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The role of mTORC1 activation in seizure-induced exacerbation of Alzheimer's disease

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The risk of seizures is 10-fold higher in patients with Alzheimer's disease than the general population, yet the mechanisms underlying this susceptibility and the effects of these seizures are poorly understood. To elucidate the proposed bidirectional relationship between Alzheimer's disease and seizures, we studied human brain samples ($n = 34$) from patients with Alzheimer's disease and found that those with a history of seizures ($n = 14$) had increased amyloid-β and tau pathology, with upregulation of the mechanistic target of rapamycin (mTOR) pathway, compared with patients without a known history of seizures ($n = 20$).

To establish whether seizures accelerate the progression of Alzheimer's disease, we induced chronic hyperexcitability in the five times familial Alzheimer's disease mouse model by kindling with the chemoconvulsant pentylenetetrazol and observed that the mouse model exhibited more severe seizures than the wild-type. Furthermore, kindled seizures exacerbated later cognitive impairment, Alzheimer's disease neuropathology and mTOR complex 1 activation. Finally, we demonstrated that the administration of the mTOR inhibitor rapamycin following kindled seizures rescued enhanced remote and long-term memory deficits associated with earlier kindling and prevented seizure-induced increases in Alzheimer's disease neuropathology.

These data demonstrated an important link between chronic hyperexcitability and progressive Alzheimer's disease pathology and suggest a mechanism whereby rapamycin may serve as an adjunct therapy to attenuate progression of the disease.

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Abbreviations: 5XFAD = five times familial Alzheimer's disease; AD+/–Sz = Alzheimer's disease group with/without seizure history; ERK = extracellular signal-regulated kinase; JNK = c-Jun N-terminal kinase; mTORC1 = mechanistic target of rapamycin complex 1; PTZ = pentylenetetrazol

Introduction

Alzheimer's disease, the most common form of dementia and an increasing source of morbidity, mortality and economic costs, is associated with neuronal hyperactivity in both human patients and animal models.[1] In patients with Alzheimer's disease, seizure incidence can be increased even early in the disease course, with further elevations in those with younger disease onset.[2] Overall, 10–22% of patients with Alzheimer's disease have unprovoked seizures.[3] Given the overlap of symptoms from these seizures (amnestic spells, fluctuations in cognition and déjà vu) and cognitive decline due to Alzheimer's disease, the seizures may be underdiagnosed.[3] While the comorbid association of Alzheimer's disease with epilepsy is well-established,[4–6] seizures in this setting are often assumed to be a by-product or complication of Alzheimer's disease.[3]

However, current literature suggests a bidirectional relationship between Alzheimer's disease and epilepsy, with seizures inducing Alzheimer's disease pathology and vice versa. Alzheimer's disease pathology is characterized by the accumulation of amyloid-β_{42} peptide, produced via the processing of the amyloid precursor protein (APP), and an increase in hyperphosphorylated tau (pTau) protein.[7] Patients with Alzheimer's disease and those with temporal lobe epilepsy exhibit learning and memory deficits, and human brain tissue from patients with temporal lobe epilepsy displays increased amyloid-β plaques and tau pathology that correlate with cognitive decline.[8–11] In addition, animal models of Alzheimer's disease or epilepsy demonstrate that both APP and soluble amyloid-β_{42} can induce synaptic dysfunction and epileptic activity, while tau pathology participates in neuronal hyperexcitability and epileptogenesis.[12–15] Consistent with these observations, Alzheimer's disease mice are more prone to seizures.[16] The five times familial Alzheimer's disease (5XFAD) mouse model used in this study overexpresses human APP with three familial Alzheimer's disease mutations (K670N/M671L, I716V and V717I) and human presenilin 1 (PSEN1) with two familial Alzheimer's disease mutations (M146L and L286V).[17] The five mutations lead to aggressive amyloid pathology and the mice consequently recapitulate the connection between hyperexcitability and Alzheimer's disease. The 5XFAD mice exhibit epileptiform spikes at 4 months,[18] impaired long-term potentiation at around 6 months (when extensive amyloid pathology is present and the cognitive impairments begin) and non-convulsive spontaneous seizures starting at 10 months of age.[16,17,19–21] Indeed, clinical studies suggest that seizures can accelerate clinical progression to dementia in the elderly[22] and similarly in mice, pilocarpine-induced epilepsy causes premature and enhanced plaque formation and altered neuronal pTau expression in the triple transgenic model of Alzhiemer's disease (3xTg).[23] Despite evidence for an interaction between epilepsy and Alzheimer's disease, there is still little known concerning the underlying mechanisms.

In addition to the uncertainty regarding the effects of seizures on Alzheimer's disease progression, there is no clear consensus on the treatment of seizures in patients with the disease.[22] Trials of conventional anti-epileptic drugs in patients with Alzheimer's disease have shown varied results, with the drugs resulting in both the mitigation and exacerbation of mood and cognitive symptoms, highlighting the necessity to explore additional targets.[24–26] One such target is the mechanistic target of rapamycin (mTOR), given that Alzheimer's disease and epilepsy share several molecular pathways upstream and downstream of mTOR complex 1 (mTORC1). Activation of mTORC1 has been implicated in epileptogenesis and its cognitive comorbidities[27–30] and has been observed in human epilepsy, including brain tissue from patients with temporal lobe epilepsy[31] and other epilepsy syndromes such as tuberous sclerosis complex.[32] In addition, mTORC1 has shown promise as a target for epilepsy in humans, with benefits shown in several mouse models, including those of temporal lobe epilepsy and tuberous sclerosis complex.[27,31,33–36] Furthermore, Alzheimer's disease pathology reveals an association of mTORC1 with increased amyloid-β_{42} production, decreased amyloid-β_{42} clearance, tau hyperphosphorylation and apoptosis.[37–40] While previous studies in Alzheimer's disease have focused on rapamycin's effects on vascular and metabolic function, none to date have explored rapamycin's interactions with seizure-induced pathophysiology in Alzheimer's disease.

We hypothesized that seizure-induced neuronal hyperexcitability contributes to Alzheimer's disease pathology progression at least in part through a mechanism involving mTORC1 pathway activation, and that, similar to preclinical epilepsy models, anti-epileptogenic treatment with the mTORC1 inhibitor rapamycin will reverse these changes. To establish whether mTORC1 is altered by seizures in human Alzheimer's disease, we examined temporal neocortex from patients with Alzheimer's disease and a history of seizures versus those without seizures for amyloid and tau burden in conjunction with mTORC1 signalling. To investigate a more causal relationship and complement these human studies, we used the 5XFAD mouse model, which allowed us to manipulate excitability along the course of the disease. We then determined whether pentylenetetrazol (PTZ)-induced chronic hyperexcitability directly affects mTORC1 activation, Alzheimer's disease pathology and cognition and examined whether long-term mTORC1 inhibition with rapamycin could mitigate seizure-induced changes in cognitive decline and Alzheimer's disease pathology. We found that seizures exacerbate neuropathology and mTORC1 activation in patients with Alzheimer's disease and 5XFAD mice, while rapamycin slows disease progression in 5XFAD mice. Taken together, these findings support our hypothesis of a bidirectional relationship between seizures and Alzheimer's disease, in part mediated by mTORC1 signalling.

Materials and methods

Study design

The primary research objectives of this study were to assess the effects of seizures on Alzheimer's disease pathology, cognitive function and mTORC1 activity in human Alzheimer's disease brain tissue and an established mouse model of Alzheimer's disease,

and to test whether mTORC1 inhibition by rapamycin could ameliorate the end points of disease progression in mice. These end points were determined prospectively based on an extensive review of the literature. In all experiments, animals were randomly assigned to experimental or treatment groups and matched for age, sex and littermate controls. All behavioural scorings and histological analyses were carried out blind to genotype and treatment groups. Sample sizes were calculated *a priori* by power analysis based on pilot data. In 5XFAD mice, 12 to 19 mice per group were used to obtain statistical significance. In our human study, 11 to 19 cases per group were used to obtain statistical significance. No animals were excluded as outliers from the reported dataset. Detailed experimental procedures, including patient clinical information, mouse kindling methods, behavioural testing and tissue analysis protocols, are provided in the Supplementary material.

Statistics

For the human study, comparisons of normally distributed data were performed using unpaired *t*-tests, and comparisons of data with non-gaussian distributions were performed using Mann-Whitney testing. For the mouse study, we performed either two-way ANOVA to assess the contributions of both kindling and genotype or three-way ANOVA, with PTZ kindling, treatment and genotype as variables. All ANOVA analyses were followed by a *post hoc* multiple comparison Tukey's test when significant interaction or main effects were found. These results are presented as box and whisker plots in the figures. Additional multiple linear regression analysis was performed for all data to determine potential sex effects. In cases where sex was a predictor or sex had an interaction effect, scatter plots were used to enable the reader to visualize the sex-related differences. Correlations were calculated using Pearson correlation coefficients. All statistical analyses and graphic representations were carried out using GraphPad Prism 8 software (San Diego, CA). We considered all results to be significant at $P \leqslant 0.05$. Statistical results obtained in the human and mice cohorts are summarized in the Supplementary Tables 2–4.

Study approval

The human study was approved by the Institutional Review Board of the University of Pennsylvania and all procedures were performed in accordance with the institutional ethical standards. Animal studies were authorized and approved by the Institutional Animal Care and Use Committee (IACUC) Office of Animal Welfare of the University of Pennsylvania.

Data availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Results

Seizures are associated with greater brain atrophy, amyloid burden, tau pathology and mTORC1 activity in patients with Alzheimer's disease

Given the cognitive dysfunction seen in patients with temporal lobe epilepsy, we hypothesized that a history of seizures would be correlated with greater neuropathology in patients with Alzheimer's disease. We first analysed clinical data from Alzheimer's disease cases without a known seizure history (AD–Sz) and patients with Alzheimer's disease who had experienced at least one seizure (AD+Sz), compared with neurologically normal control cases (Supplementary Table 1).

