The image stacks were subjected to maximum intensity projection to create 2D images and then imported into Neurolucida software (MBF Bioscience) for cell number counting. Microglia within the areas of the M1 motor cortex around layer IV [cortex (CTX)], the hippocampal radiatum layer adjacent to pyramidal CA1 (CA1), the stratum lucidum adjacent to CA3 (CA3), and dentate gyrus (DG) were quantified. Data are presented as the number of Iba1⁺ cells/mm². For the quantification of immunostaining intensity of CD68, all images were acquired and processed with identical parameter settings. Immunofluorescence intensity was quantified using the NIH image analysis software ImageJ.

Fluoro-Jade B staining. Fluoro-Jade B (FJB) staining was used to evaluate the acute excitatory injury triggered by SE. The sections were mounted on 2% gelatin-coated slides and then air dried on a slide warmer at 50°C for at least 30 min. The slides were first immersed in a solution containing 1% sodium hydroxide in 80% alcohol (20 ml of 5% NaOH added to 80 ml absolute alcohol) for 5 min. This was followed by 2 min in 70% alcohol and 2 min in distilled water. The slides were then transferred to a solution of 0.06% potassium permanganate on a shaker table for 10 min. The slides were then rinsed in distilled water for 2 min. After 20 min in 0.0004% Fluoro-Jade B staining solution (Histo-Chem Inc.), and 50 μg/ml DAPI (Sigma-Aldrich) in 0.1% acetic acid, the slides were rinsed in three changes of distilled water, for 1 min each. Excess water was removed by briefly (~15 s) draining the slides vertically on a paper towel. The slides were then placed on a slide warmer set to ~50°C until they were fully dry (5–10 min). The dry slides were cleared by immersion in xylene for at least 1 min before coverslipping with DPX (catalog #06522, Millipore Sigma). FJB-stained slices were imaged using a Zeiss AxioImager M2 Microscope system paired with Neurolucida and Stereo Investigator software packages. In brief, the Cy2 channel (for the green signal of FJB) and the DAPI channel (for DAPI) were selected. A region of interest (ROI; the whole hippocampus area) was traced by using a 5× objective lens [5×/numerical aperture (NA) 0.16]. The ROI was then scanned in a multisites prefocused model by using a 20× objective lens (20×/NA 0.80). The final images were saved in TIFF format for later analysis. The FJB⁺ cells were counted using Neurolucida with an identical preset threshold among the compared groups.

Analysis of in vivo phagocytosis. *In vivo* phagocytosis analysis was performed as previously described (Abiega et al., 2016; Zhao et al., 2018). In brief, brain sections were stained with anti-Iba1 (Wako) and DAPI (Sigma-Aldrich), and confocal image stacks were collected using a 40× oil-objective lens with a 0.2 μm interval through a 20 μm z-depth of the tissue under the tiles and stitching mode covering cortex and CA1 areas of 211.7 × 636.8 μm. Dying cells were recognized as cells showing a condensed and/or fragmented nuclear morphology after DAPI staining. To quantify microglial phagocytosis of dying cells, the enclosed pouches formed by microglial processes wrapping around dying cells

were counted. Then, phagocytosis capacity was calculated by applying the following formula: phagocytosis capacity = [(1Ph1 + 2Ph2 + 3Ph3 ... + nPhn)/MG]% (where Phn is the number of microglial cells with *n* phagocytic pouches, and MG is the total number of microglia cells). For each mouse, three coronal 35 μm sections that were ~105 μm apart (i.e., every fourth section) and from similar anatomic locations (position –2 mm from bregma based on the mouse brain atlas) were selected for histologic analysis.

Analysis of synapse density. Synapses were quantified according to the protocol previously described (Zhao et al., 2018). Brain tissues were sectioned at 15 μm thickness. Three sections at equidistant planes (100 μm apart) per mouse were used for subsequent immunostaining with antibodies against the presynapse protein VGlut2 (EMD Millipore) and the postsynapse protein Homer1 (EMD Millipore) for excitatory synapses, and the presynapse protein VCAT (Synaptic Systems) and the postsynapse protein gephyrin (Synaptic Systems) for inhibitory synapses. In brief, for staining of excitatory synapses, brain sections were incubated in blocking buffer (0.3% Triton X-100 and 10% BSA in 1× PBS) for 1 h at room temperature and then incubated in primary antibody solution (0.3% Triton X-100 and 10% BSA in 1× PBS; anti-VGlut2 1:4000, anti-Homer1 1:4000) at 4°C for 48 h. For staining of inhibitory synapses, brain sections were incubated in blocking buffer (0.2% Triton X-100 and 5% normal goat serum in 1× PBS) for 1 h at room temperature and then incubated in primary antibody solution (0.2% Triton X-100 and 3% normal goat serum in 1× PBS; anti-VGAT 1:4000, anti-gephyrin 1:4000) at 4°C for 48 h. Brain sections were washed for 20 min three times, followed by incubation with fluorophore-conjugated secondary antibodies at room temperature for 1 h. Nuclei were counterstained with DAPI (Sigma-Aldrich), and coverslips were applied with Fluoromount G (SouthernBiotech), and sealed with nail polish. The stained sections were imaged within 48 h of staining with a 63× Zeiss Pan-Apochromat Oil, 1.4 NA objective lens on a Zeiss LSM 880 with the Airyscan protocol in super-resolution mode. Two images with maximum synaptic staining within a z-stack of 21 serial optical frames (0.3 μm interval) were selected for quantification. Six single images per mouse brain were analyzed. Synapses were identified as yellow punctae, which represent the colocalization of VGlut2 (green) and Homer1 (red) or VCAT (green) and gephyrin (red). The number of synapses in the areas including the M1 motor cortex around layer IV and the hippocampal radiatum layer adjacent to pyramidal CA1 were counted using ImageJ software (version 1.51f; NIH).

Three-dimensional reconstruction of microglia. For microglial cell three-dimensional reconstruction, 35 μm coronal brain sections were stained with anti-Iba1 (Wako) as described previously (Zhao et al., 2018). Confocal image stacks were collected using a 63× oil-objective lens with a 0.2 μm interval through a 20 μm z-depth of the tissue under the tiles and stitching mode covering cortex (CTX), hippocampus CA1 and CA3, and DG areas of 256.41 × 256.41 μm. For each mouse, three coronal 35 μm sections that were ~105 μm apart (i.e., every fourth section) and from similar anatomic locations (position –2 mm from bregma based on the mouse brain atlas) were selected for imaging and further analysis using IMARIS software (Bitplane). For three-dimensional reconstruction using IMARIS software (Bitplane), microglia cells were traced/reconstructed by following the vendor instructions. In brief, for microglial cell volume evaluation, the Surfaces module of IMARIS was used to reconstruct cells and to automatically analyze cell volume. For evaluating microglia dendrite length, number of branch points, number of terminal points, and number of segments, the Filaments module of IMARIS was used to trace and reconstruct cells, and to further perform automatic analyses. All settings used for the reconstruction were identical between the control and the *mTOR^{Cx3cr1-cre}*CKO groups.

