Neuroscience

Hyperactivity of mTORC1 or mTORC2-dependent signaling causes epilepsy downstream of somatic PTEN loss

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Erin R. Cullen, Isabelle Mittelstadt, Matthew C. Weston

Department of Neurological Sciences, Larner College of Medicine, University of Vermont, Burlington VT, 05405, USA • Fralin Biomedical Research Institute at VTC, Center for Neurobiology Research, Roanoke VA, 24016, USA • School of Neuroscience, Virginia Polytechnic and State University, Blacksburg VA, 24060, USA

Abstract

Gene variants that hyperactivate PI3K-mTOR signaling in the brain lead to epilepsy and cortical malformations in humans. Some gene variants associated with these pathologies only hyperactivate mTORC1, but others, such as *PTEN*, *PIC3CA*, and *AKT*, hyperactivate both mTORC1- and mTORC2-dependent signaling. Previous work has established a key role for mTORC1 hyperactivity in mTORopathies, however, whether mTORC2 hyperactivity contributes is not clear. To test this, we inactivated mTORC1 and/or mTORC2 downstream of early *Pten* deletion in a new model of somatic Pten LOF in the cortex. Spontaneous seizures and epileptiform activity persisted despite mTORC1 or mTORC2 inactivation alone, but inactivating both mTORC1 and mTORC2 normalized pathology. These results suggest that hyperactivity of both mTORC1 and mTORC2 are sufficient to cause epilepsy, and that targeted therapies should aim to reduce activity of both complexes.

eLife assessment

This **useful** study addresses epilepsy caused by the loss of a molecule called Pten, resulting in hyperactivity of the mTOR pathway. The findings suggest that inhibiting two molecules called mTORC1 and mTORC2 can reduce epilepsy symptoms but there is much less effect when inhibited separately. The evidence supporting the conclusions is currently **incomplete**, but could be strengthened after additional experiments.

Introduction

Genetic disruptions that hyperactivate mTOR activity, termed 'mTORopathies', cause macrocephaly, intractable childhood epilepsies, and behavioral presentations including autism spectrum disorders [1-4]. The mTOR kinase can function in two complexes, mTORC1 and mTORC2, each with unique downstream targets and upstream activators [5]. A unifying feature of mTORopathies is hyperactivation of mTORC1, which has led to the hypothesis that this complex and its effectors mediate disease phenotypes [6]. A subset of mTORopathies however, (e.g. those

caused by variants in *PTEN*, *PIC3CA*, and *AKT)*, hyperactivate both mTORC1 and mTORC2 [7,8], and mTORC2 hyperactivity has also been reported in human TLE [9]. It is unclear whether mTORC2 hyperactivity contributes to, or can cause, disease phenotypes such as epilepsy.

PTEN LOF in neurons induces soma hypertrophy, increased dendritic branching, and synaptic hyperconnectivity [10,11]. These phenotypes resemble those observed in models of mTORC1-specific hyperactivation [12], and can be prevented with either the mTORC1 inhibitor rapamycin or loss of the mTORC1-specific protein Raptor [13]. Rapamycin also rescues epilepsy and mortality associated with *Pten* loss, even after epilepsy has been established, suggesting that mTORC1 hyperactivity underlies these phenotypes [14-16]. Thus, there is strong evidence that hyperactivation of mTORC1 downstream of PTEN disruption causes the macrocephaly, epilepsy, early mortality, and synaptic dysregulation observed in humans and model organisms [17].

There is also evidence against the mTORC1-centric hypothesis. Rapamycin may affect mTORC2 activity at high doses or over long periods of time [18,17], and mTORC2 regulates synapse function, neuronal size, and cytoskeletal organization independent of mTORC1 [19,20]. In a mouse model in which *Pten* was inactivated in forebrain neurons in early adulthood, mTORC2 inhibition, but not mTORC1 inhibition, rescued seizures and behavioral abnormalities. In this model, epilepsy was dissociated from macrocephaly, which was rescued by mTORC1 inhibition but not mTORC2 inhibition [21]. However, mTORC2 inactivation did not normalize spontaneous seizures or seizure susceptibility in a model of *Pten* loss in dentate granule neurons [22]. Thus, there is conflicting evidence about whether mTORC1 or mTORC2 hyperactivity causes epilepsy downstream of *Pten* loss.

