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Mechanistic target of rapamycin complex 1 and 2 in human temporal lobe epilepsy

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Abstract

Objective—Temporal lobe epilepsy (TLE) is a chronic epilepsy syndrome defined by seizures and progressive neurological disabilities, including cognitive impairments, anxiety and depression. Here, human TLE specimens were investigated focusing on the mechanistic target of rapamycin (mTOR) Complex 1 (mTORC1) and Complex 2 (mTORC2) activities in the brain, as both pathways may represent unique targets for treatment.

Methods—Surgically resected hippocampal and temporal lobe samples from therapy-resistant TLE patients were analyzed by Western blotting to quantify the expression of established mTORC1 and mTORC2 activity markers and upstream or downstream signaling pathways involving the two complexes. Histological and immunohistochemical techniques were used to assess hippocampal and neocortical structural abnormalities and cell-specific expression of individual biomarkers. Samples from patients with focal cortical dysplasia (FCD) type II served as positive controls.

Results—We found significantly increased expression of phospho-mTOR (Ser2448), phospho-S6 (Ser235/236), phospho-S6 (Ser240/244) and phospho-Akt (Ser473) in TLE samples compared to controls, consistent with activation of both mTORC1 and mTORC2. Our work identified the

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AUTHOR CONTRIBUTIONS

D.M.T. designed the experiments, acquired and analyzed data, interpreted the results and wrote the paper. L.M.J., S.G. C.A.C., K.C.L., K.A.D., and M.M.L. acquired and analyzed data, interpreted the results and participated in study design and manuscript preparation. H.S., T.H.L. and F.E.J. participated in study design, interpretation of the results and manuscript preparation.

POTENTIAL CONFLICT OF INTEREST

None of the authors has any conflict of interest to disclose.

PI3K and Ras/ERK signaling pathways as potential mTORC1 and mTORC2 upstream activators. In addition, we found that overactive mTORC2 signaling was accompanied by induction of two Akt-dependent pro-survival pathways, as evidenced by increased inhibitory phosphorylation of FoxO3a (Ser253) and GSK-3 β (Ser9).

Interpretation—Our data demonstrate that mTOR signaling is significantly dysregulated in human TLE, offering new targets for pharmacologic interventions. Specifically, clinically available drugs that suppress mTORC1 without compromising mTOR2 signaling, such as rapamycin and its analogs, may represent a new group of antiepileptogenic agents in TLE patients.

INTRODUCTION

Epilepsy is a chronic neurological disorder characterized by recurrent seizures and caused by both genetic and acquired etiologies. More than 50% of patients with epilepsy suffer from cognitive and neuropsychiatric co-morbidities, including memory and attention impairments, psychosis, anxiety and depression(1). Current drugs reduce recurrent seizures, but do not address the cognitive and psychiatric conditions, or the underlying disease pathology. Temporal lobe epilepsy (TLE) is the most common form of epilepsy in adulthood, and also the most common drug-resistant epilepsy syndrome (2). A better understanding of the molecular mechanisms underlying epileptogenesis, including the associated cognitive and neuropsychiatric co-morbidities in TLE patients may provide novel opportunities for pharmacologic intervention.

The mechanistic target of rapamycin (mTOR) plays critical roles in normal brain function, and has recently emerged as a possible key signaling pathway implicated in epileptogenesis, including seizure-induced co-morbidities (3–10), and therefore a target for such treatments. Hyperactivation of mTOR signaling has been associated with several human neurodevelopmental epilepsy disorders, such as Tuberous Sclerosis Complex (TSC) and focal cortical dysplasia (FCD) (11), but also observed in other epilepsy pathologies, including TLE and Rasmussen's encephalitis (12–14).

mTOR forms two structurally and functionally distinct complexes (Figure 1), called mTORC1 and mTORC2 (15, 16). mTORC1 signaling is inhibited by rapamycin and its derivatives, while mTORC2 is insensitive to such treatments. The rapamycin-sensitive mTORC1 promotes protein synthesis, cell growth and proliferation through activation of ribosomal protein S6 kinases (S6K1/2) and inhibition of the eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1). The mTORC1 also induces lipid and nucleotide synthesis and suppresses autophagy, a process by which unnecessary or dysfunctional cellular components are degraded (15, 16). The rapamycin-insensitive mTORC2 promotes cell survival through activation of Akt/protein kinase B (PKB) (17), which in turn phosphorylates and inactivates several pro-apoptotic factors such as the transcription factor Forkhead box class O3a (FoxO3a) and the glycogen synthase kinase 3 beta (GSK-3 β) (18, 19). In addition, mTORC2 is a key regulator of the actin cytoskeleton (16).

The activities of mTORC1 are mainly regulated by the phosphatidylinositol-3-kinase (PI3K)/Akt and Ras/extracellular signal-regulated kinase 1 and 2 (ERK1/2) signaling pathways, which can be induced by insulin, growth factors, neurotransmitters or cytokines

(15). mTORC2 is also activated by PI3K through a currently unknown mechanism (Figure 1). The induction of mTORC1 and mTORC2 signaling in neurons is activity-dependent (20). Importantly, seizures and status epilepticus (SE) increase mTORC1(3–5, 7–10, 21–24), while transiently inhibiting mTORC2 signaling (25).

Despite compelling experimental evidence supporting a role for mTORC1 in promoting epileptogenesis, its activation status in human drug-resistant TLE has been only demonstrated by immunohistochemical analysis (12–14). Furthermore, no studies to date have evaluated the activation status of mTORC2 signaling in human TLE brain, although several pre-clinical studies suggest that diminished mTORC2-dependent negative regulation of pro-apoptotic pathways may be responsible for acute seizure-induced neuronal death (25–27). Therefore, here we examined the activation status of both mTORC1 and mTORC2 in hippocampus and temporal lobe cortex from therapy-resistant TLE patients and control cases by combining quantitative Western blot analysis and immunohistochemistry. We also characterized potentially targetable upstream mechanisms leading to mTOR dysfunction, and defined downstream pro-survival signaling events linked to altered mTORC2 function. Given the central role of mTOR in oncogenesis, a growing number of promising agents targeting these pathways are entering phase I-III clinical trials for various cancers. As these agents may represent new therapeutic tools for epileptic brain disorders, there is a great need for better understanding of the mechanisms governing mTOR signal transduction in the context of chronic epilepsy.