As indicators of global brain atrophy, we compared brain weight and ordinal ratings of gross ventricular enlargement at autopsy between groups. Brain weight was decreased in patients with Alzheimer's disease compared with the controls (–229 g, i.e. –17.1%, $P < 0.0001$; Fig. 1A), and seizure history was associated with a further decrease in patients with Alzheimer's disease (–310 g, i.e. –23.1%, $P < 0.05$; Fig. 1A). Additionally, as expected due to sexual dimorphism,[41] we observed a significant effect of sex by multiple linear regression analysis: female sex [$P < 0.0001$, (–220.3, –90.3); Fig. 1A]. Similarly, ventricular enlargement was greater in patients with Alzheimer's disease, with a frequency of severe pathology of 41.2% versus 0% in controls ($P < 0.0001$; Fig. 1B) and further enlargement in the AD+Sz cases compared with the AD–Sz group with a frequency of severe pathology of 64.3% versus 25%, respectively ($P < 0.05$). None of these differences were related to age at onset of Alzheimer's disease or age at death, as there were no discernable differences in these variables between the patients with seizures and those without (Supplementary Fig. 1A and B).

We next analysed the post-mortem temporal cortex tissue from the same control and Alzheimer's disease cases for markers of Alzheimer's disease neuropathology. Soluble amyloid-β_{42} expression, evaluated by ELISA, was increased in Alzheimer's disease temporal cortex compared with the controls (+5455%, $P < 0.0001$; Fig. 1C) but did not vary between the AD+Sz and AD–Sz cases. To account for insoluble amyloid-β in diffuse and cored plaques, we also examined the extent of amyloid-β coverage by immunohistochemistry. We found increased amyloid plaque pathology in all Alzheimer's disease cases compared with the controls, both in the grey matter (+1723%, $P < 0.0001$; Fig. 1D) and subcortical white matter (+1361%, $P < 0.01$; Fig. 1E), with a significant elevation of amyloid-β coverage in the white matter of AD+Sz cases compared with AD–Sz (+299%, $P < 0.05$) but no significant differences based on seizure history in the grey matter. In parallel, we assessed tau protein accumulation in Alzheimer's disease and control cases by western blot, including total tau (Tau 5) and phosphorylated tau at [Thr212, Ser214] (AT100), [Ser202, Thr205] (AT8) and [Thr231] (AT180) residues. We observed elevated expression of total tau (+165%, $P < 0.001$; Supplementary Fig. 1C) and pTau (pTau:total tau ratio) at all epitopes examined (+9399%, +2777% and +795%, respectively, $P < 0.0001$; Fig. 1F and Supplementary Fig. 1D–E) in Alzheimer's disease temporal cortex relative to controls. Additionally, there was a significant increase of pTau AT100 in the AD+Sz group compared with the AD–Sz group (+62%, $P < 0.05$; Fig. 1F). No sex differences were found when analysing markers of Alzheimer's disease pathology.

As mTORC1 is implicated in both epileptogenesis[42] and Alzheimer's disease neuropathology,[38] we subsequently measured the level of phosphorylated S6 (pS6) [Ser235, Ser236], which is a readout of mTORC1 activation.[43] Western blot analysis demonstrated increased pS6/S6 ratios in patients with Alzheimer's disease compared with controls (+130%, $P < 0.001$; Fig. 1G), and this marker of mTORC1 activity was higher in AD+Sz compared with AD–Sz cases (+64%, $P < 0.05$). No change was observed for the other mTORC1-targeted phosphorylation sites of S6 [Ser240, Ser244] (Supplementary Fig. 1F).

Given that both mTORC1 activity and AT100 were increased in AD+Sz cases, we next performed double label immunohistochemistry for pTau AT100 and pS6 [Ser235, Ser236]. We confirmed strong pTau AT100 immunoreactivity in the temporal cortex of Alzheimer's disease cases, mostly in pathological inclusions such as neurofibrillary tangles and found that 100% of pTau AT100 inclusions occurred in cells with intracellular pS6 in both control and Alzheimer's disease cases (Fig. 1J). Conversely, 31.6% of pS6

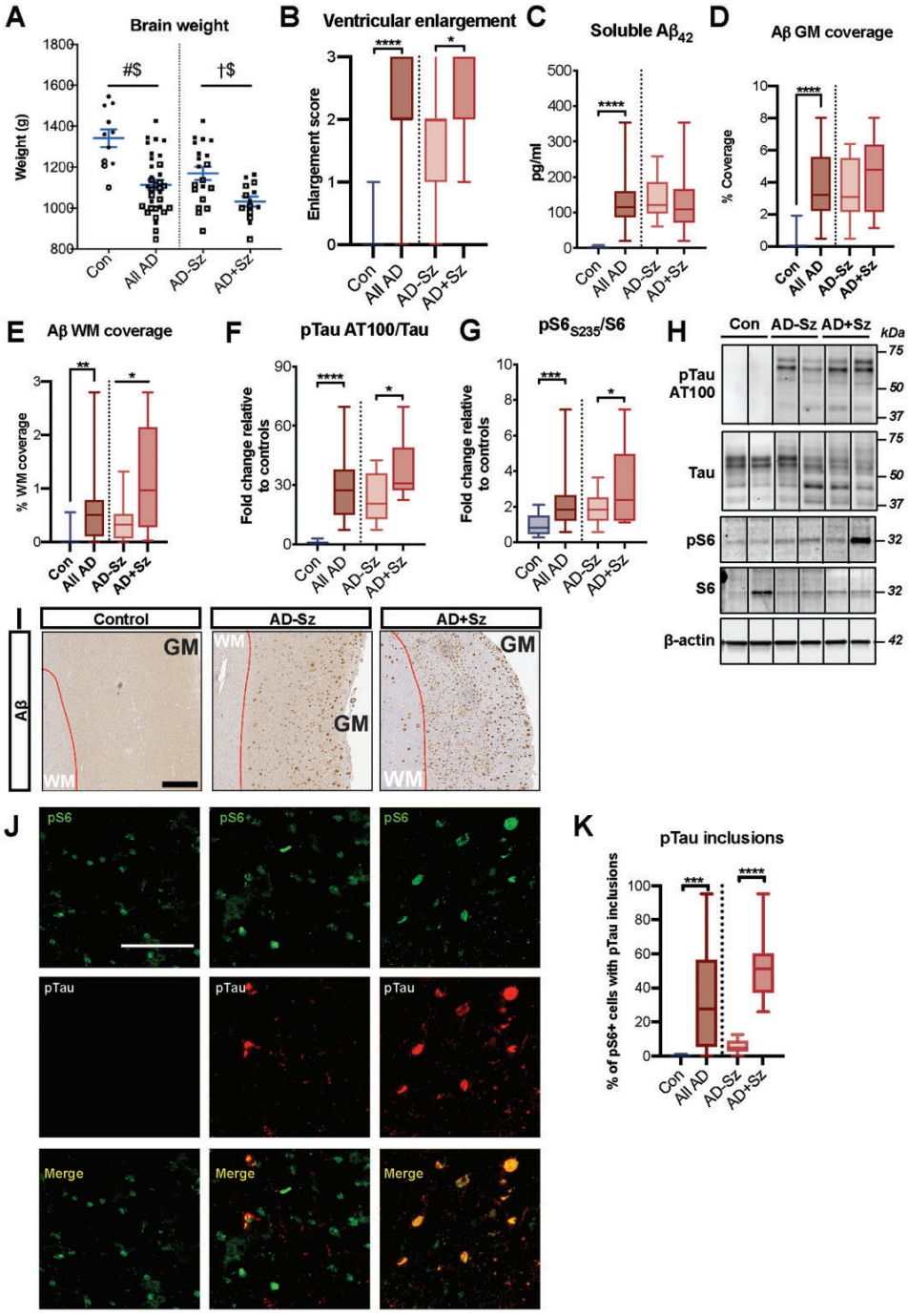

Figure 1 Seizures are associated with increased brain atrophy, neuropathology and mTORC1 activity in patients with Alzheimer's disease. Study cohorts included control cases (Con, circles, $n = 7–13$), and patients with Alzheimer's disease (AD, squares, $n = 18–34$), split into subgroups: those without (AD–Sz, $n = 9–20$) or with (AD + Sz, $n = 9–14$) known seizure history. In these patients, we evaluated: (**A**) brain weight at the time of death in grams; (**B**) ventricular enlargement pathology graded by severity (0 = none, 1 = mild, 2 = moderate, 3 = severe); (**C**) soluble human amyloid-β_{42} ($A\beta_{42}$) in pg/ml in the temporal cortex; quantification of amyloid plaque deposition in the (**D**) grey matter (GM) of the temporal cortex and (**E**) subcortical white matter (WM) from staining performed in **I**; (**F**) pTau [Thr212/Ser214] AT100/Tau in the temporal cortex; and (**G**) phosphorylated-S6 [Ser235/Ser236]/S6 in the temporal cortex. (**H**) Representative western blot images of the temporal cortex for **F** and **G** showing non-adjacent bands originating from the same blot. (**I**) Representative images of the temporal cortex from a control subject, an AD–Sz patient and an AD + Sz patient immunolabelled with amyloid-β, showing increased amyloid plaques in cases of Alzheimer's disease, with increased subcortical plaque deposition in the AD + Sz patient. Scale bar = 500 μm. (**J**) Representative images of the temporal cortex from a control subject, an AD–Sz patient and an AD + Sz patient co-immunolabelled with pTau AT100 (red) and pS6 (green), showing increased pTau AT100 accumulation in neurofibrillary tangles in cases of Alzheimer's disease co-localizing with the mTORC1 activity marker pS6. (**K**) Quantification of the percentage of cells with pTau inclusions from staining performed in **J**. Scale bar = 50 μm. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$. $^{\#}$Alzheimer's disease effect, $P < 0.0001$; $^{\$}$Sex effect, $P < 0.0001$; †Seizure effect, $P < 0.001$. Box and whisker plots display minimum, maximum and all quartiles. Scatter plots display all data-points, with mean ± standard error of the mean (SEM). Closed symbols represent males and open symbols represent females.