To address this, we suppressed mTORC1 and mTORC2 activity alongside PTEN LOF by inducing simultaneous deletion of *Raptor* or *Rictor*, whose protein products are unique and essential components of mTORC1 and mTORC2, respectively, in a model of somatic PTEN LOF in the cortex. In this model, epilepsy could be rescued by concurrent mTORC1 and mTORC2 inactivation, but persisted when either gene remained intact, suggesting that hyperactivity of either complex can lead to neuronal hyperexcitability and epilepsy.

Results

Generation of a developmental brain somatic mosaic model of PTEN LOF

To model the epileptogenic somatic mutations often observed in mTORopathies [23,24], we injected an AAV9 virus expressing GFP and Cre under control of the *hSyn* promoter into one hemisphere of the cortex of *Pten*$^{(fl/fl)}$, *Pten*$^{(fl/fl)}$-*Raptor*$^{(fl/fl)}$, *Pten*$^{(fl/fl)}$-*Rictor*$^{(fl/fl)}$, and *Pten*$^{(fl/fl)}$-*Raptor*$^{(fl/fl)}$-*Rictor*$^{(fl/fl)}$ mice at P0 (**Fig. 1A**). Green fluorescent protein (GFP) was expressed in neurons throughout the cortical layers in all experimental animals, but largely confined to one cortical hemisphere and the underlying hippocampus (**Fig. 1B**). The percentage of cells that were GFP+ was similar across groups, although there was a decrease in cell density in the Pten LOF and Pten-Rictor LOF groups [25] (**Fig. 1C**).

We analyzed the impact of Pten, Raptor, and Rictor LOF on mTORC1 and mTORC2 activity in the affected hemisphere using quantitative immunohistochemistry. Phospho-S6 levels, which report mTORC1 activity through S6K1, were increased from Controls in Pten LOF and Pten-Rictor LOF brains, but rescued in Pten-Raptor LOF brains (**Fig. 1D**$_1$). Phospho-Akt (S473) levels, which report mTORC2 activity, were increased in Pten LOF and Pten-Raptor LOF but normalized in Pten-Rictor LOF brains (**Fig. 1D**$_2$). Levels of both pS6 and pAkt were not different from Controls in