MATERIALS AND METHODS

Human Subjects

The temporal lobe epilepsy study population (Supplemental Table 1) consisted of 14 patients who had undergone surgery for resection of epileptogenic region at the University of Pennsylvania, Philadelphia, PA from March 2013 to March 2016 (10 females and 4 males). Cortical specimens from two male patients with chronic epilepsy due to hemimegalencephaly (HMEG) and focal cortical dysplasia (FCD) were also included and served as positive controls. All patients were prospectively enrolled in the study, and informed consent for use of brain tissue and for access of medical records was obtained from all individuals prior to surgery. The study was reviewed and approved by the local Institutional Review Board. The pre-surgical data and post-operative outcomes were reviewed by a board certified neurologist (K.A.D.). All surgeries were performed by the same neurosurgeon (T.H.L.) and consisted of temporal lobectomy, including *en bloc* excision of the hippocampal formation, amygdala and the parahippocampal gyrus. All surgical specimens were reviewed independently from the primary clinical review process by the same neuropathologist (M.M.L.) and the criteria for hippocampal sclerosis (HS) were based on the current International League Against Epilepsy (ILAE) classification (28). The cortical dysplasia type was assigned based on the ILAE consensus classification (29). The control group consisted of six autopsy cases without known neurologic or psychiatric history, including epilepsy (3 females and 3 males). The specimens were obtained from the Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD, and were age and region-matched to the epilepsy samples. The autopsy was

performed within 6–18 hours of death (mean 11.8 hours). We specifically limited our study to cases with a postmortem interval (PMI) less than 24 hours, as we have previously reported no effect of PMI on mTORC1 and mTORC2 activation markers within this time interval (30, 31). All control cases were assessed by the same neuropathologist and were diagnosed as histologically normal (Figure 3A–B).

Tissue preparation

At the time of surgery, hippocampal and temporal lobe specimens were divided into representative tissue blocks, fixed in 10% formalin, embedded in paraffin and used for standard histopathologic diagnostic evaluation. Subsequently, blocks with representative pathology and good tissue preservation were selected for additional immunohistochemistry. Adjacent samples were either fixed in 4% paraformaldehyde for immunofluorescence double labeling, or immediately frozen for Western blot analysis. Autopsy tissue blocks were received either fixed in 10% formalin or frozen.

Immunohistochemistry

Paraffin-embedded TLE and control samples were sectioned at 6µm. Immunohistochemistry was performed using antibodies against human non-phosphorylated neurofilament RMD020, phosphorylated neurofilament TA51, GFAP, CD68, CD163, phospho-S6 (Ser235/236), phospho-S6 (Ser240/244) and phospho-Akt (Ser473) (all listed in Supplemental Table 2). Staining was done on a Leica Bond-III™ instrument using the Bond Polymer Refine Detection System (Leica Microsystems DS9800). Heat-induced epitope retrieval was performed for 20 minutes in either ER1 (Leica Microsystems AR9961) or ER2 solution (Leica Microsystems AR9640). Slides were incubated with the primary antibodies for 15 min, followed by 8 min incubation with post primary IgG and polymeric HRP-linker antibody conjugates. Slides were then incubated with hydrogen peroxide for 5 min, followed by 10 min 3,3'-diaminobenzidine tetrahydrochloride hydrate (DAB) exposure. The nuclei were lightly counterstained with hematoxylin and slides were then dehydrated and mounted with a permanent medium. All experiments were performed at room temperature (RT). Negative controls, where the primary antibodies were omitted, were included in each run to exclude unspecific binding. The immunofluorescence double labeling was carried out on 4% paraformaldehyde (PFA)-fixed tissue to avoid potential artifacts due to tissue autofluorescence caused by formalin fixation. Fresh tissue samples were submerged in PFA overnight at 4°C, cryoprotected in 30% sucrose for at least 48 hours, embedded in Tissue-Tek OCT, sectioned at 20 µm on a Leica Cryostat CM 3050S (Leica Microsystems, Nussloch, Germany) and subjected to antigen retrieval using Citrate Buffer (pH 6.0, Abcam, Cambridge, MA) for 10 minutes in a 95°C water bath (4, 30, 31). Additional frozen tissue blocks embedded in OCT were cut at 20 µm and tissue was post-fixed by incubating the slides in 4% PFA for 10 minutes at –20°C. Sections were blocked for 1 hour at RT in phosphate buffered saline (PBS) containing 5% normal goat serum (Equitech-Bio, Kerrville, TX) and 0.1% Triton. Primary antibodies (listed in Supplemental Table 2), diluted in the same blocking solution, were applied overnight at 4°C. Additional sections were incubated without primary antibodies to exclude false positive labeling. Subsequently, slides were incubated for 1 hour at RT with the Alexa 488-conjugated rabbit and Alexa 568-conjugated mouse secondary antibodies (1:1000, Molecular Probes, Eugene, OR), then mounted with

DAPI Fluoromount-G (SouthernBiotech, Birmingham, AL). Slides were viewed using a Nikon Eclipse 80i microscope. Images were captured with either a Retiga 2000R or a Nikon DS-Fi2 digital camera, using the NIS-Elements BR software 3.0 (Micro Video Instruments).

Western blotting

Frozen TLE and control samples were homogenized in glass Dounce homogenizer on ice in lysis buffer containing 100ul of Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific, Rockford, IL) and 100 µl of 100mM PMSF. Whole cell, cytosol and nuclear fractions were prepared using standard procedures (4, 30, 31). Total protein amounts were measured with a Bradford protein assay (Bio-Rad, Hercules, CA) and samples were then diluted to equal amounts of protein in each sample in lysis buffer and 4x Laemmli Sample Buffer (Bio-Rad). Samples were run on 4–20% Tris-Glycine precast gels (Bio-Rad) at 115 V for 1.5 hours and transferred overnight at 4°C onto polyvinylidene difluoride membranes (Millipore, Temecula, CA). Membranes were then blocked in 5% nonfat dry milk (LabScientific, Highlands, NJ) and then incubated overnight at 4°C with the primary antibodies listed in Supplemental Table 2. Corresponding horseradish peroxidase-conjugated secondary antibodies (1:2000; Vector, Burlingame, CA) were applied for 1 hour at RT. Protein bands were visualized by chemiluminescence using an Image Reader LAS 3000 digital system (Fuji Film Life Sciences, Stamford, CT) and quantified with Image Quant TL software (Fuji Film Life Sciences). Actin, GAPDH and lamin A/C were used to control for differences in protein loading, and normalized values were expressed as percentage of the mean control samples run on the same blot. Phosphorylated proteins to total protein ratios were calculated in a similar manner.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism v.6.0b software (GraphPad Software Inc., San Diego, CA). Group means were compared by unpaired t tests, while Pearson correlation analysis was used to establish potential associations between variables. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Clinical and neuropathological characterization of study population