staining was found in pTau AT100-positive cells in Alzheimer's disease cases versus only 0.1% in control brains ($P < 0.001$; Fig. 1K). Moreover, seizure history was associated with an increased percentage of pS6 reactivity in cells with pTau inclusions (51.4% in AD+Sz and 5.8% in AD–Sz groups, respectively; $P < 0.0001$; Fig. 1K). The strong co-localization of pTau and pS6 indicates a potential mechanistic link between both mTORC1 activity and seizure related increases in AT100 tau phosphorylation.[44]

In addition to mTORC1, we examined two other stress kinases implicated in both Alzheimer's disease and epilepsy[8]: (i) the extracellular signal-regulated kinase (ERK) 1 and 2,[45,46] an upstream activator of mTORC1[47]; and (ii) the c-Jun-N-terminal kinase (JNK),[48,49] implicated in APP and tau phosphorylation and BACE1 (APP amyloidogenic processing enzyme) upregulation.[50,51] We found elevated phosphorylation of ERK at [Thr202, Tyr204] and JNK at [Thr183, Tyr185] in Alzheimer's disease cases when compared with the controls (+262%, $P < 0.001$ and +118%, $P < 0.0001$, respectively; Supplementary Fig. 1G and H) and additional increased phosphorylation of JNK in AD+Sz patients compared with the AD–Sz group (+38.8%, $P < 0.05$; Supplementary Fig. 1H). The elevations of activated ERK and JNK in patients with Alzheimer's disease may provide a mechanism for the elevated mTORC1 activity and Alzheimer's disease pathology, respectively. No sex differences were found when analysing the activities of mTORC1 and the stress kinases. The statistical analyses of the Alzheimer's disease and control patients are reported in their entirety in Supplementary Table 2.

5XFAD mice exhibit a hyperexcitable phenotype at the presymptomatic age of 3 months

To determine how the seizures, increased Alzheimer's disease pathology and mTORC1 activation seen in our human tissue study relate to one another during the progression of Alzheimer's disease, we used the 5XFAD mouse model to examine the effects of chronic hyperexcitability on Alzheimer's disease progression. We used a PTZ kindling seizure induction protocol, a well-established epilepsy model in which sub-convulsive doses of the chemoconvulsant PTZ are given on alternating days over a period of 2 weeks.[52] Mice were kindled at the presymptomatic age of 3–3.5 months and evaluated for the effects of hyperexcitability on behaviour and Alzheimer's disease neuropathology at 6.5–7 months (Fig. 2A).

We found that even at 3 months of age, 5XFAD mice expressed a subclinical hyperexcitable phenotype. Compared with PTZ-kindled wild-type littermates, PTZ-kindled 5XFAD mice exhibited increased seizure scores on each day of injection, with 5XFAD mice reaching, after the eighth PTZ injection, an average Racine seizure score[53] of 4.4 compared with the wild-type mice score of 3.1 (overall $P < 0.0001$; Fig. 2B). In the hour after PTZ injection, 5XFAD mice also demonstrated increased seizure severity (maximal score reached) throughout the period ($P < 0.0001$; Fig. 2C), increased total seizure burden (area under the curve, AUC) (+64%, $P < 0.001$; Fig. 2C, inset) and decreased latency to first seizure (–50%, $P < 0.0001$; Fig. 2D).

One month after the last PTZ injection (Day 45; Fig. 2B), mice were challenged with the same sub-convulsive dose of PTZ or vehicle to determine whether the overall network hyperexcitability in kindled mice was long-lasting.[52] The averaged Racine seizure scores in both the wild-type and 5XFAD mice during this challenge were comparable with the scores reached following the eighth PTZ injection performed at 3.5 months (3.2 and 4.4 versus 3.1 and 4.4, respectively), with scores significantly higher in the 5XFAD mice ($P < 0.05$; Fig. 2B).

Pentylenetetrazol kindling exacerbates cognitive impairment in both wild-type and 5XFAD mice

To determine if seizure activity in the early stages of amyloid pathology could worsen disease progression in 5XFAD mice, we first analysed hippocampal-dependent memory at 3 months after completion of PTZ kindling, when mice were 6.5–7 months old. The fear conditioning test was administered at 24 h and at 14 days to assess long term memory and remote memory, respectively, as has been previously shown to reveal deficits in 5XFAD mice.[54] All fear conditioning data were evaluated in relation to individual baseline freezing time, which did not differ across subgroups (WT-vehicle 16%; WT-PTZ 20%; 5XFAD-vehicle 18%; and 5XFAD-PTZ 20%; see Supplementary Table 3 for relevant statistics). Long-term contextual memory, evaluated by freezing behaviour 24 h post-stimuli, was diminished in 5XFAD mice independent of kindling [genotype effect, $F(1,61) = 7.189$, $P < 0.01$; Fig. 2E, left], as 5XFAD-vehicle mice performed worse than WT-vehicle (–49%, $P < 0.05$), as expected.[20] In remote recall trials, 5XFAD mice again showed impaired recall [genotype effect, $F(1,61) = 11.35$, $P < 0.01$; Fig. 2E, right]. Notably 5XFAD-PTZ mice performed significantly worse than 5XFAD-vehicle mice (–30%, $P < 0.05$; Fig. 2E), demonstrating that seizures significantly exacerbated the memory impairment [kindling effect, $F(1,61) = 4.577$, $P < 0.05$]. Working memory, evaluated by spontaneous alternation in the Y-maze, was diminished in kindled 5XFAD mice [kindling effect, $F(1,61) = 6.738$, $P < 0.05$; Fig. 2F]. Mouse self-care, evaluated by the nest building test, was also diminished in kindled mice [kindling effect, $F(1,61) = 18.28$, $P < 0.0001$; Fig. 2G], as PTZ-kindled wild-type mice had a decreased nesting score compared with vehicle-treated wild-type mice (–30%, $P < 0.001$). Unlike wild-type mice, we found no impaired nest building behaviour in the 5XFAD cohort. No significant effect of sex was found on any behavioural or kindling metric by multiple linear regression. Results of statistical analysis for this mouse cohort are reported in their entirety in Supplementary Table 3. Taken together, seizures had a deleterious effect on behaviour, including working memory and mouse self-care, regardless of genotype, with seizures worsening the deficits in remote recall only in 5XFAD mice.

Pentylenetetrazol kindling worsens amyloid pathology and neuronal death in 5XFAD mice

After behavioural assessment, 7 month old wild-type and 5XFAD mice were sacrificed, and brains were subjected to biochemical and immunohistochemical analysis, with a focus on the hippocampus, a site implicated in Alzheimer's disease pathology, and the aforementioned behavioural tests. We first analysed the effect of PTZ kindling on hippocampal amyloid pathology. PTZ kindling led to the increased accumulation of soluble human amyloid-β_{42} in the hippocampus of 5XFAD mice (+66%, $P < 0.05$; Fig. 3A), as measured by ELISA. As wild-type mice do not express human amyloid-β_{42}, we found no soluble human amyloid-β_{42} in wild-type mice. Amyloid-β_{42} levels in PTZ-kindled 5XFAD hippocampus were positively correlated with seizure severity ($r = 0.52$, $P < 0.05$; Fig. 3B). Additional ELISA analysis of soluble human amyloid-β_{42} in the cortex of 5XFAD mice demonstrated increased amyloid in female mice [$P < 0.0001$, (+354.7 pg/ml, +181.0 pg/ml)] as previously documented,[17,55] with a trend towards an effect of kindling ($P = 0.09$; Fig. 3C). Increases in soluble human amyloid-β_{42} were accompanied by similar changes in amyloid plaque pathology, evaluated by immunohistochemistry (Fig. 3D). For both the hippocampus and cortical layers IV–VI, significant predictors of increased amyloid plaque load in 5XFAD mice included: female sex [$P < 0.0001$, (51.1%, 130.6%) for hippocampus and $P < 0.01$, (5.355%, 229.1%) for cortex] and PTZ kindling [$P < 0.0001$, (66.67%,