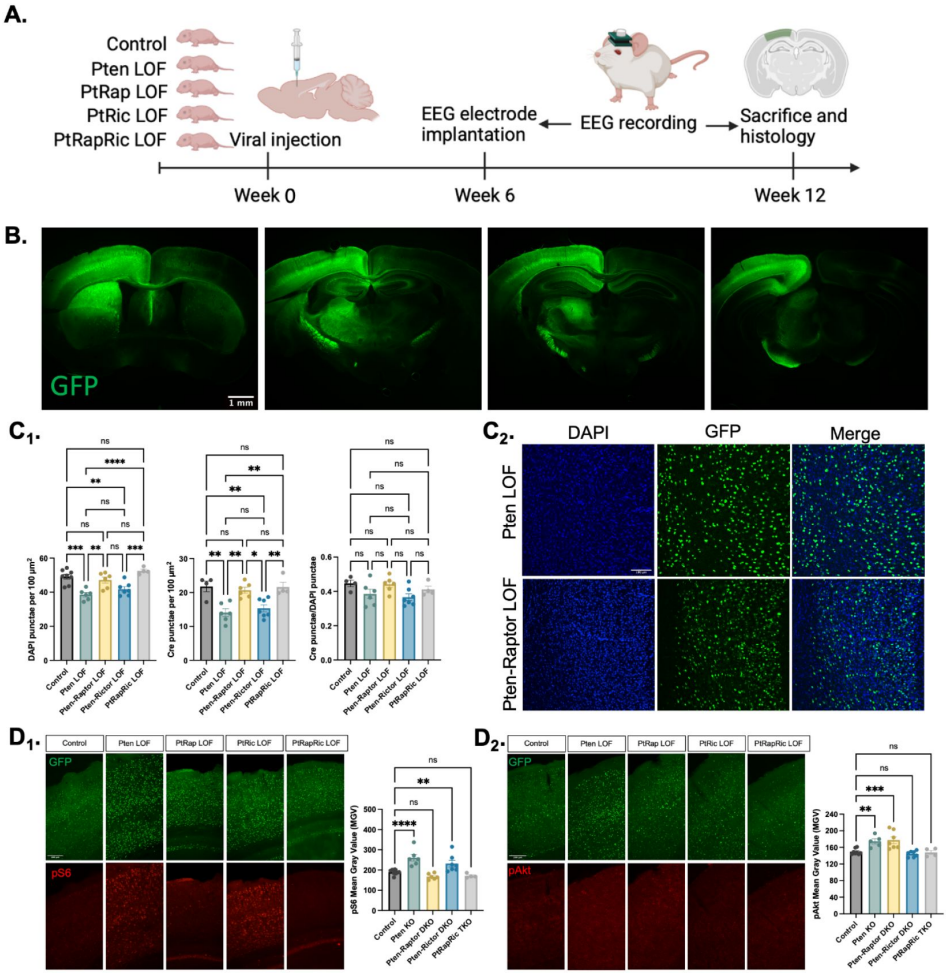

Figure 1.

Characterization of the focal cortical Pten LOF model.

A) Experimental timeline showing induction of *hSyn*-Cre-GFP or Control *hSyn*-GFP AAV at P0 and EEG recording in adulthood. B) Representative images of GFP expression in a Control mouse brain, demonstrating expression predominantly in one hemisphere of the cortex. C1) Quantification of lesion severity and neuron density in Cre-expressing animals. There were fewer Cre-expressing neurons per unit area in the *Pten* LOF and *Pten-Rictor* LOF groups, which was at least partially attributable to a decrease in cell density in these groups. No significant differences in Cre expression remained when Cre expression was calculated based on cell density rather than area. C2) Representative images of DAPI and Cre fluorescence in the cortex of *Pten* LOF and *Pten-Raptor* LOF animals. Cell density and Cre density in *Pten-Raptor* LOF animals was indistinguishable from Controls. D_1) Phospho-S6, a marker of mTORC1 activity, was increased by *Pten* LOF and reduced to control levels by concurrent *Raptor* LOF. Phospho-S6 was also increased from Control levels in *Pten-Rictor* LOF, indicating that mTORC1 hyperactivity was not normalized by *Rictor* LOF. Combined *Raptor*/*Rictor* LOF also normalized phospho-S6 expression. D2) phospho-Akt, a marker of mTORC2 activity, was increased in *Pten* LOF and normalized by *Rictor* LOF, but not by *Raptor* LOF. Combined *Raptor*/*Rictor* LOF also normalized phospho-Akt expression. Error bars show mean ± s.e.m. ns indicates p>0.05, * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001, and **** indicates p<0.0001 as assessed by one-way ANOVA with Tukey multiple comparisons correction. Diagram created with *BioRender.com (http://biorender.com/)*.

Pten-Raptor-Rictor LOF brains. These results indicate that Pten LOF hyperactivates both mTORC1 and mTORC2 in this model, and that these increases in activity are independently rescued by mTORC1 and mTORC2 inactivation.