The mean age at surgery in the TLE group (Supplemental Table 1) was 35.6 years (range 20–56 years), with an average age at seizure onset of 19.1 years (range 1–36 years) and a mean duration of epilepsy of 16.5 years (range 2–42 years). The predominant seizure types were medically intractable focal onset, impaired awareness seizures (FOIA, $n=13$), followed by focal onset, aware (FOA, $n=10$) and focal to bilateral tonic clonic seizures (FBTC, $n=10$). All but one patient experienced more than one seizure type at surgery. All TLE patients were on poly-therapy with two or more antiepileptic drugs (AEDs). All but two patients had undergone intracranial EEG (iEEG) monitoring, including depth electrode placement for seizure onset localization, which revealed either an amygdalo-hippocampal ($n=7$) or a neocortical focus ($n=5$). The majority of TLE patients (10 out of 14) presented epilepsy risk factors, including head trauma ($n=5$), a family history of epilepsy or febrile seizures ($n=5$), dual pathology (primary lesion within the ipsilateral temporal lobe or hemisphere (28) ($n=2$),

status post-craniopharyngioma resection (n=1), febrile seizures (n=1) and co-morbid autism (n=1). Hippocampal histopathology assessment revealed hippocampal sclerosis (HS) in 9 out of 14 patients, while the remaining 5 patients presented only with gliosis or mild neuronal loss and were classified as no-HS (28). The examination of the temporal lobe cortex revealed mild to moderate subpial and cortical gliosis. Seizure outcome was assessed conform to Engel's classification (32). The mean age at surgery for the FCD cohort was 46.5 years (45 and 48 years), with an average duration of epilepsy of 39.5 years (45 and 34 years). Both specimens originated from temporal lobe neocortex, and both surgeries led to Engel class I outcomes (Supplemental Table 1). The histopathological features were consistent with FCD type IIa and FCD type IIb, respectively. The mean age of the control group was 38.5 years (range 19–58 years), which did not differ significantly from the TLE group (p=0.58). All control subjects died of non-neurological causes: arteriosclerotic cardiovascular disease (n=3), asthma (n=1), acute pancreatitis (n=1) and asphyxia by hanging (n=1).

Increased mTORC1 and mTORC2 activation in human TLE

First we used Western blot analysis to assess the phosphorylation of mTOR itself using antibodies against phospho-mTOR (Ser2448). We found that the ratios of p-mTOR (Ser2448)/mTOR were significantly increased in TLE patients compared to non-epilepsy controls, both in the hippocampus (Figure 2A–B) and temporal lobe cortex (Figure 2F–G). To determine the activation state of individual complexes, we next used antibodies against established mTORC1 and mTORC2 specific substrates (15). Levels of mTORC1 activation were measured by assessing the phosphorylation of ribosomal protein S6 using antibodies against phospho-S6 (Ser235/236) and phospho-S6 (Ser240/244). Both p-S6 (Ser235/236)/S6 and p-S6 (Ser240/244)/S6 ratios were significantly increased in TLE hippocampus (Figure 2A, C–D) and temporal lobe cortex (Figure 2F, H–I), compared to controls. To determine mTORC2 activity, we measured p-Akt (Ser473) levels and similarly found significantly increased p-Akt (Ser473)/Akt ratios in TLE relative to controls, both in the hippocampus (Figure 2A, E) and cortex samples (Figure 2F, J). As expected, cortical tissue from FCD type II cases showed increased mTOR (Ser2448) phosphorylation and enhanced activation of downstream proteins related to both mTORC1 and mTORC2 pathways (Figure 2F–J) (33–40).

Next, we performed correlation analyses in TLE patients to establish potential associations of these markers with clinical variables (Table 1). Hippocampal tissue analysis demonstrated no association between epilepsy duration and the expression levels of p-mTOR (Ser2448), or specific mTORC1 and mTORC2 activation markers. In addition, no statistically significant differences were observed with respect to the underlying hippocampal pathology. However, we found that both p-S6 (Ser240/244)/S6 and p-Akt (Ser 473)/Akt levels were significantly higher when the seizure focus was located within the mesial temporal lobe structures, as opposed to the neocortex. Although we found robust mTORC1 and mTORC2 induction in temporal lobe cortex, no relationships with clinical variables were observed within this brain region.

Immunostaining of hippocampal tissue with p-S6 (Ser235/236) and p-S6 (Ser240/244) showed weak immunoreactivities throughout all hippocampal subfields in the control group (Figure 3C–D), in contrast to strong labeling of pyramidal neurons in the subiculum and CA1-CA4 regions in TLE cases (Figure 3E–F). The granule cell layer (GCL) demonstrated less reactivity for both p-S6 antibodies, consistent with previous reports (12, 14). Non-neuronal labeling was quite evident in all TLE cases (Figure 3G–O), in particular for the p-S6 (Ser235/236) antibodies. Many of these cells co-labeled with CD68 or GFAP, specific cell markers of activated microglia and astrocytes, respectively (Figure 3K and 3N – insets).

Immunohistochemistry for mTORC2 activity marker p-Akt (Ser473) demonstrated low expression levels in control hippocampal tissue (Figure 4A–C). In contrast, we saw increased nuclear and cytoplasmic neuronal labeling in TLE cases, both in non-sclerotic (Figure 4D–F) and sclerotic (Figure 4G–I) hippocampus samples. Altered p-Akt (Ser473) subcellular localization in TLE hippocampal tissue was confirmed by additional Western blotting after separation of protein extracts into cytoplasmic and nuclear fractions ($8595 \pm 552\%$ of control, $p < 0.0001$ in the cytoplasm; $325 \pm 56\%$ of control, $p < 0.05$ in the nucleus; data not shown). We also observed robust p-Akt (Ser473) immunoreactivity in activated microglia (Figure 4J–L), and to some extent in reactive astrocytes (Figure 4M–O). In the temporal lobe cortex, we saw a similar low expression for p-S6 (Ser235/236), p-S6 (Ser240/244) and p-Akt (Ser473) in controls, but increased neuronal labeling in TLE cases (data not shown). For all markers, the staining was most prominent in layer III and V pyramidal neurons. Similar to the hippocampal formation, we confirmed activated mTORC1 and mTORC2 signaling in cortical microglia and astrocytes by double label immunohistochemistry for p-S6 (Ser235/236) or p-Akt (Ser473) and the corresponding cell markers (data not shown).