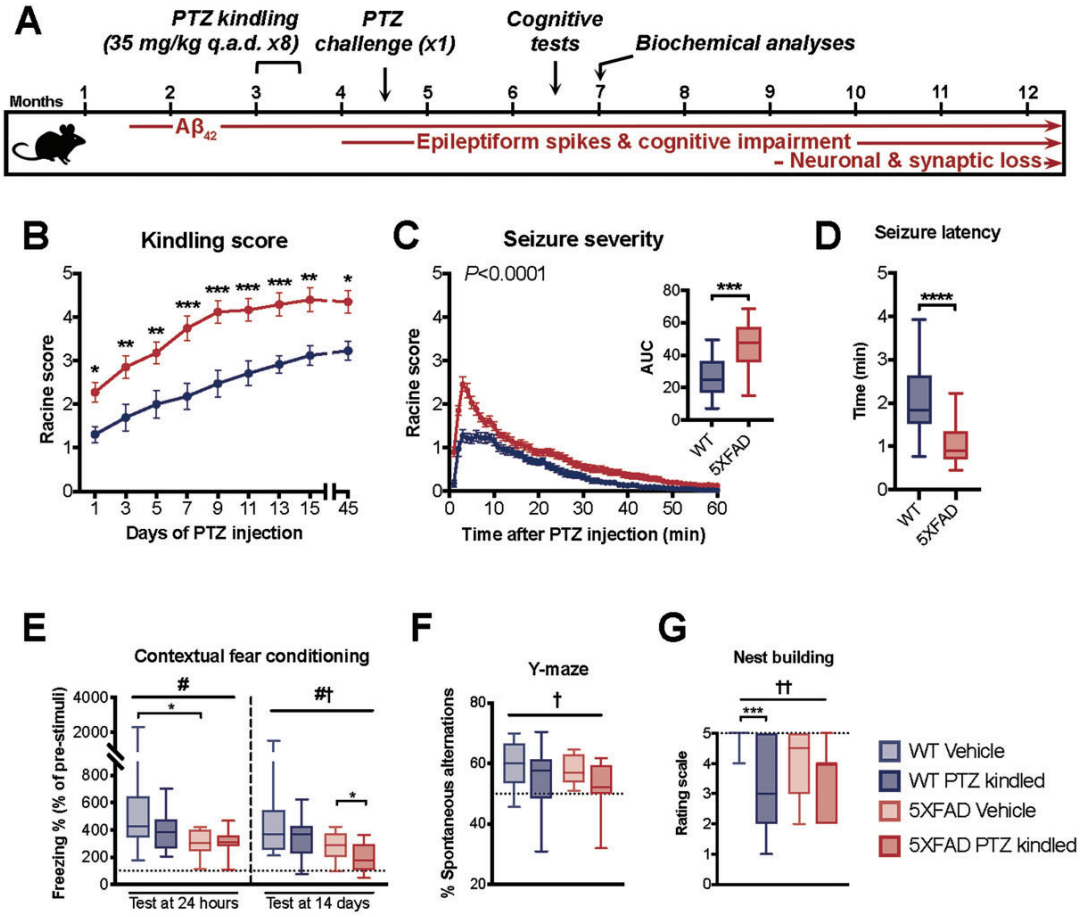

Figure 2 Hyperexcitability phenotype and seizure-induced effect on cognition in 5XFAD mice. (**A**) Experimental design in wild-type (WT) and 5XFAD mice involving PTZ kindling starting from 3 months, cognitive assessment from 6.5–7 months and euthanasia for biochemical analyses of the hippocampus at 7 months. The schematic provides additional background on the 5XFAD mice: amyloid-β_{42} accumulation starting from 1.5 months of age, epileptiform spikes and cognitive impairment starting from 4 months and neuronal and synaptic loss starting from 9 months. (**B**) Maximal Racine score reached per day of PTZ injection. (**C**) Maximal Racine score reached per minute during the 1-h post-PTZ injection video recording (average of eight PTZ injections). *Inset* represents evaluation of the area under the corresponding generated curves. (**D**) Latency to seizure in minutes. (**E**) Associative long-term (tested at 24 h post-training) and remote memory (tested at 14 days post-training) assessed with contextual fear conditioning test, measuring % of time freezing adjusted by individual baseline freezing on training day. (**F**) Spatial working memory evaluated by % spontaneous alternation in the Y-maze. (**G**) Mice self-care assessed by nest building. Box and whisker plots display minimum, maximum and all quartiles. $n = 19$ WT-vehicle, 17 WT-PTZ, 12 5XFAD-vehicle and 17 5XFAD-PTZ. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. $^{\#}$Genotype effect, $P < 0.01$; †Kindling effect, $P < 0.05$; ††Kindling effect, $P < 0.0001$.

146.9%) and $P < 0.05$ (24.79%, 202%)], respectively (Fig. 3E). Consistent with human amyloid-β_{42} increases, the endogenous mouse amyloid-β_{42}, measured by ELISA, was also elevated in the hippocampus of PTZ-kindled 5XFAD mice compared with 5XFAD-vehicle (+18%, $P < 0.05$; Fig. 3F), with both groups having significantly increased levels compared with their wild-type controls (+123% and +158%, respectively, $P < 0.0001$). To determine whether the increased expression of amyloid-β_{42} was associated with an increase in its production, we quantified hippocampal APP levels by western blot. The expression of APP was elevated in the hippocampus of 5XFAD mice compared with the wild-type [+154% and +233% in the vehicle and PTZ subgroups, respectively; genotype, $F(1,61) = 539.8$, $P < 0.001$; Fig. 3G], with kindling further increasing APP expression within the 5XFAD group (+26% versus 5XFAD-vehicle, $P < 0.0001$). However, hippocampal APP did not vary after kindling in wild-type mice. The changes in hippocampal APP expression were associated with similar increases in its phosphorylation level at [Thr668] (pAPP; Fig. 3H), a post-translational modification leading to upregulation of its processing into amyloid-β_{42}.[56] Western blot quantification demonstrated increased expression of pAPP in the hippocampus of 5XFAD mice compared with the wild-type [+88% and +164% in the vehicle and PTZ subgroups, respectively; genotype, $F(1,61) = 21.85$, $P < 0.001$], with kindling further elevating APP expression within the 5XFAD group (+27% versus 5XFAD-vehicle, $P < 0.05$; Fig. 3H). As with APP, kindling did not affect pAPP levels in wild-type mice. These results demonstrated that seizures lead to increased human and endogenous amyloid pathology in 5XFAD mice, likely through increased APP production and processing.

We next used Fluoro-Jade® B staining to examine ongoing neuronal death, as previously reported in 9-month-old 5XFAD mice.[17] Analysis revealed no Fluoro-Jade B staining in wild-type mice, regardless of kindling status (Fig. 4A). Significant predictors of increased neuronal death in the hippocampus and cortical layers IV–VI of 5XFAD mice included: female sex [$P < 0.001$, (1.67, 5.015) and $P < 0.05$ (0.3391, 3.651)] and PTZ kindling [$P < 0.001$, (0.34, 3.65) and $P < 0.001$ (1.67, 5.015); Fig. 4B]. Importantly, the number of

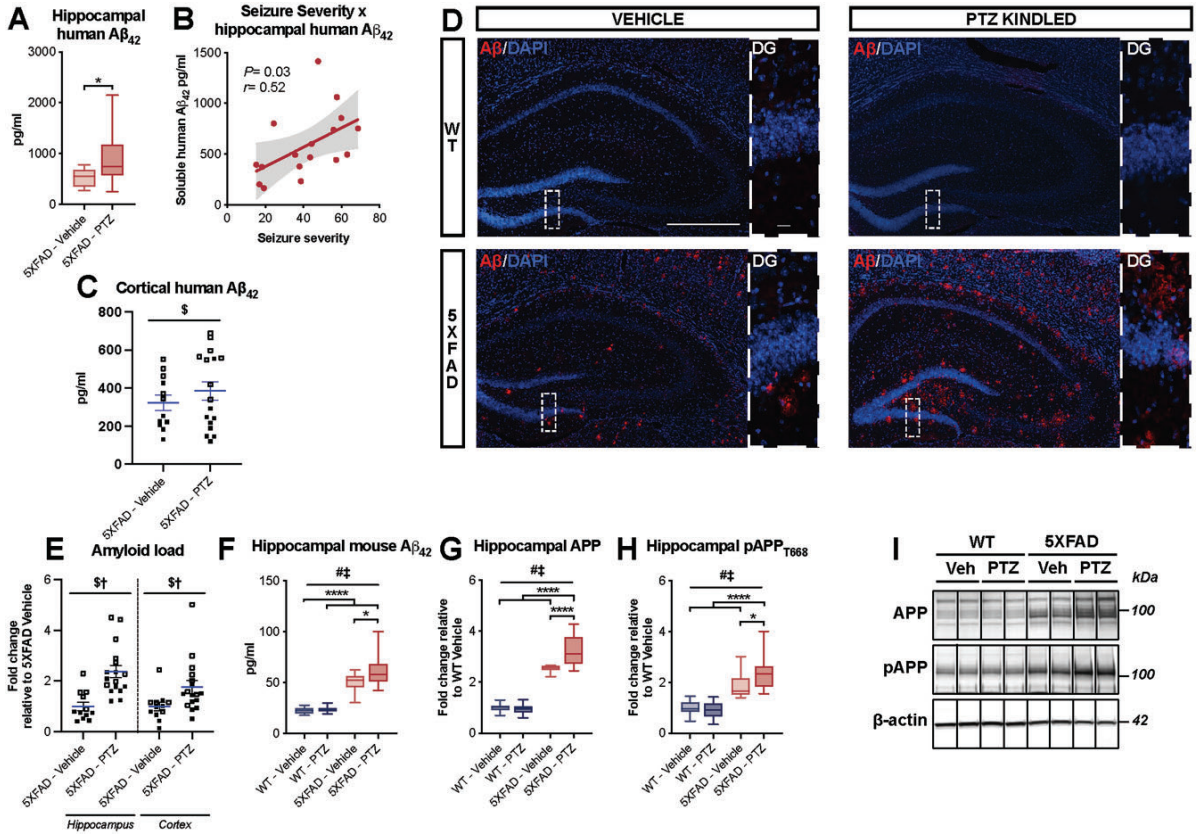

Figure 3 PTZ kindling worsens amyloid pathology in 5XFAD mice. (**A**) ELISA assessment of soluble human amyloid-β_{42} ($A\beta_{42}$) in the hippocampus of 5XFAD mice. (**B**) Pearson correlation between seizure severity and human amyloid-β_{42} concentration in pg/ml in the hippocampus of 5XFAD-PTZ mice. Grey area indicates 95% confidence interval. (**C**) ELISA assessment of soluble human amyloid-β_{42} in the cortex of 5XFAD mice. (**D**) Representative images of the hippocampus and cortex with insets of the dentate gyrus (DG) immunohistochemically (IHC) co-labelled with human amyloid-β_{42} (red) and nuclear DAPI stain (blue). Images show no expression in the wild-type mice, amyloid plaque accumulation in 5XFAD mice and increased amyloid deposition in the 5XFAD-PTZ group compared to 5XFAD-vehicle. Scale bars = 500 μm for entire images; and 20 μm for *insets*. (**E**) Corresponding immunohistochemical quantification of amyloid load (% area amyloid-β_{42}-positive) in the hippocampus (*left*) and cortical layers IV–VI (*right*) of 5XFAD mice. (**F**) ELISA assessment of hippocampal soluble mouse amyloid-β_{42} levels. Western blot quantification of (**G**) total APP and (**H**) phosphorylated APP [Thr668] in wild-type and 5XFAD hippocampus. (**I**) Representative western blot images for **G** and **H** showing non-adjacent bands originating from the same blot. Box and whisker plots display minimum, maximum and all quartiles. Scatter plots display all data-points, with mean ± SEM. Closed symbols represent males and open symbols represent females. n = 12–19 WT-vehicle, 12–17 WT-PTZ, 12 5XFAD-vehicle and 17 5XFAD-PTZ. $^{*}P < 0.05$, $^{****}P < 0.0001$. $^{\$}$Sex effect, $P < 0.0001$; †Kindling effect, $P < 0.0001$ for hippocampus, $P < 0.05$ for cortex; $^{\#}$Genotype effect, $P < 0.001$; ‡Genotype × Kindling interaction, $P < 0.05$.