TUNEL staining. TUNEL staining was performed using the TMR-red Kit (Roche) following the manufacturer instructions. In short, tissue sections were fixed with fixation solution (4% paraformaldehyde in PBS, pH 7.4, freshly prepared) for 20 min and then washed with PBS for 30 min at room temperature. The slides were incubated in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate, freshly prepared) for 2 min on ice (2–8°C). After two rinses with PBS, the brain sections were incubated with 50 μl of TUNEL reaction mixture and placed in a

Table 1. Key resources, related to experimental procedures

Reagent or resource	Source	Identifier
Antibodies		
Anti-Iba1	Wako	Catalog #019-19741; RRID:AB_839504
Anti-Iba1	Novus Biologicals	Catalog #NB100-1028; RRID:AB_521594
Anti-Ki67	Abcam	Catalog #ab15580; RRID:AB_443209
Phospho-S6 ribosomal protein (Ser240/244)	Cell Signaling Technology	Catalog #5364; RRID:AB_10694233
NeuN (D4G40) XP rabbit mAb	Cell Signaling Technology	Catalog #24307; RRID:AB_2651140
Rat anti-mouse CD68	Bio-Rad	Catalog #MCA1957; RRID:AB_322219
Anti-NeuN	Millipore	Catalog #MAB377; RRID:AB_2298772
Anti-glial fibrillary acidic protein	Millipore	Catalog #AB5541; RRID:AB_177521
Anti-VGLUT2	Millipore	Catalog #AB2251-I; RRID:AB_2665454
Anti-Homer1	Millipore	Catalog #ABN37; RRID:AB_11214387
Anti-VGAT	Synaptic Systems	Catalog #131011; RRID:AB_887872
Anti-gephyrin	Synaptic Systems	Catalog #147008; RRID:AB_2619834
Cleaved Caspase-3 (Asp175) (5A1E) rabbit mAb	Cell Signaling Technology	Catalog #9664; RRID:AB_2070042
Goat anti-rabbit IgG (H + L) secondary antibody, Alexa Fluor 488 conjugate	Thermo Fisher Scientific	Catalog #A-11034; RRID:AB_2576217
Goat anti-rabbit IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor 568	Thermo Fisher Scientific	Catalog #A-11036; RRID:AB_10563566
Goat anti-rat IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 555	Thermo Fisher Scientific	Catalog #A-21434; RRID:AB_2535855
Goat anti-mouse IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor 488	Thermo Fisher Scientific	Catalog #A-11029; RRID:AB_2534088
Goat anti-chicken IgY (H + L) secondary antibody, Alexa Fluor 488	Thermo Fisher Scientific	Catalog #A-11039; RRID:AB_2534096
Donkey anti-goat IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor Plus 488	Thermo Fisher Scientific	Catalog #A32814; RRID:AB_2762838
Donkey anti-goat IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 647	Thermo Fisher Scientific	Catalog #A-21447; RRID:AB_2535864
Anti-mouse IgG, HRP-linked antibody	Cell Signaling Technology	Catalog #7076; RRID:AB_330924
Anti-rabbit IgG, HRP-linked antibody	Cell Signaling Technology	Catalog #7074; RRID:AB_2099233
Anti-PE MicroBeads	Miltenyi Biotec	Catalog #130-105-639
Anti-Cx3cr1-PE antibody	BioLegend	Catalog #149006; RRID:AB_2564315
Chemicals, peptides, and recombinant proteins		
Rapamycin	LC Laboratories	Catalog #R-5000
BSA	Millipore Sigma	Catalog #A7030
Donkey serum	Millipore Sigma	Catalog #D9663; RRID:AB_2810235
pHrodo Green Zymosan Bioparticles conjugate for phagocytosis	Thermo Fisher Scientific	Catalog #P35365
Fluoro-Jade B	Histo-Chem Inc	Catalog #1FJB
Cresyl Violet acetate	Millipore Sigma	Catalog #C5042
Pilocarpine hydrochloride	Millipore Sigma	Catalog #P6503
(–)-Scopolamine methyl bromide	Millipore Sigma	Catalog #S8502
Diazepam	Millipore Sigma	Catalog #439-14-5
DAPI	Millipore Sigma	Catalog #D9542
Percoll	GE Healthcare	Catalog #17-0891-01
Papain	Sigma-Aldrich	Catalog #P3125
Dispase II	STEMCELL	Catalog #07913
TRizol	Thermo Fisher Scientific	Catalog #15596018
Glycogen	Roche	Catalog #10901393001
DPX	Sigma-Aldrich	Catalog #06522
RNase-free water	Thermo Fisher Scientific	Catalog #BP561-1
Critical commercial assays		
In Situ Cell Death Detection Kit, TMR red	Millipore Sigma	Catalog #12156792910
Verso cDNA Synthesis Kit	ThermoFisher	Catalog #AB1453B
PowerUp SYBR Green Master Mix	ThermoFisher	Catalog #A25777
Other materials		
Three-channel EEG electrodes		
MS columns	Plastic One	Catalog #MS333/3-AIU/SPC
Pre-separation filters (30 μm)	Miltenyi Biotec	Catalog #130-042-201
Falcon 40 μm cell strainer	Miltenyi Biotec	Catalog #130-041-407
PCR plate	Corning	Catalog #352340
Gelatin-subbed slides	Bio-Rad	Catalog #MLL9601
	SouthernBiotech	Catalog #SLD01-CS
Experimental models: organisms/strains		
<i>Tg(Cx3cr1-cre)/MW126Gsat/Mmucd</i> (Cx3cr1-cre) mice	MMRRC	Strain# 036395-UCD; RRID:MMRRC_036395-UCD
<i>B6.129S4-Mtor^{tm1.2Kaz/J}</i> (mTOR ^{fl/fl}) mice	The Jackson Laboratory	Strain #011009; RRID:IMSR_JAX:011009
C57BL/6NJ (WT) mice	The Jackson Laboratory	Strain #005304; RRID:IMSR_JAX:005304
Software and algorithms		
ImageJ	NIH	https://fiji.sc/ or https://imagej.nih.gov/ij/
GraphPad Prism 7.0	GraphPad Software	https://www.graphpad.com
Zen Black	Zeiss	https://www.zeiss.com
Zen Blue	Zeiss	https://www.zeiss.com
NeuroLucida	MBF Bioscience	http://www.mbfbioscience.com
Bitplane	IMARIS software	https://imaris.oxinst.com/
DataWave SciWorks	DataWave	http://www.datawavetechnologies.com/

Table 2. Primers used in this article, related to experimental procedures

	Sense (5'–3')	Anti-sense (5'–3')	Species
Gene			
Primers used for quantitative real-time PCR			
TNF- α	ATGGCCTCCCTCTCATCAGT	GTTTGCTACGACGTGGGCTA	Mouse
IL-1 β	CGCAGCAGCATCAACAAG	GTGCTCATGTCTCATCTG	Mouse
IL-6	ACCAGAGGAATTTTCAATAGGC	TGATGCACTTGCAAAAACA	Mouse
IFN- β	TCCGAGCAGAGATCTTCAGGA	TGCAACCACTCATTTCTGAG	Mouse
GAPDH	GACAACCTTGGCATTGTGG	ATGCAGGGATGATGTTCTG	Mouse
	Forward (5'–3')	Reverse (5'–3')	
Genotype primers			
Mouse strains			
Cx3CrCre+/-	GATCCTGGCAAATTCGGCTA	TTGCTGCATGACCGGTGCA	Mouse
mTOR flox	GATAATTGCAGATTTGGCTAGCAG	CTCCTTCTGTGACATACATTTCCT	Mouse

humidified chamber for 60 min at 37°C in the dark. The slides were washed with PBS three times for 5 min each, then counterstained with DAPI. TUNEL staining was imaged using a Zeiss LSM 880 confocal microscope with Airyscan and processed with Zen Black 2.1 or Zen Blue lite 2.3 Software (Carl Zeiss). For evaluating the ratio of TUNEL/cleaved Caspase-3⁺ cells to FJB⁺ cells, three adjacent sections of each group were picked and stained separately with FJB, TUNEL, and cleaved Caspase-3. Images were acquired and cell numbers were counted by using Neurolucida. The ratio was calculated by dividing the TUNEL/cleaved Caspase-3⁺ cell number with the FJB⁺ cell number.