The PI3K and Ras/ERK signaling pathways are induced in human TLE

The PI3K and Ras/ERK pathways represent two major signaling transduction pathways that can activate mTOR. Since both signaling pathways can be activated by seizures (4, 41), we sought to determine whether these patterns seen in animal models are also present in humans. As the protein kinase activities of both mTORC1 and mTORC2 are increased by PI3K activation, we first carried out Western blot analysis of TLE and control tissue homogenates to determine the expression of phospho-Akt (Thr308), as this site is specifically phosphorylated upon PI3K activation by phosphoinositide-dependent kinase 1 (PDK1) (15). We found a significant increase in phospho-Akt (Thr308)/Akt ratios in the TLE hippocampus (Figure 5A–B), but no significant changes in the temporal lobe cortex relative to non-epilepsy controls (Figure 5D–E). We next assessed the expression levels of phospho-ERK1/2 (Thr202/Tyr204), two residues that are specifically targeted by the ERK kinases MEK1 and MEK2, downstream from Ras (15). Western blot quantification of phospho-ERK1/2 (Thr202/Tyr204)/ERK ratios showed significant increases in TLE cases, both in the hippocampus (Figure 5A, C) and temporal lobe cortex (Figure 5D, F). As further shown in Figure 5D–F, the FCD type II cortical specimens presented a more variable PI3K and Ras/ERK induction, when compared to TLE cortex.

Subsequent correlation analyses in TLE patients demonstrated no association between hippocampal levels of p-Akt (Thr308)/Akt and epilepsy duration or underlying pathology; however, we found that hippocampal p-Akt (Thr308)/Akt levels were significantly higher in cases with mesial temporal onset, consistent with the results obtained regarding mTORC1 and mTORC2 activation markers (Table 1). In contrast, hippocampal p-ERK1/2 (Thr202/Tyr204)/ERK levels correlated strongly with the duration of epilepsy, but we found no statistically significant differences with respect to seizure focus location, or the presence or absence of HS. Again, cortical tissue analysis revealed no relationships with clinical variables.

Double label immunohistochemistry for p-Akt (Thr308) and p-ERK1/2 (Thr202/Tyr204), along with the neuronal marker NeuN, showed a similar regional and cell-specific expression pattern. In the hippocampus, we found strong labeling of the subiculum and CA1-CA4 regions, but lower expression in the GCL (Figure 5G–O; only p-ERK is shown). In the temporal lobe cortex, both antibodies showed robust labeling of layer III and V pyramidal neurons (data not shown). In addition, within both brain regions, there was widespread p-Akt (Thr308) and p-ERK1/2 (Thr202/Tyr204) expression in non-neuronal cells, in a pattern similar to mTORC1 and mTORC2 activity markers.

Decreased FoxO3a signaling in human TLE

To further investigate potential downstream effects of overactive mTORC2 signaling in TLE tissue, we next evaluated the phosphorylation status and subcellular localization of FoxO3a, a key pro-apoptotic transcription factor that is suppressed by nuclear Akt signaling. Akt phosphorylates FoxO3a at several residues, including Ser253, which results in its nuclear export and subsequent suppression of pro-apoptotic genes transcription (42). Western blot quantification of TLE, FCD and control samples demonstrated significantly increased phospho-FoxO3a (Ser253)/FoxO3a ratios in TLE hippocampus and cortex (Figure 6A–D), but we observed only a modest increase in FoxO3a phosphorylation in FCD type II cases (Figure 6C–D). Interestingly, we found no correlations between p-FoxO3a (Ser253)/FoxO3a and epilepsy duration, seizure focus location, or hippocampal pathology in our TLE cohort (Table 1).

Double labeling for NeuN and FoxO3a revealed predominant cytoplasmic expression in both non-sclerotic (Figure 6E–G) and sclerotic (Figure 6H–J) hippocampus samples, consistent with a strong anti-apoptotic response in both conditions. Besides neurons, robust cytoplasmic FoxO3a expression was also noted in glial and endothelial cells. A similar expression pattern was observed in the temporal lobe cortex (data not shown).

Suppression of GSK-3 β signaling in human TLE

GSK-3 β represents another important downstream target of Akt. Phosphorylation of constitutively active GSK-3 β at Ser9 by Akt results in GSK-3 β inactivation (18). Similar to p-FoxO3a, Western blot analysis of TLE hippocampal and cortical samples demonstrated significantly increased phospho-GSK-3 β (Ser9)/GSK-3 β levels in both brain regions (Figure 7A–D). A comparable increase in p-GSK-3 β (Ser9)/GSK-3 β levels was observed in FCD type II cortical tissue. We found no statistically significant correlations with clinical

variables in TLE patients, however hippocampal tissue analysis showed a trend towards decreased p-GSK-3 β (Ser9)/GSK-3 β expression with longer duration of epilepsy (Table 1). Immunohistochemistry for p-GSK-3 β (Ser9) and NeuN demonstrated robust neuronal and non-neuronal expression throughout all hippocampal sectors, without any noticeable differences in p-GSK-3 β (Ser9) expression pattern between the non-sclerotic (Figure 7E–G) and sclerotic (Figure 7H–J) hippocampus, but consistent with the Western blot data, diminished p-GSK-3 β (Ser9) expression with longer epilepsy history. In the temporal lobe cortex, phospho-GSK-3 β (Ser9) expression was expressed predominantly in layer III and V pyramidal neurons and in the blood vessels endothelium (data not shown).

DISCUSSION

Our study provides the first quantitative analysis of numerous steps in mTOR-related signaling in human therapy-resistant TLE, as opposed to previous work, which was limited to immunohistochemical analyses of mTORC1 downstream signaling (12–14), without quantitative comparisons of major components of the mTORC1 and mTORC2 pathways. Here we show that both complexes are highly activated in TLE, and that these changes are not confined to the primary seizure focus, but involve the anterior temporal lobe structures more broadly. Nevertheless, our data show that activation of both mTORC1 and mTORC2 is more prominent in the brain regions where the electrographic seizure activity is greatest. In addition, our results establish for the first time that two neuroprotective signaling pathways downstream from mTORC2, the Akt/FoxO3a and the Akt/GSK-3 β pathways, are strongly induced in TLE. Our work also identifies PI3K and Ras/ERK1/2 signaling pathways as upstream activators of mTORC1 and mTORC2, and as potential new targets for treatment. Importantly, our data demonstrate that many of the same molecular changes reported in pre-clinical studies during different stages of epileptogenesis are still present in human chronic TLE. The duration of epilepsy varied greatly among our patients (2–42 years), yet we found no correlation between epilepsy duration and mTORC1 or mTORC2 activity levels, suggesting an extended window for antiepileptogenic and neuroprotective therapeutic interventions. We also acknowledge several inherent limitations, common to all epilepsy human tissue studies. First, our study cannot establish whether these differences represent intrinsic brain properties that precede the development of epilepsy, or reflect molecular responses to seizures. In addition, we cannot exclude independent effects of anti-epileptic drug treatment, as due to the small number of cases we were unable to analyze this statistically.