Raptor and/or Rictor LOF attenuate Pten LOF-induced macrocephaly

Macrocephaly and neuronal hypertrophy are well-characterized sequelae of deficient PTEN signaling in patients and mouse models [26,27]. In mouse models of Pten LOF, these phenotypes can be rescued by rapamycin treatment or Raptor LOF [21,28,17,13]. Here, cortical thickness was increased in Pten LOF and rescued to Control levels by Raptor LOF, Rictor LOF, or combined Raptor-Rictor LOF (**Fig. 2A-B**). In individual neurons Pten LOF caused a ≈100% increase in mean soma size versus Control. Pten-Rictor LOF significantly reduced soma size from Pten LOF levels, but still showed a 60% increase over Controls. Soma size in Pten-Raptor LOF and Pten-Raptor-Rictor LOF neurons did not significantly differ from Controls (**Fig. 2C**).

Concurrent mTORC1/2 inactivation, but neither alone, rescues epilepsy and interictal EEG abnormalities in focal Pten LOF

Next, we measured epileptic brain activity with video-EEG to determine the ability of mTORC1 or mTORC2 activity to rescue this key feature of mTORopathies, Generalized seizures (GS) were observed in 0/9 Control, 4/7 Pten LOF, 2/6 Pten-Raptor LOF, 2/7 Pten-Rictor LOF, and 0/6 Pten-Raptor-Rictor LOF animals. GS events were longer-lasting in Pten-Raptor LOF animals than in Pten LOF or Pten-Rictor LOF animals (**Fig. 3A**). GS did not appear to be correlated with mTOR pathway activity (**Supplementary Figure 2**).

The most striking and consistent type of epileptic brain activity in the Pten LOF animals was frequent 5-7 Hz spike trains lasting 3 seconds or more, which fit previous characterizations of spike- and-wave discharges (SWDs) in rodents [29]. SWDs were observed in all Pten LOF, Pten-Raptor LOF, and Pten-Rictor LOF animals, and in 3/9 Control and 3/6 Pten-Raptor-Rictor LOF animals. The frequency of SWDs in Pten LOF, Pten-Raptor LOF, and Pten-Rictor LOF animals was significantly higher than Controls, but Pten-Raptor-Rictor LOF completely blocked this increase. Pten-Rictor LOF animals had a lower frequency of SWD events than Pten LOF animals (**Fig. 3B**), indicating a partial rescue. Taken together, these data indicate inactivating mTORC1 provides no protection against *Pten* loss-induced epilepsy, and may even exacerbate it. mTORC2 inactivation provides some protection, but only concurrent mTORC1 and mTORC2 inactivation can prevent it.

In addition to epileptic activity, we also found that Pten LOF caused obvious alterations in features of the interictal EEG. To quantify these changes, we measured EEG coastline, absolute mean amplitude, and power spectra of the EEG. Coastline, mean amplitude, and total power were all significantly increased by Pten LOF, and these changes were not significantly reduced by Pten-Raptor or Pten-Rictor LOF. Pten-Raptor-Rictor LOF, however, reduced these increases to Control levels. Correspondingly, EEG power was increased in Pten LOF animals (**Fig. 4A**; **Table 1A**). This increase was normalized by Pten-Raptor-Rictor LOF, but not by Pten-Raptor LOF or Pten-Rictor LOF. Pten-Raptor-Rictor LOF did not fully rescue a rightward shift in normalized EEG band power (**Fig. 4B**; **Table 1B**).

Discussion

Convergent discoveries have positioned mTORopathies as prime candidates for a precision medicine approach [30,31]. There has been some clinical success with rapamycin analogues [32], but preclinical animal studies have suggested ways to further improve the selectivity and