Nissl (cresyl violet) staining. Brain sections were mounted onto gelatin-subbed slides (SouthernBiotech) and dried at room temperature for 2 h. Before staining, the cresyl violet staining solution was made by combining 30 ml of the cresyl violet stock solution (0.2 g cresyl violet-acetate in 150 ml of distilled water; Sigma-Aldrich) with 300 ml buffer solution, pH 3.5 (282 ml of 0.1 M acetic acid combined with 18 ml of 0.1 M sodium acetate). The cresyl violet staining solution was prewarmed in an incubator for at least 1 h at 60 °C before staining. Then, the brain sections were stained as follows: (1) xylene, 5 min; (2) 95% alcohol, 3 min; (3) 70% alcohol, 3 min; (4) deionized distilled water, 3 min; (5) cresyl violet staining solution at 60°C, 10 min; (6) distilled water, 3 min; (7) 70% alcohol, 3 min; (8) 95% alcohol, 1 min; (9) 100% alcohol, 3 dips; (10) xylene, 5 min; and (11) xylene, 30 min. After finishing all the steps, the slides were covered with DPX (Sigma-Aldrich) and sealed with nail polish. For imaging of Nissl-stained slides, the color mode of the Zeiss AxioImager M2 Microscope system paired with Neurolucida was selected. An ROI (the whole hippocampus area) was traced using a 5× objective lens (5×/NA 0.16). After switching the objective lens to 20× (20×/NA 0.80), white balance and background subtraction were performed and the ROI was then scanned using a multisite prefocused model. The final images were saved in TIFF format for later analysis.

In vitro phagocytosis assay. Cultured microglia were seeded onto poly-D-lysine-coated 35 mm culture dishes with glass bottoms at 0.5×10^5 cells/dish (MatTek) 1–2 d before the assay. pHrodo Green Zymosan Bioparticles (Thermo Fisher Scientific) were dissolved at a concentration of 0.5 mg/ml in phenol red-free DMEM (Thermo Fisher Scientific), vortexed, and sonicated to homogeneously disperse the particles immediately before the assay. Before live imaging, the culture medium was removed and the culture dish was washed once with prewarmed phenol red-free DMEM, and then 100 μ l of bioparticle suspension was added to the area with the glass bottom. The microscope stage incubator was preset to 37°C and the environmental chamber was filled with 5% CO₂. Immediately following the addition of the bioparticles, a series of image frames was acquired at 1 frame/min for 61 frames (Zhao et al., 2018).

Purification of microglia from mouse brains. Mice were anesthetized with pentobarbital (100 mg/kg, i.p.) and quick perfused with 30 ml of PBS without Ca²⁺ and Mg²⁺. Mouse brains were dissected into 5 ml round-bottom tubes (1 brain/tube; Falcon) prefilled with 1 ml serum-free medium (DMEM/F12 with 4.5 mg/ml glucose) and 1 ml of dissociation medium (DMEM/F12 plus 1 mg/ml papain and 1.2 U/ml dispase II, and DNase I to 20 U/ml) prepared immediately before use, and homogenized using a 3 ml syringe plunger (BD Bioscience). After adding an

additional 2 ml of dissociation medium, the suspension was transferred into a new 15 ml tube and incubated at 37°C for 10 min. Then, 3 ml of neutralization medium (DMEM/F12 with 4.5 mg/ml glucose and 10% FBS) was added to each tube to stop the digestion. The suspension was further dissociated by pipetting up and down for 10–15 rounds with a 1 ml pipette tip touching the bottom of the 15 ml tube, and then passed through a 30 μ m cell strainer (Miltenyi Biotec). Myelin was removed by adding an equal volume of 70% Percoll followed by centrifugation at $800 \times g$ at 4°C for 15 min. Cells were washed once with FACS buffer (1% BSA, 0.1% sodium azide, 2 mM EDTA in PBS, pH 7.4) and then processed for further purification of microglia. We used anti-Cx3cr1-PE antibody (BioLegend) to bind microglia, followed by the addition of anti-PE MicroBeads (Miltenyi Biotec) to capture bound cells. The entire procedure was based on the manufacturer instructions. Briefly, dissociated cells ($\sim 10^7$) were resuspended in 50 μ l of FACS buffer followed by the addition of 1 μ l of anti-mouse CD16/CD32 (BioLegend) and incubation on ice for 10 min to block Fc receptors. The cells were then incubated with primary PE-conjugated antibody according to the manufacturer recommendations. After washing the cells twice with FACS buffer, the cells were resuspended in 80 μ l FACS buffer and 20 μ l anti-PE MicroBeads, and incubated at 4°C for 15 min. The cells were further diluted in 500 μ l of FACS buffer and passed through a magnetic MS column (Miltenyi Biotec). After three washes of the column with FACS buffer, the MS column was moved away from the magnetic field to elute the cells from the column with 1 ml FACS buffer.

RNA isolation, RT-PCR, and real-time PCR. Total RNA was extracted from purified microglia and cultured microglia using TRIzol Reagent (Thermo Fisher Scientific) according to the manufacturer instructions. To isolate RNA from brain tissues, mouse brains were first perfused with 50 ml of PBS before dissection of the cortex and hippocampus. Dissected cortical and hippocampal tissues were briefly homogenized in TRIzol Reagent, followed by RNA extraction. To better recover total RNA from purified microglia, 0.5 μ l RNase-free glycogen at 20 μ g/ml (Roche) was added to 0.5 ml of TRIzol extraction volume before RNA precipitation with isopropanol. RNA pellets were resuspended in 50 μ l of RNase-free water (Thermo Fisher Scientific) and incubated at 55°C for 10 min. RNA concentrations were determined by using a SmartSpec Plus Spectrophotometer (Bio-Rad). cDNA was synthesized from 0.2–1 μ g of total RNA via reverse transcription using a Verso cDNA Synthesis Kit (Thermo Fisher Scientific) in a total volume of 20 μ l. The cDNA templates were further diluted two to three times in water. Two microliters of diluted templates were used for real-time PCR. RT-PCR was performed in a 96-well PCR plate (Bio-Rad) using a SYBR Green qPCR Master Mix Kit (Applied Biosystems) in a Step One Plus Real-time PCR System (Applied Biosystems). Each sample was evaluated in triplicate. The CT value was used to calculate the fold change of RNA abundance after normalization to GAPDH. See Table 2 for all primer sequences.

Video/EEG recording of spontaneous seizures. Video/EEG recording was done according to previously published work (McMahon et al., 2012; Zhao et al., 2018). 1 week after SE, mice were implanted epidurally with three-channel EEG electrodes (Plastics One). After another week of recovery from the electrode implantation surgery,

Figure 1. Activation of mTOR in microglia following SE. **A**, Confocal images acquired from CTX, hippocampus CA1 and CA3, and the DG. Scale bar, 20 μ m. **B**, **C**, Quantification of p-S6/Iba1 double-positive microglial cells (**B**) and CD68/Iba1 double-positive microglial cells (**C**) in sham-treated ($n = 4$; 2 males and 2 females), SE-3d ($n = 5$; 2 males and 3 females), and SE-3d + rapamycin ($n = 4$; 2 males and 2 females) mice. Data are presented as the mean $\pm$ SEM. Sample comparison was made among three groups (sham, SE, and SE + rapamycin groups) and analyzed with one-way ANOVA (P1), followed by Tukey's multiple-comparisons test (P2, comparison between control and SE; P3, comparison between SE and SE + rapamycin).