The mTORC1-dependent protein synthesis and its role in synaptic development and plasticity are well established, suggesting that altered mTORC1 signaling in TLE may have a profound impact on brain function and contribute to some of its cognitive comorbidities. Developing neurons require mTORC1 activity for dendritic and axonal growth (30, 43–45) and for the establishment of proper excitatory/inhibitory (E/I) balance (31, 46–48). Neurons also require mTORC1 activity for long term synaptic potentiation (LTP) and long term depression (LTD), two activity-driven processes involved in learning and memory (49–52).

The mTORC1 is activated in experimental epilepsy models, most prominently in the hippocampus, but also in other areas of the brain, such as neocortex and amygdala (4, 8–10,

12, 21–23). Activation of mTORC1 occurs within hours following SE induction, and depending on the model, persists up to several weeks or even months after SE (5, 8–10, 12). The initial increase in mTORC1 activity (the first 24h) occurs mostly in neurons, and is followed by sustained activation in glial cells (4, 5, 10, 22).

The synaptic re-organization of the hippocampal circuitry, in particular those connections involving the hippocampal dentate GCL, is thought to play a causative role in TLE (53, 54). Several TLE models have shown upregulated mTORC1 activity in the GCL (12, 22, 55), suggesting that mTORC1 may promote cellular hypertrophy, induce sprouting of GC axons (mossy fibers), enhance basal dendrites formation and lead to aberrant hippocampal neurogenesis, which in turn may promote epileptogenesis. Indeed, rapamycin can effectively suppresses post-SE GCL hypertrophy and mossy fiber sprouting, preventing the establishment of recurrent excitation in the dentate gyrus and the development of epilepsy (8–10, 21, 56, 57). Remarkably, delayed rapamycin treatment, initiated after the establishment of epilepsy is still able to reduce seizure activity and mossy fiber sprouting (9), but the seizure-suppressing effects are transient. In addition, rapamycin can prevent the sprouting of CA3/mossy cells axons onto the proximal dendrites of granule cells (58), suggesting much broader drug effects on post-SE hippocampal synaptic reorganization. Noteworthy, rapamycin can also suppress the sprouting of inhibitory hilar neurons axons, which may possibly have negative net effects on hippocampal hyperexcitability (59). Besides inhibiting mossy fiber sprouting, rapamycin has little or no effect on other SE-induced GCL phenotypes, including basal dendrite formation and ectopic granule cell generation (57, 60, 61), which may explain the negative results on seizure prevention in some models (57, 60).

Another potential mechanism by which increased activation of mTORC1 within the temporal lobe structures may promote epileptogenesis is by altering the expression and function of neurotransmitter receptors and ion channels, as previous studies have demonstrated that rapamycin treatment prevented seizure-induced enhancement of glutamatergic function (4) and reversed several ion channels abnormalities in hippocampal neurons (5).

Besides altered protein synthesis, defects in autophagic clearance of cellular proteins and organelles due to mTORC1 activation may also be involved in epileptogenesis, although the exact downstream mechanisms remain to be elucidated. Impaired autophagy may contribute to neurodegeneration and cell death (62), or may increase neurotransmitter release, as local presynaptic autophagy plays an important role in degradation of synaptic vesicles (63).

The observation that reactive astrocytes and activated microglia have sustained p-S6 expression within the temporal lobe structures (in particular within the sclerotic hippocampus) (13), suggests a possible role for glial mTORC1 signaling in promoting epileptogenesis. Indeed, rapamycin was able to reduce SE-induced hippocampal astrogliosis and microgliosis (5), while deletion of mTOR in reactive astrocytes in mice with chronic epilepsy effectively suppressed seizures, without affecting mossy fiber sprouting (64).

Dysfunction of mTORC1 signaling has also been implicated in the pathophysiology of several cognitive disorders (62, 65, 66), and there is evidence to suggest that it may also contribute to epilepsy co-morbidities. Indeed, chronic rapamycin treatment markedly diminished aggressive behavior and cognitive impairments in several TLE models (5, 6). Similarly, in models of early life seizures, short-term mTORC1 inhibition was not only antiepileptogenic, but also highly effective in preventing the associated cognitive decline and autistic-like behavior (4, 7).

While there is abundant data implicating mTORC1 in epilepsy and its co-morbidities, along with extensive pre-clinical experience with mTORC1 inhibitors, information regarding mTORC2 and its relevance to epileptogenesis is still relatively sparse. The mTORC2 pathway seems to be particularly important for neuronal survival under a wide variety of circumstances, an effect mainly mediated by inhibitory phosphorylation of FoxO3a (25, 67, 68). Indeed, cells with disruption of mTORC2 signaling are more sensitive to apoptosis (67), while constitutive mTORC2 activation in the majority of cancers confers therapy resistance (69). Besides promoting FoxO3a-dependent anti-apoptotic responses, mTORC2 signaling may likely induce a number of neuroprotective mechanisms through the negative regulation of GSK-3 β . GSK-3 β phosphorylates tau protein making it insoluble, which can lead to neuronal death and degeneration (70). While hyperphosphorylation of tau is mainly known as an Alzheimer's disease-associated phenotype, recent studies have revealed pathologic tau phosphorylation in several other neurologic diseases, including epilepsy (71–73). In addition, GSK-3 β can inhibit the antioxidant cell responses by directly phosphorylating the transcription factor Nrf2, a master regulator of redox homeostasis (74), and promote pro-inflammatory and pro-apoptotic responses in the brain (75, 76).

Our novel finding that mTORC2 signaling is upregulated in TLE is of particular interest, as seizure-induced oxidative stress, neuroinflammation, neuronal death and degeneration have been postulated as important mechanisms underlying the epileptogenic process (77, 78). Our data shows that in human TLE tissue, activated Akt translocates to the nucleus, where it phosphorylates FoxO3a leading to its nuclear export. Interestingly, neither the degree of mTORC2 activity, nor of FoxO3a inhibition correlated with epilepsy duration or the severity of hippocampal pathology, suggesting that FoxO3a-independent mechanisms may have contributed to cell death in our cases (79, 80).

In addition, we found that GSK-3 β is highly inhibited in neurons and glia by the mTORC2-Akt regulatory pathway, and therefore neurons may be protected from tau-induced neurodegeneration, oxidative stress and excessive production of pro-inflammatory mediators. However, we saw a trend towards decreased GSK-3 β phosphorylation with longer epilepsy duration, which suggests that overtime GSK-3 β inhibition may become insufficient. Therefore, therapies directed towards further activation of mTORC2 in TLE may be critical for improved neuronal survival, and therefore may represent a novel strategy for disease-modification.