Fluoro-Jade B-positive cells in PTZ-kindled 5XFAD hippocampus were positively correlated with seizure severity ($r = 0.61$, $P < 0.05$; Fig. 4C). In addition to Fluoro-Jade B, we performed quantitative immunohistochemistry for neuronal marker NeuN to determine cumulative neuronal loss. In the hippocampus, we found that both PTZ-kindled and vehicle-treated 5XFAD mice had lower neuronal counts compared with their wild-type controls (–20% and –19%, respectively, $P < 0.05$; Fig. 4D). In cortical layers IV–VI, the 5XFAD-vehicle mice also had lower neuronal counts compared with their wild-type controls (–23%, $P < 0.05$), which was also observed in the PTZ-kindled 5XFAD mice compared with their wild-type controls, but this did not reach statistical significance (–20%, $P = 0.07$). No significant NeuN differences were found between vehicle and PTZ-kindled 5XFAD mice in the hippocampus or cortical layers IV–VI.

Increased neuronal death in PTZ kindled 5XFAD mice may further exacerbate hyperexcitability in these mice both globally through the disruption of neuronal circuits, and locally through the loss of specific inhibitory cell populations. Thus, we quantified parvalbumin-expressing cells, accounting for the majority of GABAergic interneurons,[57] to determine if there was a loss of this subpopulation. We found lower parvalbumin cell counts in the hippocampus of 5XFAD mice compared with the wild-type mice ($P < 0.05$, –22.5%; Supplementary Fig. 2A), with no effect of PTZ kindling. However, when examining cortical layers IV–VI, there were lower parvalbumin cell counts in 5XFAD mice compared with the wild-type mice ($P < 0.05$, –15.82%), female mice compared with male mice ($P < 0.05$, –14.51%) and PTZ-kindled mice compared with saline-treated mice ($P < 0.05$, –18.96%, Supplementary Fig. 2B).

Deficits in contextual fear conditioning memory may not only be due to neuronal death but also impaired neurogenesis;[58,59] hence, we stained for doublecortin to measure the neurogenesis in the hippocampus of 5XFAD and wild-type mice. While there was decreased neurogenesis in the hippocampus of 5XFAD mice compared with the wild-type ($P < 0.05$, –29%; Supplementary Fig. 2C), notably, there was no effect of PTZ kindling on doublecortin cell counts in either group.

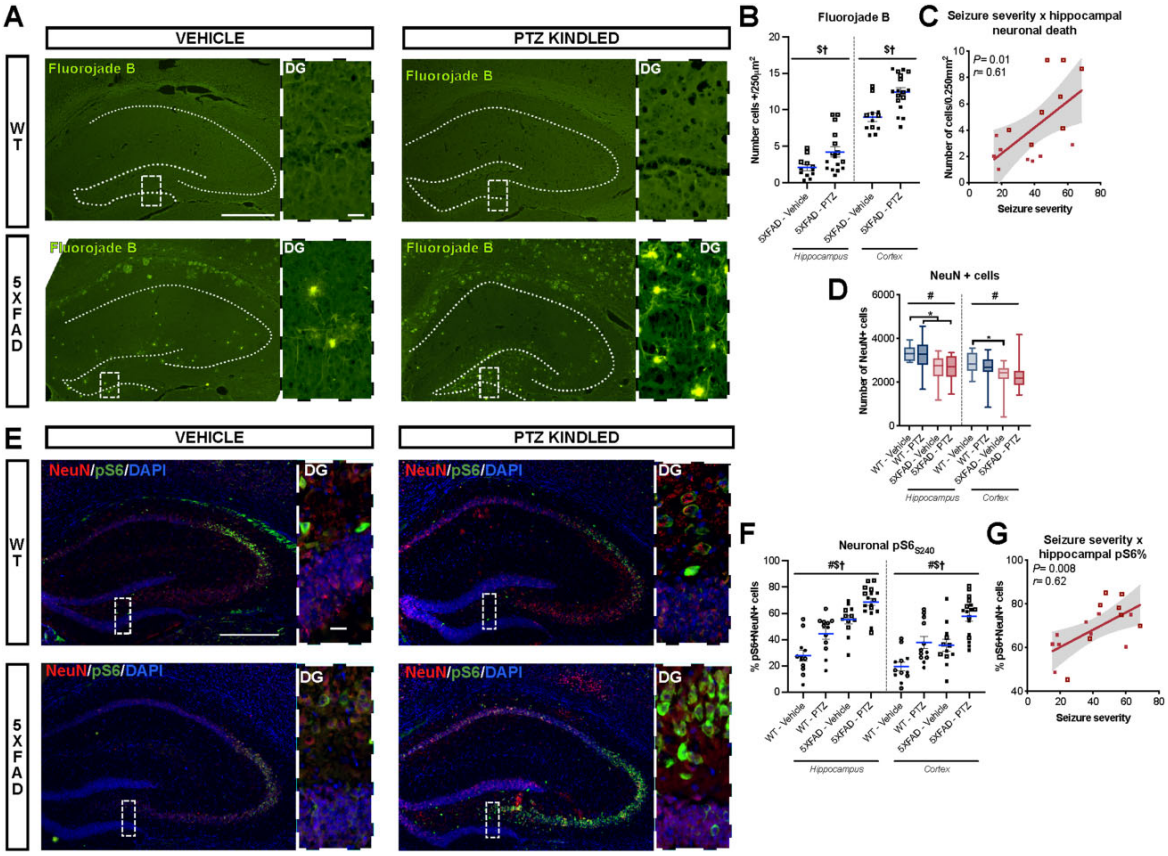

Figure 4 PTZ kindling worsens neuronal death and upregulates mTORC1 activity in 5XFAD mice. (**A**) Representative images of the hippocampus and cortex with insets of the dentate gyrus (DG) stained with Fluoro-Jade B. Images show no neuronal death in the wild-type mice, moderate neuronal death in 5XFAD mice and severe neuronal death in the 5XFAD-PTZ group compared with 5XFAD-vehicle. Scale bars = 500 µm for whole images; and 20 µm for *insets*. (**B**) Corresponding quantification of hippocampal (*left*) and cortical (*right*) Fluoro-Jade B staining, expressed as the number of positive cells per 0.25 mm^2. (**C**) Pearson correlation between seizure severity and the number of Fluoro-Jade B-positive cells in 5XFAD-PTZ hippocampus. (**D**) Immunohistochemical quantification of the number of neurons (NeuN-positive cells) in wild-type and 5XFAD hippocampus (*left*) and cortical layers IV–VI (*right*). (**E**) Representative images of the hippocampus and cortex with insets of the DG from wild-type and 5XFAD mice, co-labelled with NeuN (red), pS6 [Ser240/Ser244] (green) and nuclear DAPI stain (blue), showing increased pS6 staining in neuronal cell bodies in 5XFAD mice compared with the wild-type, as well as an increase in PTZ groups compared with the vehicle-treated mice in both genotypes. Scale bars = 500 µm for entire images; and 20 µm for *insets*. (**F**) Corresponding quantitative analysis of phosphorylated S6, expressed as a % of phosphorylated S6-positive cells relative to the number of NeuN-positive neurons per analysed area of the hippocampus (*left*) and cortical layers IV–VI (*right*). (**G**) Pearson correlation between seizure severity and the percentage of pS6-positive cells in the hippocampus of the 5XFAD-PTZ group. Scatter plots display all data-points, with mean ± SEM. Closed symbols represent males and open symbols represent females. Grey area in Pearson correlations indicates 95% confidence interval. n = 12–19 WT-vehicle, 12–17 WT-PTZ, 12 5XFAD-vehicle and 17 5XFAD-PTZ. *$P < 0.05$, ****$P < 0.0001$. $^{\$}$Sex effect, $P < 0.05$; †Kindling effect, $P < 0.05$; $^{\#}$Genotype effect, $P < 0.001$.

Pentylenetetrazol kindling causes upregulation of mTORC1 activity in 5XFAD mice

As mTORC1 activity was elevated in the human study, we performed immunohistochemistry for NeuN and pS6 [Ser240, Ser244], a more specific readout of mTORC1 activation.[43] Phosphorylated S6 was observed in all areas of 5XFAD hippocampus and cortical layers IV–VI and almost exclusively in neurons (Fig. 4E).[31] The percentage of S6-positive neurons (the number of pS6-positive/NeuN-positive cells relative to the total number of NeuN-positive neurons) revealed mTORC1 activity increases in the hippocampus and cortical layers IV–VI in female mice ($P < 0.05$, +5.9% and $P < 0.05$, +17.2%), 5XFAD mice ($P < 0.0001$, +71.6% and $P < 0.0001$, +69.5%) and PTZ-kindled mice ($P < 0.01$, +36.4% and $P < 0.0001$, +80.06%; Fig. 4F). This sex effect of mTORC1 corroborated with the findings that female sex was an independent predictor of both neuronal death and amyloid plaque load, suggesting that amyloid plaque deposition in female 5XFAD mice may further upregulate mTORC1 activity. Again, the percentage of S6-activated hippocampal neurons was positively correlated with seizure severity in the PTZ-kindled 5XFAD mice ($r = 0.62$, $P < 0.01$; Fig. 4G), strengthening the relationship between seizures and mTORC1 activity.

The hippocampal expression of stress kinases ERK and JNK, both involved in the activated mTORC1 pathway,[43,60] demonstrated elevated ERK activity in 5XFAD-vehicle mice compared with WT-vehicle mice (+112%, $P < 0.05$), further exacerbated by kindling (+105% in 5XFAD-PTZ versus WT-PTZ, $P < 0.001$ and +60.5% in 5XFAD-PTZ versus 5XFAD-vehicle, $P < 0.05$; Supplementary Fig. 3B), but no significant changes in JNK activity in 5XFAD mice or with PTZ kindling (Supplementary Fig. 3C). Hence, these increases in ERK activity may contribute to mTORC1 activation in 5XFAD mice and with PTZ kindling.