Figure 2. Characterization of microglial morphology in $mTOR^{C3cr1-creCKO}$ mice. **A**, Brain coronal sections from control and $mTOR^{C3cr1-creCKO}$ mice were stained with anti-Iba1 (green) and DAPI (blue). Representative images with entire cortical and hippocampal fields and high magnification of images from CTX and CA1. Scale bars: whole scale, 200 μ m; ROI, 20 μ m. **B**, Confocal images (green + blue) and Imaris three-dimensional reconstructed images (cyan) from CTX, CA1, CA3, and DG of control and $mTOR^{C3cr1-creCKO}$ mice. Scale bar, 20 μ m. **C**, Imaris-based automatic quantification of microglial cell volume, dendrite length, number of branch points, number of terminal points, and number of segments in control ($n = 5$; 2 males and 3 females) and $mTOR^{C3cr1-creCKO}$ mice ($n = 4$; 2 males and 2 females). Data are presented as the mean $\pm$ SEM. A two-tailed unpaired t test was used for the comparison between control and $mTOR^{C3cr1-creCKO}$ mice.

the electrodes were connected to the video/EEG system (DataWave Technologies) and monitored 24 h/d for up to 60 d. All video/EEG data were analyzed by trained researchers blinded to the genotypes. EEG seizure events were characterized by the sudden onset of high-

frequency and high-amplitude (greater than twofold background) activity and a duration >10 s, along with characteristic postictal suppression. All electrographic seizures were verified behaviorally by video data to exclude movement artifacts.

Figure 3. Microglial proliferation and activation in control and *mTOR^{Cx3cr1-cre}CKO* mice following SE. **A**, Full montage of confocal images showing acute injury (FJB⁺, green), microglial density (Iba1⁺, green), and CD68 expression (CD68⁺, red) in the hippocampus of control and *mTOR^{Cx3cr1-cre}CKO* mice either sham-treated or 1–7 d post-SE. Scale bar, 200 μ m. **B**, Representative confocal images acquired from the CA1 of control and *mTOR^{Cx3cr1-cre}CKO* mice either sham-treated or 1–7 d post-SE. Scale bar, 20 μ m. **C**, Representative confocal images showing Ki67 expression (Ki67⁺, red) in the CA1 of control and *mTOR^{Cx3cr1-cre}CKO* mice either sham-treated or 1–7 d post-SE. Scale bar, 20 μ m. **D**, Average doses of pilocarpine that induced stage 4/5 seizures in control and *mTOR^{Cx3cr1-cre}CKO* mice. **E**, **F**, Quantification of Iba1⁺ microglia and CD68 immunofluorescence intensity in control and *mTOR^{Cx3cr1-cre}CKO* mice either sham-treated or 1–7 d post-SE. Sample sizes in control groups: sham (*n* = 6; 3 males and 3 females), SE-1d (*n* = 6; 3 males and 3 females), SE-3d (*n* = 6; 3 males and 3 females), and SE-7d (*n* = 6; 3 males and 3 females); and in the *mTOR^{Cx3cr1-cre}CKO* groups: sham (*n* = 4; 2 males and 2 females), SE-1d (*n* = 5; 2 males and 3 females), SE-3d (*n* = 6; 3 males and 3 females), and SE-7d (*n* = 4; 2 males and 2 females). **G**, Quantification of Ki67/Iba1 double-positive microglial cells in control and *mTOR^{Cx3cr1-cre}CKO* mice either sham-treated or 1–7 d post-SE. Sample sizes in control groups: sham (*n* = 6; 3 males and 3 females), SE-1d (*n* = 6; 3 males and 3 females), SE-3d (*n* = 6; 3 males and 3 females), and SE-7d (*n* = 6; 3 males and 3 females); and in the *mTOR^{Cx3cr1-cre}CKO* groups: sham (*n* = 4; 2 males and 2 females), SE-1d (*n* = 5; 2 males and 3 females), SE-3d (*n* = 6; 3 males and 3 females), and SE-7d (*n* = 4; 2 males and 2 females). Data are presented as the mean $\pm$ SEM. A two-tailed unpaired *t* test was used for the comparison between control and *mTOR^{Cx3cr1-cre}CKO* mice.

Statistical analysis. Data were analyzed using GraphPad Prism 7 software with appropriate tests for comparisons between the control and *mTOR^{Cx3cr1-cre}CKO* mice. G-Power was used for power analysis. Sample sizes were calculated to determine the minimum number of animals for adequate study power to detect the differences among groups. For immunohistological analysis, we observed that the readouts for the differences of the number of p-S6⁺ microglia, the density of microglia, and phagocytosis activity between the compared groups (i.e., sham-treated vs SE and control vs *mTOR^{Cx3cr1-cre}CKO*) are consistent and robust. Our preliminary calculation found the coefficient of determination to be 0.9–0.95. We set α at 0.05, and power at 95%. A sample size of four to five is adequate. One-way ANOVA, followed by Tukey's multiple-comparisons test was used to test the significance of differences among three experimental groups. Student's *t* test was used to test the significance of differences between the control group and the *mTOR^{Cx3cr1-cre}CKO* group. Two-way ANOVA, followed by Sidak test, was used for the comparison of the time course for *in vitro* phagocytosis between the control and

mTOR^{Cx3cr1-cre}CKO groups. For analysis of spontaneous seizures, we set the coefficient of determination at 0.6, yielding a sample size of 21. The χ^2 test was used to test the percentage difference of SRs between the control and *mTOR^{Cx3cr1-cre}CKO* groups. The Mann–Whitney test was used for testing the difference in seizure duration and number of cumulative seizure events between control and *mTOR^{Cx3cr1-cre}CKO* mice. A *p* value of <0.05 was considered significant.

All key resources related to the experimental procedures are listed in Tables 1 and 2.

Results

Increased levels of pS6 in activated microglia

Previous studies revealed that the levels of p-S6, an *in vivo* mTOR activation marker, are elevated following SE induced by either pilocarpine or kainic acid in animal models of TLE (Zeng

Figure 4. mTOR deletion decreases microglial phagocytosis activity *in vivo* following SE and *in vitro* phagocytosis of Zymosan bioparticles. **A**, Representative confocal images acquired from the CA1 of control and mTOR^{Cx3cr1-cre}CKO mice either sham treated or 1–7 d post-SE. Microglia were labeled by Iba1 (green). Scale bar, 20 μ m. **B**, Representative images from *in vitro* live imaging of microglial phagocytosis of pHrodo Green Zymosan Bioparticles (pHrodo⁺, green). Scale bar, 10 μ m. **C**, Quantification of microglial phagocytosis capacity in control and mTOR^{Cx3cr1-cre}CKO mice either sham treated or 1–7 d post-SE. Sample sizes in control groups: sham ($n = 6$; 3 males and 3 females), SE-1d ($n = 6$; 3 males and 3 females), SE-3d ($n = 6$; 3 males and 3 females), and SE-7d ($n = 6$; 3 males and 3 females); and in the mTOR^{Cx3cr1-cre}CKO groups: sham ($n = 4$; 2 males and 2 females), SE-1d ($n = 5$; 2 males and 3 females), SE-3d ($n = 6$; 3 males and 3 females), and SE-7d ($n = 4$; 2 males and 2 females). Data are presented as the mean $\pm$ SEM. A two-tailed unpaired *t* test was used for the comparison between control and mTOR^{Cx3cr1-cre}CKO mice. **D**, Quantification of the number of phagocytotic particles per microglial cell at each time point in cultured microglia prepared from control and mTOR^{Cx3cr1-cre}CKO mice ($n = 40$ for each time point). Data are presented as the mean $\pm$ SEM. Two-way ANOVA, followed by Sidak test, was used for the comparison of *in vitro* phagocytosis between the control and mTOR^{Cx3cr1-cre}CKO groups at each time point.