To date, there are several studies reporting altered mTORC2 activity in response to seizures and SE. However, it appears that mTORC2 activity levels are highly dependent on the severity and type of insult, in contrast to mTORC1, which is activated post-SE across

models. For example, brief seizure episodes that are not associated with significant neuronal injury, such as those induced by pentylenetetrazole, result in mTORC2 activation, as evidenced by rapid increases in Akt Ser473 phosphorylation (41). In contrast, in the kainic acid or pilocarpine models, two paradigms that result in acute and chronic injury to the hippocampus, there is an initial robust suppression of mTORC2 activity, persisting up to 1–3 days post-injury, which correlates temporally with elevated Bim expression, a read-out of FoxO3a transcriptional activity, increased apoptotic cell death and GSK-3 β –dependent tau hyperphosphorylation (25–27, 81, 82). At the same time, brain regions that typically do not undergo cell death and degeneration in these models (such as hippocampal CA1 and neocortex) display a strong induction of mTORC2 (27).

In contrast, during the latent period and the chronic epilepsy stages, there is increased mTORC2 activity (5, 83, 84), suggesting that endogenous mechanisms directed to prevent seizure-induced neuronal loss and limit disease progression might be activated at later stages. The mechanism of this “acquired” mTORC2-mediated neuroprotection in chronic epilepsy is not known, but there is evidence that prolonged synaptic activity, in particular activation of synaptic NMDA receptors can induce such changes (85, 86), raising the intriguing possibility that milder seizures may render temporal lobe structures less susceptible to further neuronal loss.

In addition to conferring neuroprotection, mTORC2 regulates several proteins involved in actin cytoskeleton rearrangement, including the protein kinase C- α (PKC- α) and Ras-related C3 botulinum toxin substrate 1 (Rac1) (87, 88). Importantly, by modulating actin dynamics, mTORC2 promotes LTP and memory formation, which further underscores the importance of preserving or activating mTORC2 to enhance memory, or reverse memory loss in TLE (87, 89).

Another important finding of our study is the robust activation of ERK signaling in both hippocampus and temporal cortex of patients suffering from therapy-resistant TLE, consistent with previous studies showing upregulated ERK signaling in several neocortical epilepsy syndromes, including TSC and FCD (90–92). ERK is upstream of mTORC1 and both kinases are simultaneously induced at activated synapses to regulate local protein synthesis (20). In addition to activating mTOR, ERK also regulates the activity of cAMP response element binding protein (CREB), and therefore may be critically involved in seizure-induced dysregulated gene expression (91). In animal models, ERK signaling is activated in a time dependent manner following seizures (4, 84, 93), and augmented ERK signaling has been directly linked with increased seizure susceptibility (94). Our finding that levels of activated ERK positively correlate with epilepsy duration, suggests that adjuvant therapies directed towards ERK inhibition may be of great clinical benefit, especially at later stages.

In this study, FCD samples were used as a positive control group and, indeed, our study confirms previous reports showing strong induction of both mTORC1 and mTORC2 in these cases (33–40, 90). However, our FCD cohort data also revealed several distinct features with respect to upstream and downstream mechanisms. The upstream PI3K signaling did not appear to be activated, but rather suppressed in our FCD cohort, as suggested by lower p-Akt

(Thr 308) expression, which apparently contradicts previous studies (33, 37–40). However, the small number of samples analyzed here may be responsible for these differences. We also found that mTORC2 induction in FCD was accompanied by strong GSK-3 β inhibition, but it seemed insufficient to cause a robust suppression of FoxO3a signaling. A possible explanation for this discrepancy is that in FCD type II specimens, p-Akt (Ser473) is mainly expressed in the neuronal cytoplasm and not in the nucleus (33, 35, 38, 39).

Conclusion

Epileptogenesis involves activation of numerous signaling pathways that ultimately result in synaptic reorganization. The mTORC1 and mTORC2 pathways are clearly engaged in human TLE epileptogenesis. The strong neuroprotective mechanisms triggered by mTORC2 activation is an argument against using a total mTOR blockade by second-generation inhibitors targeting the mTOR catalytic site (dual mTORC1 and mTORC2 inhibitors). Our results are suggesting high responsiveness to mTORC1 inhibitors (rapamycin and rapalogs), which are already used for cancer therapy and are currently under investigation in TSC patients for seizure suppression. However, the chronic use of high dose rapalogs, in addition to blocking mTORC1 signaling, may also compromise mTORC2 function. Therefore, alternative approaches to selectively modulate mTORC1 and mTORC2 downstream activities, such as the use of p70S6K inhibitors, autophagy inducers, or GSK-3 β inhibitors, alone or in combination, seem to be preferable treatment options for epilepsy. Finally, negative regulation of selective pathways upstream of mTORC1 and mTORC2, such as accomplished by ERK inhibitors, may also represent promising approaches for treatment of refractory epilepsy in some patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Main upstream regulators and downstream effectors of mTORC1 and mTORC2 signaling pathways relevant to epilepsy

Neurotransmitters, growth factors, cytokines and hormones (insulin) activate their membrane-bound receptors to recruit the phosphatidylinositol-3-kinase (PI3K)/Akt and Ras/extracellular signal-regulated kinase 1 and 2 (ERK1/2) signaling pathways and activate mTORC1 and mTORC2. The PI3K pathway activates mTORC1 via phosphoinositide-dependent kinase 1 (PDK1)-mediated phosphorylation of Akt on its activation loop (Thr308). PI3K also activates mTORC2 through an unknown mechanism. Ras activates mTORC1 by a parallel pathway via activation of MEK1/2 (ERK kinases) and phosphorylation of ERK. The mTOR protein is part of both complexes and can undergo phosphorylation at multiple sites, including Ser2448. The mTORC1 controls protein synthesis through activation of ribosomal S6 kinases 1/2 (S6K1/2) and inhibition of eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1). S6K1/2 activate the ribosomal protein S6 via phosphorylation at Ser235/236 and Ser240/244. Of note, Ser 235/236 may also be phosphorylated by the p90 ribosomal S6 kinase (RSK), further downstream from ERK. The mTORC2 controls several neuroprotective signaling pathways via phosphorylation of Akt at Ser473, which together with the PDK1-mediated phosphorylation at Thr308 results in full Akt activation. Once activated, Akt translocate to various subcellular compartments, including the nucleus, where it phosphorylates the pro-apoptotic transcription factor Forkhead box class O3a (FoxO3a). Phosphorylation at Ser253 by Akt leads to rapid translocation of FoxO3a from the nucleus to the cytoplasm, preventing its binding to the promoter of apoptotic genes, with a net pro-survival effect. In the cytoplasm, Akt can phosphorylate the glycogen synthase kinase 3 beta (GSK-3β) at Ser9 inhibiting its kinase activity, and preventing the phosphorylation of further downstream targets, including the microtubule-binding protein tau and the transcription factor nuclear factor E2-related factor 2 (Nrf2).