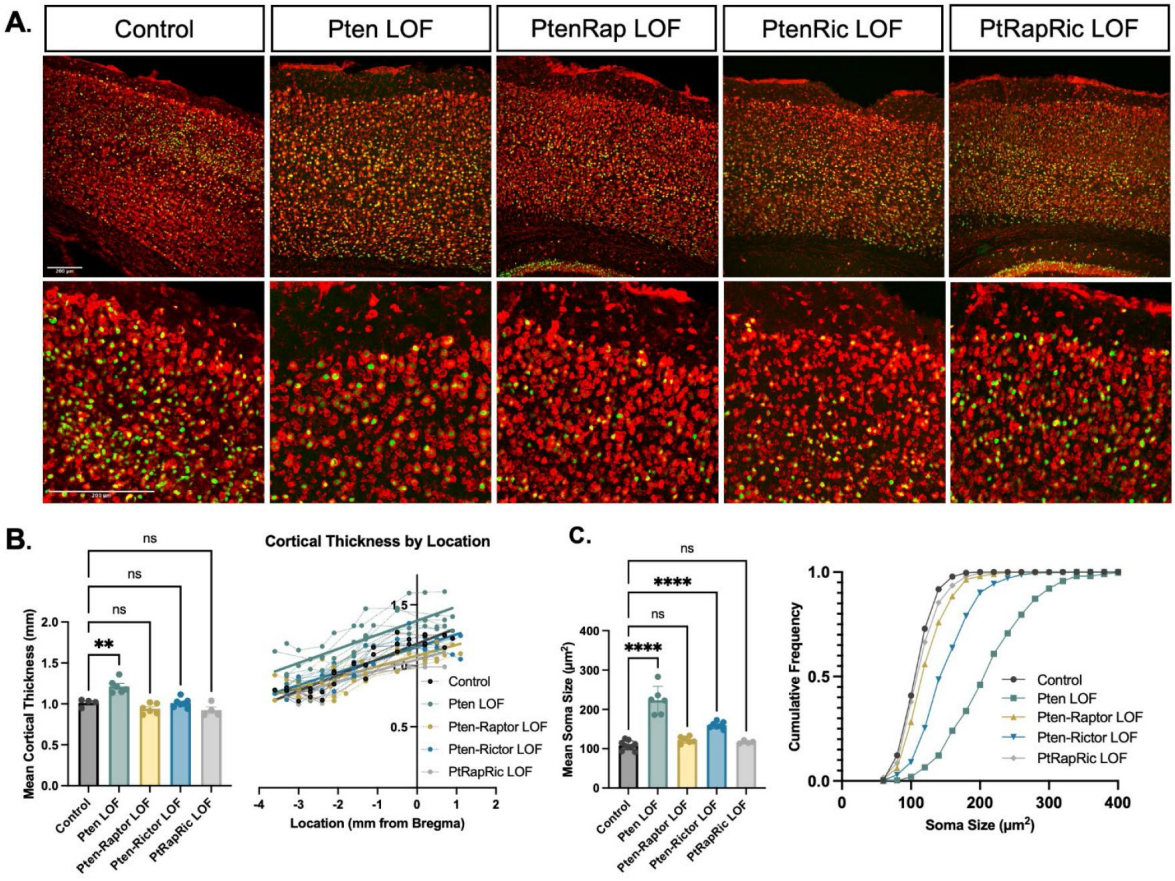

Figure 2.

mTORC1 inhibition or dual mTORC1/2 inhibition rescues, and mTORC2 inhibition partially rescues, abnormalities in cortical morphology induced by focal Pten LOF.

A) Example images showing fluorescent Nissl stain (red) and GFP expression (green) in cortical neurons. The top row shows the cortical thickness in all 5 groups and the bottom row shows zoomed in images depicting the differences in soma size across groups. B) The mean cortical thickness was increased in *Pten* LOF throughout the cortex. *Pten-Raptor, Pten-Rictor*, and *Pten-Raptor-Rictor* LOF cortical thickness did not differ significantly from Controls. C) The mean soma size was strongly increased in *Pten* LOF and to a smaller extent in *Pten-Rictor* LOF. *Pten-Raptor* LOF and *Pten-Raptor-Rictor* LOF groups did not differ significantly from Controls. Error bars show mean ± s.e.m. ns indicates p>0.05, * indicates p<0.05, ** indicates p<0.01, and **** indicates p<0.0001 as assessed by one-way ANOVA with Tukey multiple comparisons correction.