et al., 2009; Huang et al., 2010; Brewster et al., 2013). In the present study, we performed immunohistochemistry analysis to characterize the expression of p-S6 in the cortex and hippocampus. Microglia of sham-treated animals expressed very low levels of p-S6 (Fig. 1A,B). However, we observed robust p-S6 expression in morphologically activated microglia in both the cortex and hippocampus 3 d post-SE (Fig. 1A,B). Nearly 50% of Iba1⁺ cells were p-S6⁺. Treatment with the mTOR inhibitor rapamycin markedly reduced p-S6 expression (Fig. 1A,B), consistent with the finding that mTOR signaling is activated in microglia in response to neuronal injury induced by SE (Brewster et al., 2013). CD68 is an indicator of microglia activation. Rapamycin also reduced the induction of CD68 (Fig. 1A,C).

mTOR deletion impairs activation and proliferation of microglia

To examine the role of mTOR in microglia, we generated mTOR^{Cx3cr1-cre}CKO mice to delete mTOR in microglia by crossing mTOR^{fl/fl} and Cx3cr1-cre lines. Our recent studies have verified that the Cx3cr1-cre line is microglial specific (Zhao et al., 2018, 2019). In our previous study, excessive activation of microglial mTOR in TSC1KO mice caused a marked change in morphology and increased proliferation of microglia (Zhao et al., 2018). In mTOR^{Cx3cr1-cre}CKO mice, microglia with mTOR deletion became less ramified and displayed moderate morphologic

changes, including a smaller cell volume, shorter dendrites, and fewer branches and terminal segment points (Fig. 2A–C), consistent with a role of mTOR in regulating cell growth and size. However, there was no significant change in the density of microglia (Figs. 2A, 3A,B,E).

Having demonstrated that mTOR signaling is activated in microglia following SE, we next evaluated how mTOR-deficient microglia respond to neuronal injury induced by SE was induced by pilocarpine in mTOR^{Cx3cr1-cre}CKO mice and littermate controls. To determine how microglial mTOR signaling acts in response to excitatory injury, we carefully performed dosing of pilocarpine. Our goal was to induce comparable seizures in both groups so that we could determine how control and mTOR^{Cx3cr1-cre}CKO mice respond to excitatory injury. Mice were treated with a single dose of pilocarpine at a concentration of 200 mg/kg followed by 50 mg/kg every 15 min via intraperitoneal injection until they developed SE. mTOR^{Cx3cr1-cre}CKO mice appeared to require a moderately lower dose of pilocarpine to induce SE compared with their littermate controls (Fig. 3D). Nevertheless, all mice experienced 4 h of SE and then were treated with a single dose of diazepam to terminate the seizures. Mouse brains were harvested on days 1, 3, and 7 post-SE. Mouse brain sections were first stained with FJB to evaluate the extent of neuronal injury (Fig. 3A,B). Mouse brains that displayed comparable FJB staining in control and mTOR^{Cx3cr1-cre}CKO mice

Figure 5. Impact of microglial mTOR deletion on the inflammatory response following SE. **A**, quantitative real-time PCR (qRT-PCR) analysis of cytokine expression in hippocampal tissues harvested from control and *mTOR^{Cx3cr1-cre}CKO* mice either sham treated or 1–7 d post-SE. Sample sizes in the control groups are: sham ($n = 7$; 4 males and 3 females), SE-1d ($n = 8$; 4 males and 4 females), SE-3d ($n = 5$; 3 males and 2 females), and SE-7d ($n = 6$; 3 males and 3 females); and in the *mTOR^{Cx3cr1-cre}CKO* groups: sham ($n = 4$; 2 males and 2 females), SE-1d ($n = 7$; 4 males and 3 females), SE-3d ($n = 5$; 3 males and 2 females), and SE-7d ($n = 4$; 2 males and 2 females). **B**, Cytokine expression in purified microglia from control and *mTOR^{Cx3cr1-cre}CKO* mice either sham treated or 1–7 d post-SE. Sample sizes in the control groups are: sham ($n = 9$; 5 males and 4 females), SE-1d ($n = 8$; 4 males and 4 females), SE-3d ($n = 7$; 3 males and 4 females), and SE-7d ($n = 8$; 4 males and 4 females); and in the *mTOR^{Cx3cr1-cre}CKO* groups: sham ($n = 4$; 2 males and 2 females), SE-1d ($n = 4$; 2 males and 2 females), SE-3d ($n = 4$; 2 males and 2 females), and SE-7d ($n = 5$; 2 males and 3 females). A two-tailed unpaired *t* test was used for the comparison between control and *mTOR^{Cx3cr1-cre}CKO* mice. Data are presented as the mean $\pm$ SEM.

were paired, and brain sections adjacent to the sections were used for FJB staining for further analysis of microglia activation. This approach ensures that the animals experienced a similar level of injury because we are examining how microglia respond to neuronal injury. In control mice, we observed that microglia became morphologically less ramified and began to show a significant increase in microglial density on day 3 post-SE, and continued to increase on day 7 post-SE (Fig. 3*A,B,E*). In contrast, in *mTOR^{Cx3cr1-cre}CKO* mice, there was only a moderate increase in microglial density on days 3 and 7 post-SE. In control mice, CD68 induction in microglia became prominent within day 1 post-SE, reached a peak on day 3 post-SE, and then declined on day 7 post-SE (Fig. 3*A,B,F*). However, in *mTOR^{Cx3cr1-cre}CKO* mice, the induction of CD68 was very minimal on day 1 post-SE, moderate on day 3 post-SE, and largely resolved by day 7 post-SE. These data suggest that mTOR deletion prevents microglia from becoming activated and proliferative in response to excitatory neuronal injury.

We next performed Ki67 staining to evaluate the proliferation of microglia. In control mice, we observed a significant increase of Ki67 staining on day 1 post-SE, reaching a peak on day 3 post-SE, and declining on day 7 post-SE (Fig. 3*C,G*). However, in *mTOR^{Cx3cr1-cre}CKO* mice, we found a very moderate increase of Ki67 staining on days 3 and 7 post-SE. These data suggest that mTOR-deficient microglia have lost the proliferative response to excitatory neuronal injury.

mTOR deletion impairs microglial phagocytosis

Microglia are the principal phagocytotic cells that engulf and clear dying neurons (Sierra et al., 2013; Wyatt-Johnson and Brewster, 2020). We next evaluated the effect of mTOR deletion on microglial phagocytosis. In response to neuronal injury, microglia migrate close to dying neurons. Their processes wrap around the neurons to form a phagocytotic cup and engulf

neurons. We quantified the number of phagocytotic cups as described previously (Sierra et al., 2010; Abiega et al., 2016; Zhao et al., 2018). We observed an increase of phagocytotic cups on days 1–7 post-SE in control mice (Fig. 4*A,C*). However, phagocytotic cup formation was markedly reduced in *mTOR^{Cx3cr1-cre}CKO* mice, suggesting that mTOR deficiency impairs phagocytosis (Fig. 4*A,C*). The reduction in microglial phagocytosis activity *in vivo* is not attributable to the reduced microglial density, as the data are presented as the number of cups per microglial cell. We next performed an *in vitro* phagocytosis assay to evaluate phagocytotic activity in cultured microglia, an assay that we described previously (Zhao et al., 2018). We found that mTOR deletion significantly reduced the phagocytosis of Zymosan particles by microglia (Fig. 4*B,D*). Together, our data suggest that mTOR deletion downregulates microglial phagocytosis.