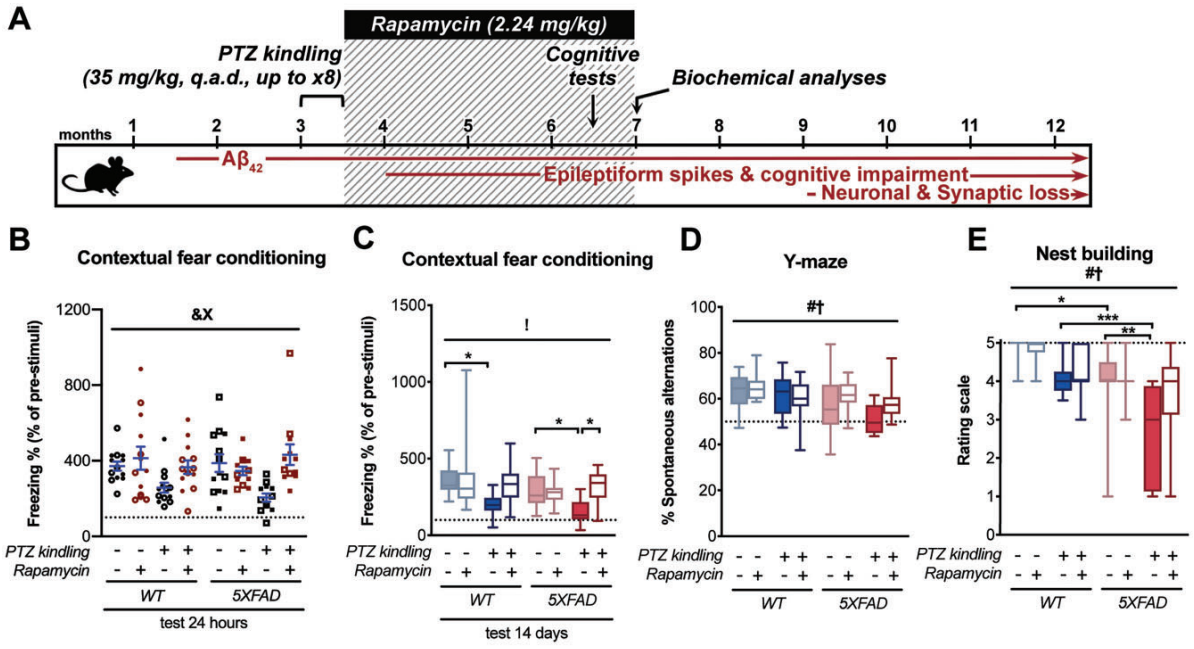

Figure 5 Inhibition of mTORC1 rescued long term and remote memory deficits in 5XFAD mice following PTZ kindling. (**A**) Experimental design in wild-type (WT) and 5XFAD mice, involving PTZ kindling starting from 3 months, rapamycin treatment starting from 3.5 months, cognitive assessment from 6.5–7 months and euthanasia for biochemical analyses at 7 months. (**B**) Associative long-term (test at 24 h post-training) and (**C**) remote memory (test at 14 days post-training) assessed with contextual fear conditioning test measuring % of time freezing relative to freezing pre-stimuli on training day. (**D**) Spatial working memory evaluated by % spontaneous alternation in the Y-maze. (**E**) Self-care assessed by nest building. Box and whisker plots display minimum, maximum and all quartiles. Scatter plots display all data-points, with mean ± SEM. Closed symbols represent males and open symbols represent females. $n = 12–13$ for each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. $^{!}$Treatment × Kindling interaction, $P < 0.01$; $^{\&}$Genotype × Sex interaction, $P < 0.01$; ^{X}Genotype × Kindling × Treatment interaction, $P < 0.01$; †Kindling effect, $P < 0.05$; $^{\#}$Genotype effect, $P < 0.01$.

Given that mTOR is implicated in the phosphorylation of tau, we quantified hippocampal levels of the tau and phosphorylated tau markers that were examined in our human cohort. We found no genotype or kindling related differences in total tau, AT8/tau, AT100/tau or AT180/tau ratios (Supplementary Fig. 3). These results were unsurprising, as 5XFAD is a model of amyloid pathology and not sporadic Alzheimer's disease, as in the human cases.

Inhibition of mTORC1 rescues seizure-induced deficits of associative long-term and remote memory in 5XFAD mice

To investigate the relationship between mTORC1 activation, seizure activity, Alzheimer's disease neuropathology and cognition, we subjected 3-month-old 5XFAD and wild-type mice to a modified PTZ kindling protocol, followed by chronic low-dose rapamycin treatment (2.24 mg/kg)[61–64] or control feed treatment from 3.5 months to 7 months of age (Fig. 5A). In this cohort, the PTZ procedure was altered to minimize kindling-related deaths. Mice were removed from kindling once they reached a Racine score of 5 on three consecutive treatment days, and no PTZ challenge was given at 1 month post-kindling. This modified protocol confirmed our previous finding of hyperexcitability in 5XFAD mice, as demonstrated by significant increases in daily kindling scores (overall $P < 0.0001$; Supplementary Fig. 4A), increased seizure severity ($P < 0.001$; Supplementary Fig. 4B), elevated cumulative seizure severity (AUC, +53%, $P < 0.0001$; Supplementary Fig. 4C) and decreased seizure latency (–36.8%, $P < 0.0001$; Supplementary Fig. 4D) in 5XFAD mice compared with wild-type controls.

The chronic rapamycin treatment was well-tolerated as no mice died prior to planned euthanasia at 7 months. Additionally, in males, rapamycin attenuated the weight loss seen in transgenic Alzheimer's disease mice.[63] Male control-fed 5XFAD mice had significantly lower weights at time of death than both control-fed wild-type mice and rapamycin-fed 5XFAD mice (–12.5% and –13.6%, respectvely, $P < 0.05$), while control-fed wild-type mice and rapamycin-fed 5XFAD mice exhibited no such differences (Supplementary Fig. 4E). Meanwhile, the weights of female mice at time of death showed no differences between groups, regardless of genotype (5XFAD or wild-type) or treatment (rapamycin or control) (Supplementary Fig. 4F).

Behavioural testing, started at 6.5 months of age, consisted of long term and remote memory (fear conditioning testing at 24 h and 14 days, respectively), working memory (spontaneous alteration in Y-maze) and nest building assessment, as described above. Rapamycin treatment had its strongest rescue effect on long term memory in 5XFAD-PTZ-kindled mice, leading to a Genotype × Kindling × Treatment interaction [$P < 0.01$, (80.36, 335.9); Fig. 5B]. Similarly, kindling led to significant deficits in remote memory in 5XFAD mice (–47.5%, $P < 0.05$), which was rescued by rapamycin treatment (+110.1%, $P < 0.05$ for remote memory; Fig. 5C). The Y-maze testing also indicated negative effects of 5XFAD genotype [$F(1,93) = 8.738$, $P < 0.01$] and kindling [$F(1,93) = 6.882$, $P < 0.05$] on working memory, with no significant rapamycin effect (Fig. 5D). Similarly, nest building assessment showed deleterious effects of genotype [$F(1,93) = 38.62$, $P < 0.0001$] and kindling [$F(1,93) = 27.41$, $P < 0.0001$] but no rapamycin rescue (Fig. 5E). Interestingly, female sex was associated with decreased long term contextual memory only in 5XFAD mice, leading to a Sex × Genotype interaction [$P < 0.01$, (62.85, 281.4); Fig. 5B]. This

was the only behavioural testing impacted by sex. Statistical analysis is reported in its entirety in Supplementary Table 4 for the rapamycin therapeutic cohort. Of note, the genotype effect for nest building and Y-maze tests, as well as the sex effect to worsen long-term memory in 5XFAD mice, were only seen in the second cohort, possibly due to larger sample sizes.

Inhibition of mTORC1 protects against seizure-induced amyloid pathology and neuronal death in 5XFAD mice

After behavioural assessment, we performed western blot analysis of the hippocampus for pS6 [Ser240, Ser244] in relation to total S6, to evaluate the degree of mTORC1 inhibition (Fig. 6A and B). We found that rapamycin treatment decreased mTORC1 activity [rapamycin, $P < 0.05$, $F(1,85) = 4.41$], with its most robust effect on mTORC1 activity in kindled mice [Rapamycin × Kindling, $P < 0.05$, $F(1,85) = 5.517$; Fig. 6B]. Western blot analysis of the hippocampus for pS6 at [Ser235, Ser236] yielded similar results, rapamycin treatment decreased mTORC1 activity (rapamycin, $P < 0.0001$, –22.94%), with its most robust effect on mTORC1 activity in 5XFAD-kindled mice ($P < 0.05$, –36.65%; Supplementary Fig. 4G). As chronic rapamycin treatment has been known to also reduce mTORC2 activity,[65] we next examined one of its readout, the phosphorylation of glycogen synthase kinase-3β (GSK-3β) at Ser9[66] in the hippocampus of rapamycin- and control-treated mice. Rapamycin did not have an effect on hippocampal mTORC2 activity, however female mice exhibited decreases in mTORC2 activity, possibly due to negative regulation from mTORC1 ($P < 0.05$, –23.6%; Supplementary Fig. 4H).