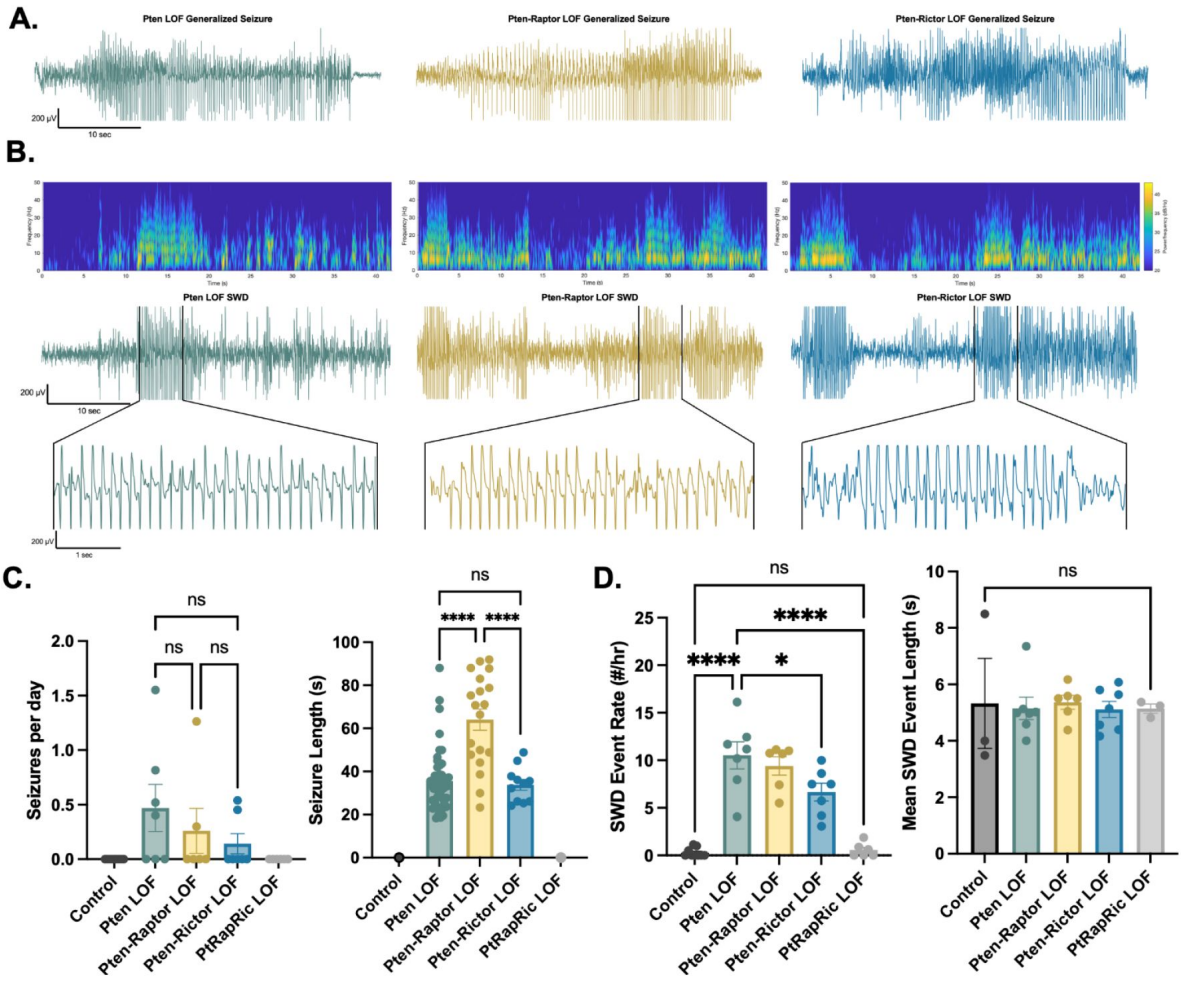

Figure 3.