Microglial mTOR deletion impairs the microglial inflammatory response to excitatory injury

We evaluated cytokines in hippocampal tissues. We observed significant induction of TNF- α , IL-1 β , IL-6, and IFN- β on days 1 and 3 post-SE in hippocampal tissues from control mice (Fig. 5*A*). However, the induction of TNF- α and IL-1 β was significantly reduced in *mTOR^{Cx3cr1-cre}CKO* mice. In contrast, the levels of IL-6 and IFN- β were elevated in *mTOR^{Cx3cr1-cre}CKO* mice when compared with control mice. We next analyzed these cytokines in purified microglia (Fig. 5*B*). In the sham-treated mice, there was no difference in the levels of TNF- α , IL-1 β , IL-6, and IFN- β cytokines in microglia prepared from control and *mTOR^{Cx3cr1-cre}CKO* mice. We observed that TNF- α was induced on days 1 and 3 post-SE, and IL-1 β was induced mainly on day 7 post-SE. These inductions were significantly reduced in *mTOR^{Cx3cr1-cre}CKO* mice. Interestingly, there was no induction of IL-6 and IFN- β in microglia. Our data suggest that the elevated level of IL-6 is mainly induced by nonmicroglial cells.

Microglial mTOR deletion exacerbates the loss of neurons

Again, we first performed FJB staining to evaluate neuronal injuries in the hippocampal CA1 region in both control and $mTOR^{Cx3cr1-cre}CKO$ mice. Mouse brain sections that displayed similar levels of injury were paired for immunohistochemistry studies. Astrocytes typically hyperproliferate following excitatory injury. Accordingly, we evaluated the density of GFAP⁺ astrocytes. Excessive activation of microglial mTOR in TSC1KO mice caused marked proliferation of astrocytes (Zhao et al., 2018). We observed a marked increase in astrocyte density in the cortex and hippocampus following SE in both the control and $mTOR^{Cx3cr1-cre}CKO$ mice, reaching a peak at approximately day 3 post-SE (Fig. 6A–C). There was no significant difference between the control and $mTOR^{Cx3cr1-cre}CKO$ groups.

In our previous study, excessive activation of microglial mTOR in TSC1KO mice altered the density of synapses (Zhao et al., 2018). We evaluated the impact of mTOR deletion on neurons. We found no significant difference in synapse density in the cortex and hippocampus between control and $mTOR^{Cx3cr1-cre}CKO$ mice (Fig. 7A,B). Pyramidal neurons began to be TUNEL⁺ at day 1 post-SE, reaching a peak by day 7 in both the control and $mTOR^{Cx3cr1-cre}CKO$ mice (Fig. 8A,B,D). However, there were significantly more TUNEL⁺ pyramidal cells in $mTOR^{Cx3cr1-cre}CKO$ mice at days 3 and 7 post-SE compared with the controls. Concurrent with increased levels of TUNEL⁺ neurons at days 3 and 7 post-SE, microglia proliferation was reduced in $mTOR^{Cx3cr1-cre}CKO$ mice (Figs. 3A,B,E, 8D,E). TUNEL positivity is an indirect indicator of apoptotic cell death whereas cleaved Caspase-3 staining is a more relevant marker of apoptosis. We found that cleaved Caspase-3 staining was largely negative in the pyramidal layers, except for very weak staining on day 7 in $mTOR^{Cx3cr1-cre}CKO$ mice (Fig. 8A,B,D). We next evaluated the density of neurons in the hippocampal CA1 region 20 d post-SE. As expected, SE caused the loss of pyramidal neurons in control mice and a significant reduction in $mTOR^{Cx3cr1-cre}CKO$ mice (Fig. 8C,F). These data suggest that microglial mTOR deletion increases neuronal loss.

Microglial mTOR deletion promotes epileptogenesis

To evaluate the impact of microglial mTOR deletion on epileptogenesis, epidural electrodes were implanted 1 week post-SE. Seizure activities were recorded continuously beginning 2 weeks post-SE for 2 months (Fig. 9A,B). We recorded 24 $mTOR^{Cx3cr1-cre}CKO$ mice and 33 littermate controls that experienced SE for 4 h. We found that 84.8% of the

Figure 6. Impact of microglial mTOR deletion on astrocyte proliferation following SE. **A**, Full montage of confocal images showing astrocyte proliferation (GFAP⁺, green) in the cortex and hippocampus of control and $mTOR^{Cx3cr1-cre}CKO$ mice either sham treated or 1–7 d post-SE. Scale bar, 200 μ m. **B**, Representative confocal images acquired from the CTX and CA1 of control and $mTOR^{Cx3cr1-cre}CKO$ mice either sham treated or 1–7 d post-SE. Scale bar, 20 μ m. **C**, Quantification of GFAP⁺ astrocytes in control and $mTOR^{Cx3cr1-cre}CKO$ mice either sham treated or 1–7 d post-SE. Sample sizes in control groups: sham ($n = 6$; 3 males and 3 females), SE-1d ($n = 6$; 3 males and 3 females), SE-3d ($n = 6$; 3 males and 3 females), and SE-7d ($n = 6$; 3 males and 3 females); and in the $mTOR^{Cx3cr1-cre}CKO$ groups: sham ($n = 4$; 2 males and 2 females), SE-1d ($n = 5$; 2 males and 3 females), SE-3d ($n = 6$; 3 males and 3 females), and SE-7d ($n = 4$; 2 males and 2 females). A two-tailed unpaired t test was used for the comparison between control and $mTOR^{Cx3cr1-cre}CKO$ mice. Data are presented as the mean $\pm$ SEM.

control animals developed SRSs compared with 100% of the $mTOR^{Cx3cr1-cre}CKO$ animals (Fig. 9C); the difference is statistically significant. The duration of seizures was prolonged in $mTOR^{Cx3cr1-cre}CKO$ mice compared with control mice (Fig. 9D). Furthermore, $mTOR^{Cx3cr1-cre}CKO$ mice experienced more frequent seizures than control mice (Fig. 9E). We also recorded 8

Figure 7. Impact of microglial mTOR deletion on density of synapses. **A**, Representative super-resolution images of coimmunostaining of VGlut2 (red)/Homer1 (green) and vGAT (green)/gephyrin (red) in the M1 motor cortex around layer IV (CTX) and the hippocampal (HIP) radiatum layer adjacent to pyramidal CA1 (CA1) of control and $mTOR^{Cx3cr1-cre}CKO$ mice. Synapses were identified as yellow dots, reflecting colocalization of the presynaptic markers vGAT and vGAT with the postsynaptic markers Homer1 and gephyrin. Scale bar, 5 μm . **B**, Quantification of the excitatory and inhibitory synapse densities in the CTX and HIP of wild-type ($n = 5$; 2 males and 3 females) and $mTOR^{Cx3cr1-cre}CKO$ ($n = 5$; 2 males and 3 females) mice. Data are presented as the mean $\pm$ SEM. A two-tailed unpaired t test was used for the comparison between control and $mTOR^{Cx3cr1-cre}CKO$ mice.

$mTOR^{Cx3cr1-cre}CKO$ and 13 control mice that had not been treated with pilocarpine. None of them developed SRSs. Together, our data suggest that microglial mTOR deletion exacerbates the development of spontaneous seizures.