Figure 2. The expression of mTORC1 and mTORC2 activation markers is increased in hippocampus and temporal lobe cortex from patients with therapy-resistant TLE compared to controls

A–E. Hippocampal tissue analysis showing (A) representative Western blot images for individual proteins and calculated ratios, expressed as % of control mean, for (B) phospho-mTOR (Ser2448)/mTOR (1640 ± 156 % in TLE, $n=8$ vs. 100 ± 13 % in controls, $n=5$), (C) phospho-S6 (Ser235/236)/S6 (823 ± 52 % in TLE, $n=11$ vs. 100 ± 13 % in controls, $n=5$), (D) phospho-S6 (Ser240/244)/S6 (1381 ± 294 % in TLE, $n=11$ vs. 100 ± 20 % in controls, $n=5$), and (E) phospho-Akt (Ser473)/Akt (1981 ± 362 % in TLE, $n=11$ vs. 100 ± 43 % in controls, $n=5$). **F–J.** Temporal lobe cortex analysis showing (F) the representative Western blot bands and quantitative analysis for (G) phospho-mTOR (Ser2448)/mTOR (447 ± 36 % in TLE, $n=12$ vs. 100 ± 24 % in controls, $n=5$ and 411 ± 97 % in FCD, $n=2$), (H) phospho-S6 (Ser235/236)/S6 (1383 ± 301 % in TLE, $n=10$ vs. 100 ± 13 % in controls, $n=6$ and 508 ± 283 % in FCD, $n=2$), (I) phospho-S6 (Ser240/244)/S6 (2098 ± 399 % in TLE, $n=13$ vs. 100 ± 12 % in controls, $n=6$ and 1234 ± 154 % in FCD, $n=2$), and (J) phospho-Akt (Ser473)/Akt (2106 ± 162 % in TLE, $n=10$ vs. 100 ± 25 % in controls, $n=6$ and 955 ± 199 % in FCD, $n=2$). *, **, ****: Denotes $p < 0.05$, $p < 0.01$ and $p < 0.0001$, respectively (unpaired t tests comparing non-epilepsy control and TLE groups). Error bars indicate S.E.M.

Figure 3. The mTORC1 activity markers phospho-S6 (Ser 235/236) and phospho-S6 (Ser 240/244) are highly upregulated in the TLE hippocampus

A–B. Representative hippocampal serial sections from a 19 year old control case stained with (A) hematoxylin & eosin (H&E) and (B) immunohistochemically labeled with non-phosphorylated neurofilament RMD020 indicating the regions of interest used for all analyses: subiculum (SUB), Cornu ammonis sectors (CA1-CA4) and the dentate granule cell layer (GCL). **C–D.** Control hippocampus showing low expression of mTORC1 activation markers (C) phospho-S6 (Ser 235/236) and (D) phospho-S6 (Ser 240/244) throughout all regions (same patient as shown in A–B). **E–F.** High expression levels of (E) phospho-S6 (Ser 235/236) and (F) phospho-S6 (Ser 240/244) in the SUB and CA1-CA4 regions of a 29 years old male with TLE and ILAE HS Type 3. **G–O.** Representative images of the dentate gyrus from a 38 years old female diagnosed with TLE without HS. Serial sections immunolabeled with (G, J, M) neurofilament RMD020, (H, K, N) phospho-S6 (Ser 235/236) and (I, L, O) phospho-S6 (Ser 240/244) showing preserved neuronal architecture and strong neuronal and glial p-S6 (Ser 235/236) and p-S6 (Ser 240/244) labeling within the CA4 and GCL regions (J-L represent enlarged images of the CA4 subfields from G–I; M–O represent enlarged areas of the GCL from G–I). Insets show co-localization of phospho-S6 (Ser 235/236) (green) with (K) CD68 (red) and with (N) GFAP (red). The nuclear DAPI staining appears in blue. Scale bars represent approximately 2000µm for A–F, 250µm for G–I, 50µm for J–O and 10µm for insets in K and N.

Figure 4. Increased expression of mTORC2 activity marker phospho-Akt (Ser473) in the TLE hippocampus

A–C. Representative sections of a control hippocampus (same case as shown in Figure 3A–D) immunolabeled with phospho-Akt (Ser473) demonstrating low expression within (A) CA1, (B) CA4 and (C) GCL. **D–F.** Region-matched representative images of a non-sclerotic TLE hippocampus (same patient as shown in Figure 3G–O) depicting increased nuclear and cytoplasmic phospho-Akt (Ser473) expression. **G–I.** Representative images of (G) CA1, (H) CA4 and (I) GCL hippocampal subfields of a 20 years old female with TLE and ILAE HS Type 1 showing the same prominent nuclear phospho-Akt (Ser473) labeling pattern. **J–L.** Representative sections of a non-sclerotic hippocampus from a 45 year old female with TLE double labeled with (J) CD68 (red) and (K) phospho-Akt (Ser473) (green) revealing a high degree of co-localization. **M–O.** Double label experiments using (M) GFAP (red) and (N) phospho-Akt (Ser473) antibodies (green) showing occasional p-Akt expression in hippocampal astrocytes (same case as shown in J–L). The nuclei stained with DAPI appear blue. Scale bars represent 50µm in A–I and 20µm in J–O.

Figure 5. Differential expression of PI3K and Ras activation markers phospho-Akt(Thr308) and phospho-ERK(Thr202/Tyr204) in hippocampus and temporal lobe cortex from patients with therapy-resistant TLE relative to controls

A–C. Analysis of hippocampal tissue homogenates showing (A) representative Western blot images and calculated ratios, expressed as % of control mean, for (B) phospho-Akt(Thr308)/Akt ($182 \pm 15\%$ in TLE, $n=11$ vs. $100 \pm 23\%$ in controls, $n=5$) and (C) phospho-ERK(Thr202/Tyr204)/ERK ($5195 \pm 378\%$ in TLE, $n=10$ vs. $100 \pm 38\%$ in controls, $n=5$).