We next analysed the interaction between chronic hyperexcitability induced by PTZ kindling and rapamycin on amyloid pathology and neurodegeneration in 5XFAD and wild-type mice. We found that hippocampal soluble human amyloid-β_{42} levels, as measured by ELISA, increased with PTZ kindling in control-fed 5XFAD mice (+94%, $P < 0.05$; Fig. 6C) but not in the rapamycin-treated 5XFAD mice. Again, no human amyloid-β_{42} was detected in wild-type mice, regardless of PTZ or rapamycin treatment. Amyloid-β_{42} levels in PTZ-kindled 5XFAD hippocampus were positively correlated with seizure severity ($r = 0.84$, $P < 0.001$; Fig. 6D). Amyloid load, assessed by immunohistochemistry (Fig. 6E), also demonstrated an increase with PTZ kindling in the hippocampus of control-fed 5XFAD mice (+134%, $P < 0.05$; Fig. 6F), in contrast to rapamycin treated 5XFAD group, where kindling showed no significant effect on amyloid load. Notably, rapamycin treatment did not decrease hippocampal amyloid load in non-kindled 5XFAD mice (Fig. 6F). In addition, rapamycin did not have an effect on the amyloid load in cortical layers IV–VI of PTZ-kindled 5XFAD mice (Supplementary Fig. 4I). Neuronal death, measured by quantification of Fluoro-Jade B-positive cells, was increased by PTZ kindling in control-fed 5XFAD mice (+106%, in the hippocampus, $P < 0.05$; Fig. 6G) and attenuated by rapamycin treatment. This pattern was particularly noticeable in the CA1 and CA2 regions where there was an appreciable effect of rapamycin treatment [$F(1,36) = 4.55$, $P < 0.05$] and kindling [$F(1,36) = 9.46$, $P < 0.01$]. We again confirmed the absence of neuronal death in wild-type animals (Fig. 6I). Mirroring the data presented in our non-therapeutic cohort, female sex in 5XFAD mice was a predictor of increased neuronal death [$P < 0.05$, (0.1055, 4.596); Fig. 6G]. As in our measurements of amyloid load, rapamycin did not have a noticeable effect on neuronal death in cortical layers IV–VI of PTZ-kindled 5XFAD mice (Supplementary Fig. 4J).

Discussion

In this study, we sought to investigate the connection between Alzheimer's disease and epilepsy, with a special emphasis on interdependent mechanisms, and to explore common therapeutic targets. Our data from studies of brain samples from patients with Alzheimer's disease and 5XFAD mice support such a bidirectional relationship between hyperexcitability and Alzheimer's disease, with each contributing to the development and progression of the other. In addition, we show that mTORC1 represents an effective target to disrupt this positive feedback loop.

While previous clinical observations have suggested that seizures can accelerate the progression of Alzheimer's disease,[2,22] this relationship was not well-established and remains disputed.[4–6,67] By examining tissue and clinical data from patients with Alzheimer's disease, we found that seizure history is associated with more severe pathological features of Alzheimer's disease, specifically more severe brain atrophy and ventricular enlargement, and increased phosphorylated tau AT100 and amyloid plaque pathology in the subcortical white matter. The increased tau hyperphosphorylation in Alzheimer's disease cases due to seizures is consistent with the presence of tau pathology in patients with temporal lobe epilepsy[8,11] and the role of tau in promoting neuronal excitability.[12,13,68] Alzheimer's disease-associated neurodegeneration is traditionally thought to preferentially target cortical grey matter, but several studies focusing on white matter deterioration[69,70] have demonstrated the presence of amyloid-β in this region.[71] This study is the first to suggest that seizures exacerbate white matter amyloid-β load, providing a novel link between hyperexcitability and Alzheimer's disease-related white matter degeneration. The lack of differences in soluble amyloid-β and grey matter plaque pathology within the temporal cortex of patients with Alzheimer's disease with and without seizures is likely because the patients with Alzheimer's disease were end-stage with advanced amyloid pathology. Amyloid-β_{42} deposition in the temporal cortex reaches a plateau very early in the disease progression,[72] making it difficult to detect potential subtle differences at later stages. Indeed, this dissociation points to the need to include seizure history in future longitudinal studies testing *in vivo* biomarkers of Alzheimer's disease progression such as CSF levels of tau and amyloid-β. We avoided this potential confound in the mouse model by performing experiments at earlier stages of disease progression.

In parallel, we demonstrated that 5XFAD mice present a hyperexcitable phenotype (decreased seizure latency and a faster and more severe kindling) during the period of onset and development of Alzheimer's disease pathology and that seizures during this critical time window have detrimental long-term effects on cognitive behaviour, amyloid and plaque pathology and neuronal death but not neurogenesis. However, these findings should be interpreted with caution, acknowledging that the seizures induced in 5XFAD mice were generalized tonic-clonic, while typically the seizures in patients with Alzheimer's disease are focal. As protocols for focal seizures in mouse models become established, more directed studies may be possible and may identify more subtle changes.

PTZ kindling at a pre-symptomatic stage resulted in the exacerbation of remote contextual memory, self-care and working memory deficits, despite the fact that, overall, our 5XFAD mice showed only subtle behavioural deficits, consistent with previous studies.[17,21] In line with the more severe atrophy observed in patients with Alzheimer's disease and seizures, kindled 5XFAD mice exhibited higher levels of neuronal death. Additionally, we found a positive correlation between neuronal death and the seizure severity in PTZ-kindled 5XFAD mice. Given that PTZ itself does not directly induce cell death, as confirmed by the absence of Fluoro-Jade B

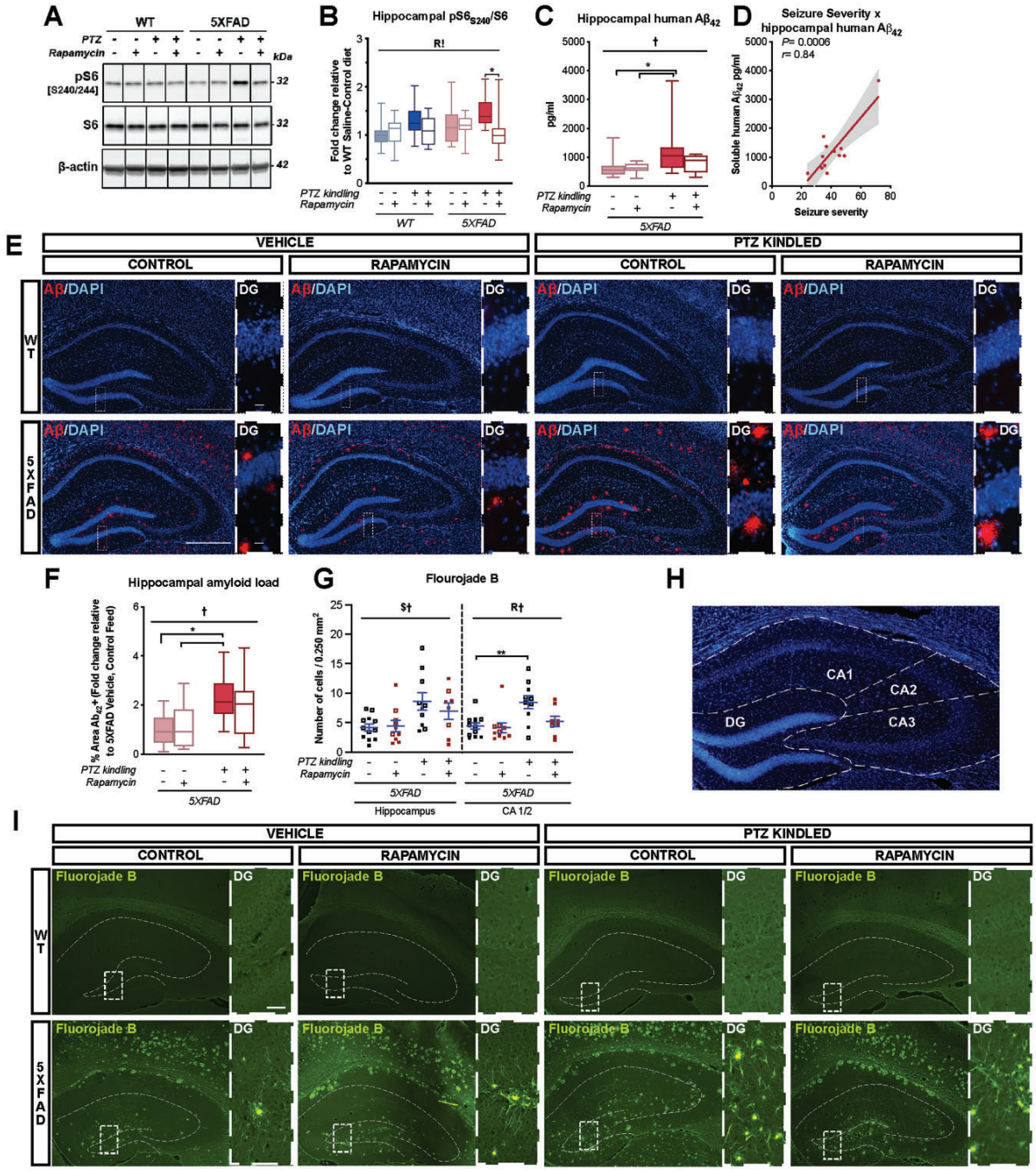

Figure 6 Inhibition of mTORC1 protects against the effect of seizures on amyloid pathology and neuronal death in 5XFAD mice. (**A**) Representative western blot images for **B** showing non-adjacent bands originating from the same blot. (**B**) Western blot quantification of phosphorylated $S6_{S240}$/total S6. (**C**) ELISA assessment of hippocampal soluble human amyloid-β_{42} in pg/ml. (**D**) Pearson correlation between seizure severity and hippocampal amyloid-β_{42} concentration. (**E**) Representative images of the hippocampus and cortex with insets of the dentate gyrus (DG) immunohistochemically (IHC) co-labelled with human amyloid-β_{42} (red) and DAPI stain (blue). (**F**) Corresponding quantification of hippocampal amyloid load. (**G**) Quantification of Fluoro-Jade B-positive cells per 0.25 mm^2 in the 5XFAD hippocampus (*left*) and the CA1/2 region (*right*). (**H**) Representative hippocampal section labelled with nuclear DAPI stain showing various regions of interest. (**I**) Representative images of the hippocampus and cortex with insets of the dentate gyrus stained with Fluoro-Jade B. Scale bars = 500 μm for entire images; and 20 μm for *insets*. Box and whisker plots display minimum, maximum and all quartiles. Scatter plots display all data-points, with mean ± SEM. Closed symbols represent males and open symbols represent females. $n = 12–13$ for each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. $^{\$}$Sex effect, $P < 0.05$; $^{\&}$Genotype × Sex interaction, $P < 0.05$; ^{X}Genotype × Kindling × Treatment interaction, $P < 0.05$; †Kindling effect, $P < 0.01$; ^{R}Treatment effect, $P < 0.05$.