Discussion

In the present study, we revealed that mTOR-deficient microglia are less responsive to excitatory injury compared with wild-type microglia. However, microglial mTOR-deficient mice displayed a significant increase of neuronal loss and developed much more severe spontaneous seizures, suggesting a protective role of microglial mTOR in mitigating neuronal loss and attenuating epileptogenesis in response to excitatory injury.

mTOR-deficient microglia have a defective proliferative response to excitatory injury

Despite the presence of severe hippocampal pyramidal neuronal injuries, mTOR-deficient microglia did not hyperproliferate in the injured pyramidal layers. Thus, the response of mTOR-deficient microglia to excitatory injury was substantially muted. Previous studies reported that microglial proliferation is inhibited by rapamycin (Brewster et al., 2013; Nguyen et al., 2015; van Vliet et al., 2016). However, it was unclear from those studies whether rapamycin acts directly on microglia or indirectly through neurons or astrocytes. Our data suggest that the inhibition of microglial mTOR is sufficient to suppress the proliferative response to excitatory injury. A recent study revealed that microglial deletion of raptor, a key component in the mTORC1 complex, reduces Iba1⁺ cells in a focal ischemia model (Li et al., 2016), consistent with our finding of a critical role for mTOR in the proliferative response of microglia to excitatory injury. Mechanistically, it is unclear how mTOR inactivation leads to a significant loss of the proliferative response of microglia to excitatory injury. mTORC1 complex regulates cell growth, which

could explain the reduced length of the microglial processes we observed. As microglial processes are moderately shortened in mTOR-deficient microglia, this could limit their contacts with neurons and compromise their ability to detect injuries. However, we found that microglia density is not significantly reduced within and around the hippocampal pyramidal layer. These data suggest that, instead of there being reduced physical contacts with neurons per se, some other mechanisms for injury detection is perhaps compromised in mTOR-deficient microglia. For example, microglial phagocytosis activity is severely impaired. It is conceivable that phagocytosis of cell debris released from dead or dying neurons may be necessary for microglia to acquire a reactive phenotype. Recent studies revealed that some receptors regulate the microglial response to excitatory injuries following SE. The down-regulation of colony-stimulating factor-1 activity attenuates microglial proliferation after seizures or appears to be antiepileptic (Srivastava et al., 2018; Feng et al., 2019). Microglial P2Y12 receptors also regulate the interaction of microglia and neurons (Eyo et al., 2014; Mo et al., 2019). It will be interesting to see whether either the expression or activation of these receptors is changed in $mTOR^{Cx3cr1-cre}CKO$ mice and to determine whether these changes are responsible for the altered microglial response to excitatory injuries. Apart from the lack of proliferative response, mTOR-deficient microglia could be more susceptible to dying after SE in the $mTOR^{Cx3cr1-cre}CKO$ mice, resulting in a reduced density of microglia. Akt is a key component of mTORC2 signaling, which is relatively rapamycin insensitive, but is still inhibited at higher concentrations of rapamycin (Sarbasov et al., 2005; Foster and Toschi, 2009). Akt plays a critical role in cell proliferation and survival (Luo et al., 2003). Future study will determine whether microglial viability is reduced as a result of deficiency in mTOR/Akt signaling.

mTOR deficiency impairs microglial phagocytosis

Microglia are professional phagocytes in the CNS. They play an important role in modifying neuronal circuits during

Figure 8. Impact of microglial mTOR deletion on neuronal loss following SE. **A**, Full montage of confocal images covering the entire hippocampus field showing FJB staining (FJB⁺, green), TUNEL staining (TUNEL⁺, red) and cleaved Caspase-3 expression (Cleaved-Cas3⁺, green) in control and $mTOR^{Cx3cr1-cre}CKO$ mice either sham treated or 1–7 d post-SE. Scale bar, 200 μ m. Images were acquired by tiling and stitching of individual high-resolution images captured with a 40 $\times$ objective lens. The overall sampling depth is 15 μ m. Scale bar, 200 μ m. **B**, Representative confocal images acquired from the CA1 of control and $mTOR^{Cx3cr1-cre}CKO$ mice either sham treated or 1–7 d post-SE. Scale bar, 20 μ m. **C**, Representative images showing Nissl staining of control and $mTOR^{Cx3cr1-cre}CKO$ mice either sham treated or 20 d post-SE. Scale bar, 100 μ m. **D**, Quantification of TUNEL and cleaved Caspase-3⁺ cells in control and $mTOR^{Cx3cr1-cre}CKO$ mice either sham treated or 1–7 d post-SE. Sample sizes in control groups: sham ($n = 6$; 3 males and 3 females), SE-1d ($n = 6$; 3 males and 3 females), SE-3d ($n = 6$; 3 males and 3 females), and SE-7d ($n = 6$; 3 males and 3 females); and in the $mTOR^{Cx3cr1-cre}CKO$ groups: sham ($n = 4$; 2 males and 2 females), SE-1d ($n = 5$; 2 males and 3 females), SE-3d ($n = 6$; 3 males and 3 females), and SE-7d ($n = 4$; 2 males and 2 females). **E**, The time course of fold changes of TUNEL⁺ cells (solid lines) and microglial density (dash lines) in control (gray) and $mTOR^{Cx3cr1-cre}CKO$ mice (red). **F**, Cell counts along the hippocampal pyramid layers of control and $mTOR^{Cx3cr1-cre}CKO$ mice either sham treated or 20 d post-SE. Sample sizes in the controls are: sham ($n = 6$; 3 males and 3 females) and SE-20d ($n = 6$; 3 males and 3 females); and in $mTOR^{Cx3cr1-cre}CKO$ mice: sham ($n = 6$; 3 males and 3 females) and SE-20d ($n = 6$; 3 males and 3 females). Data are presented as the mean $\pm$ SEM. A two-tailed unpaired t test was used for the comparison between control and $mTOR^{Cx3cr1-cre}CKO$ mice.

Figure 9. Impact of microglial mTOR deletion on the development of spontaneous recurrent seizures. **A**, Schematic depiction of timelines of the video/EEG recording procedures. **B**, Representative electrographic traces of SRSs in sham- and pilocarpine-treated control and $mTOR^{Cx3cr1-cre}CKO$ mice ($n=24$; 15 males and 9 females). None of the sham-treated control ($n=13$; 7 males and 6 females) and $mTOR^{Cx3cr1-cre}CKO$ ($n=8$; 4 males and 4 females) mice developed SRSs. $p < 0.0001$ for comparison in four groups, and $p = 0.0459$ between pilocarpine-treated control and $mTOR^{Cx3cr1-cre}CKO$ mice by χ^2 test. **D**, **E**, Seizure duration (**D**) and cumulative seizure events (**E**) in sham-treated control and $mTOR^{Cx3cr1-cre}CKO$ mice and pilocarpine-treated control and $mTOR^{Cx3cr1-cre}CKO$ mice. Data are presented as the mean $\pm$ SEM. $p < 0.0001$ by Mann–Whitney test between pilocarpine-treated control and $mTOR^{Cx3cr1-cre}CKO$ mice.

development as well as in epilepsy, by phagocytosis of synapses and neurons (Paolicelli et al., 2011; Schafer et al., 2012; Sierra et al., 2013; Brown and Neher, 2014; Abiega et al., 2016; Wyatt et al., 2017; Hammond et al., 2018; Schartz et al., 2018; Wyatt-Johnson and Brewster, 2020). We found that one of the major phenotypic changes in mTOR-deficient microglia is the loss of the ability to engulf injured neurons as well as a bacterial mimetic (Zymosan; Preissler et al., 2015). Mechanistically, mTOR signaling has been implicated in microglial phagocytosis (Shen et al., 2016). In addition, several other signaling pathways (i.e., complement C1q–C3 signaling, expression of P2Y12 receptors, fractalkine signaling, and release of ATP) have been reported to be upregulated following SE (Eyo et al., 2014, 2016, 2018; Abiega et al., 2016; Schartz et al., 2018). It will be interesting to see whether these signaling pathways are altered, resulting in impaired microglial phagocytosis in $mTOR^{Cx3cr1-cre}CKO$ mice. In addition, mTOR signaling regulates the cytoskeleton and related small G-protein activity (Sarbasov et al., 2004; Larson et al., 2010), which play a critical role in regulating cell migration and phagocytosis. This could be one of the mechanisms underlying impaired phagocytosis. Although phagocytosis activity in mTOR-deficient microglia is impaired, we did not see a significant change of synapse density in $mTOR^{Cx3cr1-cre}CKO$ mice. A possible explanation is that the engulfment of dying neurons may involve mechanisms that could be different from synapse pruning in the unperturbed brain, which perhaps reflects the difference in “find-me, eat-me, digest-me, and don’t-eat-me” signaling (Wyatt-Johnson and Brewster, 2020). Quantifying phagocytotic cups was used as a means to evaluate *in vivo* phagocytosis activity. We acknowledge that some of the phagocytotic cups could be microglia wrapping around injured neurons, but not necessarily reflecting complete phagocytosis per se.