Combined mTORC1 and mTORC2 inactivation, but neither alone, rescues epilepsy in the focal Pten LOF model.

Spontaneous seizures and interictal spike activity were assessed in mice expressing *Pten* LOF, *Pten-Raptor* LOF, *Pten-Rictor* LOF, *Pten-Raptor-Rictor* LOF, and Controls. A) Representative traces of GS in a subset of animals in *Pten* LOF, *Pten-Raptor* LOF, and *Pten-Rictor* LOF groups. B) Spectrograms (top) and traces depicting example SWD event trains. C) Summary data showing the number of GS per day and GS length. GS events were significantly longer in the *Pten-Raptor* LOF group than the other groups. In the Pten LOF group, 19% of GS events (9/48) exceeded 45 seconds in length, and these events were observed in 2/4 Pten LOF GS+ animals. 79% of GS events (15/19) in the Pten-Raptor LOF group exceeded 45 seconds, and these events were observed in 2/2 GS+ Pten-Raptor LOF animals. 1/11 GS events in the Pten-Rictor LOF group exceeded this threshold. D. Summary data showing the SWD rate and length in all animals. Error bars show mean ± s.e.m. ns indicates p>0.05, * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001, and **** indicates p<0.0001 as assessed by one-way ANOVA with Tukey multiple comparisons correction.

Table 1A: Total Power

Band	2-way ANOVA p-value						
	Control vs. Pten LOF	Control vs. Pten-Raptor LOF	Control vs. Pten-Rictor LOF	Control vs. Pten-Raptor-Rictor LOF	Pten LOF vs. Pten-Raptor LOF	Pten LOF vs. Pten-Rictor LOF	Pten LOF vs. Pten-Raptor-Rictor LOF
1	0.7215	0.9996	0.9976	0.7021	0.8249	0.9347	0.1485
2	0.0833	0.8162	0.8509	0.7749	>0.9999	0.276	0.043
3	0.0657	0.4328	0.5564	0.9949	0.5893	0.6387	0.0543
4	0.1205	0.2658	0.3091	>0.9999	0.6358	0.79	0.1219
5	0.0834	0.1019	0.1838	0.9946	0.7452	0.6116	0.1144
6	0.0508	0.0779	0.127	0.8868	0.8606	0.6302	0.0988
7	0.0395	0.0829	0.0875	0.6727	0.9963	0.8508	0.1137
8	0.0321	0.0749	0.0746	0.6353	>0.9999	0.9834	0.1093
9	0.0236	0.0727	0.0766	0.8311	0.9923	0.9993	0.0576
10	0.0156	0.0605	0.0609	0.9112	0.9096	0.9974	0.0297
11	0.0148	0.0469	0.0336	0.8046	0.7577	0.9889	0.0275
12	0.017	0.0376	0.0186	0.5816	0.7035	0.9837	0.0321
13	0.0219	0.0431	0.0138	0.5078	0.6552	0.9572	0.0379
14	0.0322	0.0298	0.0109	0.5927	0.4738	0.9128	0.0486
15	0.047	0.0172	0.0087	0.7629	0.35	0.8344	0.0616
16	0.0497	0.0133	0.0081	0.5221	0.2767	0.7401	0.0669
17	0.0386	0.0092	0.0104	0.4807	0.2108	0.6195	0.0524
18	0.0284	0.0104	0.0129	0.4624	0.1732	0.4864	0.0386
19	0.0241	0.0166	0.0139	0.607	0.1619	0.3975	0.0315
20	0.0221	0.0132	0.0179	0.3517	0.1556	0.368	0.0342
21	0.021	0.008	0.0176	0.3236	0.1487	0.3703	0.0334
22	0.0238	0.0078	0.0188	0.3161	0.1752	0.4039	0.0382
23	0.0279	0.0093	0.021	0.3728	0.2164	0.4387	0.0434
24	0.0303	0.0131	0.0247	0.4069	0.2433	0.4609	0.0463
25	0.0314	0.0191	0.0253	0.3983	0.2521	0.4648	0.0479
26	0.0366	0.0144	0.026	0.4339	0.2631	0.4714	0.0552
27	0.0399	0.0079	0.0264	0.4669	0.2576	0.4914	0.0599
28	0.0463	0.0064	0.0273	0.4853	0.2697	0.5059	0.0687
29	0.0559	0.0122	0.0282	0.4671	0.3075	0.5461	0.0831
30	0.0598	0.0141	0.0287	0.4514	0.3209	0.5616	0.0894