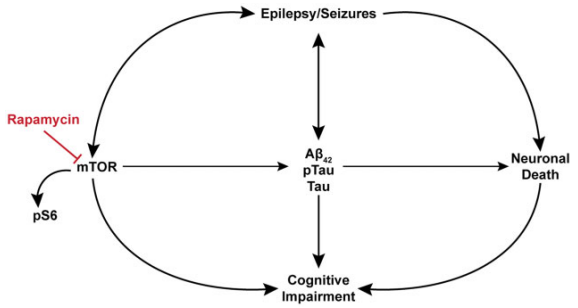

Figure 7 Proposed mechanism of the bidirectional relationship of seizures and Alzheimer's disease with contributions of mTOR. A diagram that summarizes and synthesizes the findings from our work in this paper and previous work in models of Alzheimer's disease and epilepsy. In this paper, we demonstrated that epilepsy/seizures can result in increased mTOR activity, amyloid-β (Aβ) and tau pathology and neuronal death. Previous work in epilepsy models has demonstrated that mTOR is involved in epileptogenesis, and our work here shows that mTOR is also increased by seizures. The mTOR pathway has been implicated in amyloid-β deposition via autophagy, and in this paper was shown to be co-localized at sites of pTau inclusions. Finally, amyloid pathology, tau pathology, mTOR and neuronal death all contribute to cognitive impairment in patients with Alzheimer's disease and seizures.

staining in our PTZ-kindled wild-type cohort, the increased neuronal death observed in kindled 5XFAD mice suggested an acceleration of Alzheimer's disease neurodegeneration, without affecting neurogenesis. These seizure-associated differences were accompanied by exacerbated markers of Alzheimer's disease pathology, consistent with a previous study of pilocarpine-induced seizures in the 3xTg Alzheimer's disease mice.[23] The observed PTZ-induced increases in amyloid-β_{42} in the 5XFAD mice may be due to increased APP processing, given the increased APP expression and phosphorylation. Amyloid-β_{42} is produced in response to synaptic activity,[73,74] and seizures are known to upregulate its production.[75] This may explain the strong positive relationship we found between amyloid-β_{42} levels and seizure severity in 5XFAD mice. The reverse is also true, as amyloid-β_{42} is known to induce epileptic events.[76] Further studies involving pretreatment of 5XFAD mice with amyloid reducing therapies prior to kindling may elucidate whether amyloid-β reductions are sufficient to reverse the hyperexcitability seen in 5XFAD mice, lending support to amyloid reducing therapies to lower future seizure risk in familial patients with Alzheimer's disease, a population especially at risk of seizures.[2] While we found increased levels of total and pathological phosphorylated tau in patients with Alzheimer's disease and seizures, we did not find similar changes in the 5XFAD mouse, and this may in part be due to the fact that this is an amyloid mouse model. Further studies using a tauopathy rodent model of Alzheimer's disease may help validate the patterns of expression and hyperphosphorylation of tau demonstrated in the patients with Alzheimer's disease included in this study. Overall, our mouse data were similar in hippocampus and cortex but more pronounced in hippocampus. This could be due to the progression of amyloid pathology in 5XFAD, where it first appears, starting in deep layers of the cortex and in the subiculum before spreading to the superficial cortical layers and hippocampus until 10 months of age in males and up to 14 months in females.[17,77] The effects of seizures may be more noticeable at a histopathological level in brain structures that have not yet reached the amyloid plateau.

Given that hyperexcitability and epilepsy can lead to neurodegeneration and vice versa, it is a challenge to determine the initiating factor in Alzheimer's disease. mTORC1 represents one of possibly many shared downstream effectors of both Alzheimer's disease and epilepsy that may prove to be a novel therapeutic target. Alzheimer's disease and seizures have additive effects on mTORC1 activation, a pattern that we observed in both patients with Alzheimer's disease and 5XFAD mice, where seizure severity was positively correlated with mTORC1 activity. Our human temporal cortex dataset showed an increase in pS6 [Ser235, Ser236] but not pS6 [Ser240, Ser244], both readouts of mTORC1 activation via p70S6K activity. However, Ser235 and Ser236 sites can be phosphorylated by other activity dependent kinases such as p90 ribosomal S6 kinase (RSK) via ERK[43] that we showed being activated in Alzheimer's disease temporal cortices. Therefore, the ERK/RSK pathway might be another common factor in this Alzheimer's disease-epilepsy bidirectional relationship and future human studies should further assess the relative contribution of mTORC1/p70S6K and ERK/RSK pathways.

Seizure-dependent increases in mTORC1 activity may be initiated by neurotransmitters and cytokines released during seizures that activate mTORC1 in an activity-dependent manner.[78] Activated mTORC1 can in turn phosphorylate tau on AT100 epitopes, which we found to be specifically upregulated and localized with pS6 in patients with Alzheimer's disease and seizures.[37,38,40] Activated mTORC1 can also cause amyloid-β_{42} accumulation via the downregulation of autophagy[79] or via *BACE1* translational upregulation.[38] As upregulation of the mTORC1 pathway can lead to cell proliferation,[80] it is also possible that mTORC1 overactivation is a compensatory response to neuronal loss in Alzheimer's disease brains.

The protein complex mTORC1 is a potent therapeutic target in epilepsy, and the FDA-approved drug rapamycin has shown benefit in numerous studies in Alzheimer's disease and epilepsy mouse models.[8,27,36,62,63,81–83] Rapamycin is not an anti-seizure medication, as it has no immediate effects on neuronal excitability. Its actions are various and include restoration of autophagy-related pathways, impaired in both epilepsy[84] and Alzheimer's disease,[85] and suppression of neuroinflammation, known to be induced via mTORC1 in both conditions.[86,87] Here, we showed that the inhibition of mTORC1 by rapamycin reversed the effects of seizures on cognition and protected against the deleterious effects of seizures on Alzheimer's disease neuropathology in 5XFAD mice. Rapamycin significantly improved long-term and remote memory in kindled 5XFAD mice, while Alzheimer's disease neuropathology was already set. The ability of rapamycin to rescue memory deficits in 5XFAD-kindled mice is likely due to the collective impact on neuronal death, amyloid pathology and mTORC1 signalling itself. In mouse models of Alzheimer's disease, mTORC1 activity has shown a strong correlation with cognitive performance[88] and in a model of tuberous sclerosis complex, rapamycin treatment was able to rescue cognitive function independent of seizure suppression.[89] Given the selective cognitive benefit of rapamycin in mice with high elevations in mTORC1 activity (PTZ-kindled 5XFAD mice), rapamycin or its analogues may selectively help patients with Alzheimer's disease and seizures, where mTORC1 activity was demonstrated as the highest amongst our Alzheimer's disease cases. The use of rapamycin or its analogues in patients with Alzheimer's disease with subclinical and/or clinical seizures may offer significant benefits, as the appearance of seizures often occurs early in the disease course and, thus, may slow the progression of Alzheimer's disease.[90] The low-dose rapamycin regimen in our study decreased mTORC1 activity in kindled mice sufficiently to normalize mTORC1 activity heightened by both seizures and Alzheimer's disease, which may allow for lower dosing and fewer of the side effects commonly associated with higher doses.[34] It is interesting to note that while we did find behavioural rescue, the effects on Alzheimer's disease pathology were rather modest. This

may represent a limitation of low-dose rapamycin to alter Alzheimer's disease protein accumulation, but does, at the least, suggest symptomatic relief. It is also possible that other kinases that Alzheimer's disease and epilepsy converge upon, such as ERK and JNK, which also were elevated in this study, may provide additional therapeutic targets.

While our study suggests a critical role for the mTORC1 pathways in the exacerbation of Alzheimer's disease by seizures, it is also well known that excitatory:inhibitory imbalances are operative in both diseases. In our study, naïve 5XFAD mice showed a decreased parvalbumin immunoreactivity, reflecting a deficit of GABA inhibition.[91–95] Similarly, decreased ratios of α1/α3 $GABA_AR$ subunits and increased ratios of NKCC1:KCC2, resulting in depolarizing GABA, have been noted in both human cases and mouse model studies of epilepsy[91–94,96] as well as in Alzheimer's disease mouse models.[97–101] The activity of mTORC1 was shown to be correlated and co-localized with elevated NKCC1:KCC2 ratios in patients with tuberous sclerosis complex.[93] $GABA_AR$ loss has been linked to seizures and cognitive impairment in the human APP mouse model of Alzheimer's disease[101] and could then also be responsible for such changes in our 5XFAD model. Thus, regulators of excitatory:inhibitory imbalance may also represent a mechanism by which rapamycin reversed certain neuropathological and cognitive outcomes in our study (Fig. 7).

In summary, the connection between seizures and exacerbation of Alzheimer's disease described here, in conjunction with our previous study demonstrating Alzheimer's disease-like pathology in patients with temporal lobe epilepsy,[8] suggests a bidirectional nature of epilepsy and neurodegeneration. Our data also underscore the potential deleterious effects of seizures in Alzheimer's disease and confirm the importance of further clinical trials regarding the treatment of seizures in patients with Alzheimer's disease. Past studies have shown that traditional antiepileptic drugs have varied results,[24,25] and a Cochrane review found only one randomized control trial on the use of these drugs in patients with Alzheimer's disease, a study they deemed to show 'very low quality' evidence.[26] In evaluating treatment candidates for seizures in Alzheimer's disease, adjunct therapies such as rapamycin should be included. Chronic low dose rapamycin provided prevention against the effects of seizures on Alzheimer's disease pathology in the 5XFAD mice. Complementing our study, rapamycin has attenuated other aspects of neuropathology in other preclinical trials of Alzheimer's disease[8,62,63] and shows promise as a clinical treatment for epilepsy, particularly in patients with tuberous sclerosis complex.[36] While clinical trials have not been conducted on rapamycin for Alzheimer's disease, preliminary studies show that it is a safe option in the elderly population.[102] These *in vivo* preclinical and early clinical studies, along with the data presented here, suggest that clinical trials should be conducted to examine the use of rapamycin as an adjunct therapy in patients with Alzheimer's disease who demonstrate seizure activity, given its ability to mitigate the bidirectional relationship between Alzheimer's disease and seizures.

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Competing interests

The authors have declared no conflicts of interest.

Supplementary material

Supplementary material is available at *Brain* online.

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