mTOR deficiency reduces the microglial inflammatory response

mTOR deficiency reduces induction of the proinflammatory cytokines TNF- α and IL-1 β in microglia. Mechanistically, reduced induction of proinflammatory cytokines appears to echo low expression of the microglial activation marker CD68 in $mTOR^{Cx3cr1-cre}CKO$ mice. It is conceivable that the attenuated proinflammatory response observed in microglia of $mTOR^{Cx3cr1-cre}CKO$ mice is because of an intrinsic change of the inflammatory pathway in microglia as a result of mTOR deficiency or to curtailed exposure of microglia to inflammatory stimuli elicited by excitatory injuries (i.e., reduced contact or compromised phagocytosis of dead/dying neurons). Interestingly, microglial mTOR deficiency increases the induction of IL-6 in nonmicroglial cells. The exact source of IL-6 induction and its biological significance needs to be clarified in future studies.

Microglial mTOR deficiency exacerbates neuronal loss

We observed a significant increase in the loss of neurons following SE in $mTOR^{Cx3cr1-cre}CKO$ mice. However, mTOR inhibition by rapamycin was reported to attenuate the neuronal loss (Zeng et al., 2009; van Vliet et al., 2012). Several factors could contribute to these disparate outcomes. Rapamycin could inhibit mTOR in both neurons and microglia, whereas in our study mTOR activity was restrictively removed in microglia. In addition, $mTOR^{Cx3cr1-cre}CKO$ mice became more susceptible to SE compared with their littermate controls. We cannot rule out the possibility that severe seizures in $mTOR^{Cx3cr1-cre}CKO$ mice could account for the increased excitatory injury, leading to greater loss of neurons. However, in the present study, both control and $mTOR^{Cx3cr1-cre}CKO$ mice were subjected to careful dosing and experienced similar levels of SE. Also, the levels of initial

excitatory injury were comparable based on FJB and TUNEL staining at day 1 post-SE, but a significant increase in TUNEL staining was observed at days 3 and 7 post-SE in the mTOR^{Cx3cr1-cre}CKO mice, indicating that neuronal injury continues to exacerbate even 24 h after the initial excitatory injury, becoming more severe in mTOR^{Cx3cr1-cre}CKO mice than in control mice. At the same time, the proliferation and activation of microglia were significantly muted in mTOR^{Cx3cr1-cre}CKO mice. It is conceivable that engulfing or wrapping around the dying neurons before their lysis could be key to effectively containing the propagation of excitatory damage, thereby better maintaining CNS homeostasis. Compromised microglial phagocytosis could also lead to the accumulation of dying neurons in mTOR^{Cx3cr1-cre}CKO mice. A previous study reported that pharmacological inhibition of microglial proliferation by a colony-stimulating factor-1 inhibitor saves neurons from excitatory toxicity (Feng et al., 2019). In the present study, neuronal loss was exacerbated in mTOR^{Cx3cr1-cre}CKO mice, in which microglial proliferation was significantly muted. The discrepancy could reflect the involvement of different signaling pathways, resulting in opposite effects. While apoptotic neuronal death has long been reported in excitatory loss in epilepsy, we did not see any increase of cleaved caspase-3 staining, nor any increase of cleaved caspase-3 by Western blot analysis (data not shown). Our observation is in line with previous studies that cleaved caspase-3 is nearly undetectable in the pilocarpine model (Varvel et al., 2016) and its positivity is <10% in TUNEL⁺ neurons in a kainate model (Araki et al., 2002). All of these studies suggest that pyramidal cell death may involve mechanisms other than classic apoptosis (Dingledine et al., 2014). SE triggers a significant infiltration of Cx3cr1⁺ monocytes into the brain, despite the fact that resident microglia are mainly proliferated in the hippocampus (Varvel et al., 2016; Tian et al., 2017; Feng et al., 2019). We acknowledge that the overall effect could be a mix of inputs from both microglia and macrophages.

A counterintuitive role of microglial mTOR in epileptogenesis of acquired epilepsy

Studies over the past decade have brought compelling evidence that aberrant activation of the mTOR pathway is strongly epileptogenic. Gain-of-function mutations, due either to inherited mutation or to somatic mutation, account for the abnormal brain development that forms the basis of genetic epilepsy in humans (Wong, 2013; D’Gama et al., 2015; Jansen et al., 2015; Crino, 2016; Talos et al., 2018). Modeling hyperactivation of mTOR either in neurons or glial cells in various animal models is also epileptogenic (Uhlmann et al., 2002; Meikle et al., 2007; Orlova et al., 2010; Feliciano et al., 2011; Sunnen et al., 2011; Carson et al., 2012; McMahon et al., 2012; Zhang et al., 2016; Zhao et al., 2018). In addition, the mTOR inhibitor rapamycin attenuates the development of epilepsy, reduces seizure frequency, and suppresses mossy fiber sprouting (Buckmaster et al., 2009; Zeng et al., 2009; Raffo et al., 2011; Sunnen et al., 2011; Huang et al., 2012; Talos et al., 2012). However, the effect of rapamycin is not always consistent (Buckmaster et al., 2009; Zeng et al., 2009; Gericke et al., 2020). The broad presence of mTOR signaling in brain cells could prevent mTOR inhibitors from achieving a net therapeutic effect. Notably, previous studies have focused on gain-of-function mTOR mutations, so it will be interesting to determine the impact of loss-of-function mTOR mutations in epilepsy.

While microglial-specific inactivation of mTOR activity provides a much finer granularity of mTOR signaling in epileptogenesis, genetic approaches also have some inherent limitations,

such as a change in baseline. In the present study, we did not use the Cx3cr1-CreER inducible line because this line has spontaneous leakage in microglia before tamoxifen treatment (Zhang et al., 2018; Zhao et al., 2018, 2019). Microglial deletion of mTOR causes a moderate increase of seizure susceptibility to pilocarpine. Therefore, we carefully performed pilocarpine dosing so as to induce comparable levels of initial excitatory injuries between control and mTOR^{Cx3cr1-cre}CKO mice. We acknowledge that, apart from an epileptogenic role of the muted response of mTOR-deficient microglia to excitatory injury, we cannot rule out a contribution from changes to neurons as a result of microglial mTOR deletion. However, both possibilities point to the same conclusion: that deficiency of mTOR signaling in microglia increases seizure susceptibility.

In summary, our study revealed that the activation of microglial mTOR is neuronal protective and antiepileptogenic in an acquired epilepsy model. Our finding is a significant departure from the prevailing tenet that excessive activation of mTOR is epileptogenic and explains why the effect of rapamycin is not consistent or even paradoxical in previous studies. The results from our study could better guide the use of rapamycin in treating or preventing epilepsy.

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