D–F. Temporal lobe cortex analysis showing (D) representative bands for individual subunits and quantification for (E) phospho-Akt(Thr308)/Akt ($114 \pm 11\%$ in TLE, $n=10$ vs. $100 \pm 8\%$ in controls, $n=6$ and $68 \pm 43\%$ in FCD, $n=2$), and (F) phospho-ERK(Thr202/Tyr204)/ERK ($183 \pm 15\%$ in TLE, $n=9$ vs. $100 \pm 3.8\%$ in controls, $n=6$ and $463 \pm 349\%$ in FCD, $n=2$). **, ***, ****: Denotes $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively (unpaired t tests comparing non-epilepsy control and TLE groups). Error bars indicate S.E.M. **G–O.**

Representative images of a non-sclerotic hippocampus from a 45 years old patient with therapy-resistant TLE double labeled with NeuN (red) and phospho-ERK(Thr202/Tyr204) (green) demonstrating robust neuronal p-ERK(Thr202/Tyr204) labeling within the (G–I) CA1 and (J–L) CA4 regions, in contrast to lower expression in (M–O) GCL. Numerous cellular profiles with either glial or endothelial cell morphology are strongly p-ERK(Thr202/Tyr204) positive throughout hippocampus. The nuclear DAPI staining is shown in blue. Scale bars represent $20\mu\text{m}$.

Figure 6. Suppression of FoxO3a signaling in TLE hippocampus and temporal lobe cortex
A–B. Western blot analysis of hippocampal tissue samples showing (A) representative bands and (B) quantitative analysis of phospho-FoxO3a (Ser253)/ FoxO3a ($1823 \pm 306\%$ in TLE, $n=8$ vs. $100 \pm 40\%$ in controls, $n=5$). **C–D.** Analysis of temporal lobe cortex showing (C) representative blot images and (D) expression levels of phospho-FoxO3a (Ser253)/ FoxO3a in TLE, non-epilepsy control and FCD cases ($317 \pm 53\%$ in TLE, $n=13$ vs. $100 \pm 12\%$ in controls, $n=5$ and $118 \pm 32\%$ in FCD, $n=2$). *, **: Denotes $p < 0.05$ and $p < 0.01$, respectively (unpaired t tests comparing non-epilepsy control and TLE groups). Error bars indicate S.E.M. **E–G.** Representative images of a non-sclerotic hippocampus from a 45 years old female patient with therapy-resistant TLE double labeled with NeuN (red) and FoxO3a (green) demonstrating robust cytoplasmic FoxO3a expression in both neurons and glia. **H–J.** Representative photomicrographs depicting the hippocampal CA1 region of a 55 years old female diagnosed with TLE and ILAE HS Type 2 double labeled with NeuN and FoxO3a showing almost exclusive cytoplasmic FoxO3a expression in remnant dystrophic neurons and glia. The nuclear DAPI staining appears in blue. Scale bars represent 20µm.

Figure 7. Induction of Akt/GSK-3β signaling in TLE hippocampus and temporal lobe cortex from patients with therapy-resistant TLE relative to controls

A–B. Western blot analysis of hippocampal tissue samples showing (A) representative bands for individual proteins and (B) quantitative analysis of phospho-GSK-3β (Ser9)/GSK-3β ($4393 \pm 1575\%$ in TLE, $n=7$ vs. $100 \pm 8\%$ in controls, $n=5$). **C–D.** Temporal lobe cortex analysis presenting (A) representative Western blot images and (B) corresponding quantitative analysis for phospho-GSK-3β (Ser9)/GSK-3β in TLE, control and FCD cases ($4257 \pm 781\%$ in TLE, $n=9$ vs. $100 \pm 32\%$ in controls, $n=6$ and $3770 \pm 1577\%$ in FCD, $n=2$). *, ***: Denotes $p < 0.05$, $p < 0.001$, respectively (unpaired t tests comparing non-epilepsy control vs. TLE groups). Error bars indicate S.E.M. **E–G.** Representative images of the hippocampal CA1 region from a patient diagnosed with TLE without HS and a seizure history of 21 years double labeled with NeuN (red) and phospho-GSK-3β (Ser9) (green) demonstrating intense p-GSK-3β (Ser9) in neurons and glia (same case as shown in Figure 6E–G). **H–J.** Photomicrographs of the sclerotic CA1 region from a TLE patient presenting with a seizure history of 42 years double labeled with NeuN and p-GSK-3β (Ser9). Note diminished labeling of surviving CA1 neurons and glia (same case as shown in Figure 6H–J). The nuclear DAPI staining is shown in blue. Scale bars represent 20 μm.

TABLE 1

Statistical table.

Clinical Variable Versus Molecular Marker	Epilepsy duration		Seizure Focus Location <i>Hippocampal (H) vs Extrahippocampal (EH)</i>		Hippocampal Pathology <i>HS vs no-HS</i>
	Hippocampus	Neocortex	Hippocampus	Neocortex	Hippocampus
p-mTOR (Ser2448)/mTOR	p= 0.633 t= 0.201	p= 0.546 t= 0.194	p= 0.338	p= 0.203	p= 0.257
p-S6 (Ser235/236)/S6	p= 0.143 t= 0.472	p= 0.924 t= -0.035	p= 0.969	p= 0.281	p= 0.071
p-S6 (Ser240/244)/S6	p= 0.240 t= 0.387	p= 0.969 t= 0.011	p= 0.011 (*) 2078 ± 406% (H) 552 ± 233% (EH)	p= 0.104	p= 0.194
p-Akt (Ser473)/Akt	p= 0.990 t= 0.004	p= 0.228 t= 0.419	p= 0.049 (*) 2691 ± 131% (H) 1088 ± 586% (EH)	p= 0.920	p= 0.345
p-Akt (Thr308)/Akt	p= 0.545 t= -0.205	p= 0.928 t= 0.033	p= 0.003 (**) 232 ± 13% (H) 141 ± 15% (EH)	p= 0.215	p= 0.811
p-ERK1/2 (Thr202/Tyr204)/ERK1/2	p= 0.032 (*) t= 0.676	p= 0.506 t= -0.256	p= 0.254	p= 0.279	p= 0.792
p-FoxO3a (Ser253)/FoxO3a	p= 0.954 t= -0.024	p= 0.270 t= -0.330	p= 0.589	p= 0.298	p= 0.330
p-GSK-3β (Ser9)/GSK-3β	p= 0.145 t= -0.611	p= 0.829 t= -0.085	p= 0.804	p= 0.782	p= 0.283

Statistical tests included Pearson correlation analyses (for epilepsy duration) and unpaired t tests (for seizure focus location and hippocampal pathology). Values represent group means ± S.E.M, expressed as % of control.