Table 1B: Relative Power

Band	2-way ANOVA p-value						
	Control vs. Pten LOF	Control vs. Pten-Raptor LOF	Control vs. Pten-Rictor LOF	Control vs. Pten-Raptor-Rictor LOF	Pten LOF vs. Pten-Raptor LOF	Pten LOF vs. Pten-Rictor LOF	Pten LOF vs. Pten-Raptor-Rictor LOF
1	<0.0001	<0.0001	0.0033	<0.0001	0.4967	0.0572	0.9743
2	<0.0001	0.9986	<0.0001	<0.0001	0.0009	0.3614	0.9977
3	<0.0001	<0.0001	<0.0001	<0.0001	0.9337	0.9777	0.2279
4	<0.0001	<0.0001	<0.0001	<0.0001	0.997	0.8997	0.9044

Table 1.

EEG power

5	0.5938	0.4988	<0.0001	0.1852	>0.9999	0.0063	0.9593
6	0.9826	0.0834	0.189	0.5724	0.3384	0.0796	0.9046
7	0.6153	<0.0001	0.9878	0.0043	<0.0001	0.8905	<0.0001
8	0.0053	0.0074	>0.9999	0.0208	<0.0001	0.0114	<0.0001
9	0.0065	0.9007	0.9998	0.832	0.0006	0.0053	0.0003
10	0.3806	0.9964	0.8873	0.9994	0.2612	0.0714	0.3248
11	>0.9999	0.8412	0.1377	0.9282	0.9154	0.2466	0.9685
12	0.7573	0.4594	0.0076	0.6587	0.9923	0.2905	0.9999
13	0.2732	0.3278	0.002	0.5321	>0.9999	0.5486	0.994
14	0.086	0.6136	0.0015	0.6451	0.8414	0.8216	0.819
15	0.0278	0.8819	0.0033	0.7984	0.3346	0.9894	0.4322
16	0.0089	0.9533	0.0105	0.9064	0.1119	0.9998	0.1569
17	0.0059	0.9659	0.0539	0.9184	0.0744	0.9312	0.1137
18	0.0062	0.9594	0.1831	0.9362	0.0828	0.7147	0.1027
19	0.0162	0.9653	0.4248	0.9619	0.1467	0.6116	0.152
20	0.0594	0.9772	0.6562	0.9777	0.2931	0.6739	0.2915
21	0.1876	0.9896	0.8068	0.9836	0.5003	0.8099	0.5378
22	0.397	0.9948	0.9024	0.9905	0.706	0.9022	0.7437
23	0.5863	0.996	0.9476	0.9958	0.8445	0.9485	0.8465
24	0.7443	0.998	0.972	0.998	0.9163	0.975	0.9174
25	0.8341	0.9992	0.9844	0.9988	0.9447	0.9851	0.9517
26	0.8939	0.9997	0.9931	0.9992	0.9617	0.9887	0.972
27	0.9375	>0.9999	0.9965	0.9995	0.9721	0.9934	0.9851
28	0.9652	>0.9999	0.9981	0.9996	0.9815	0.9967	0.993
29	0.9801	>0.9999	0.9988	0.9996	0.9879	0.9983	0.9972
30	0.9886	>0.9999	0.9993	0.9995	0.9922	0.9991	0.9991

Table 1. (continued)

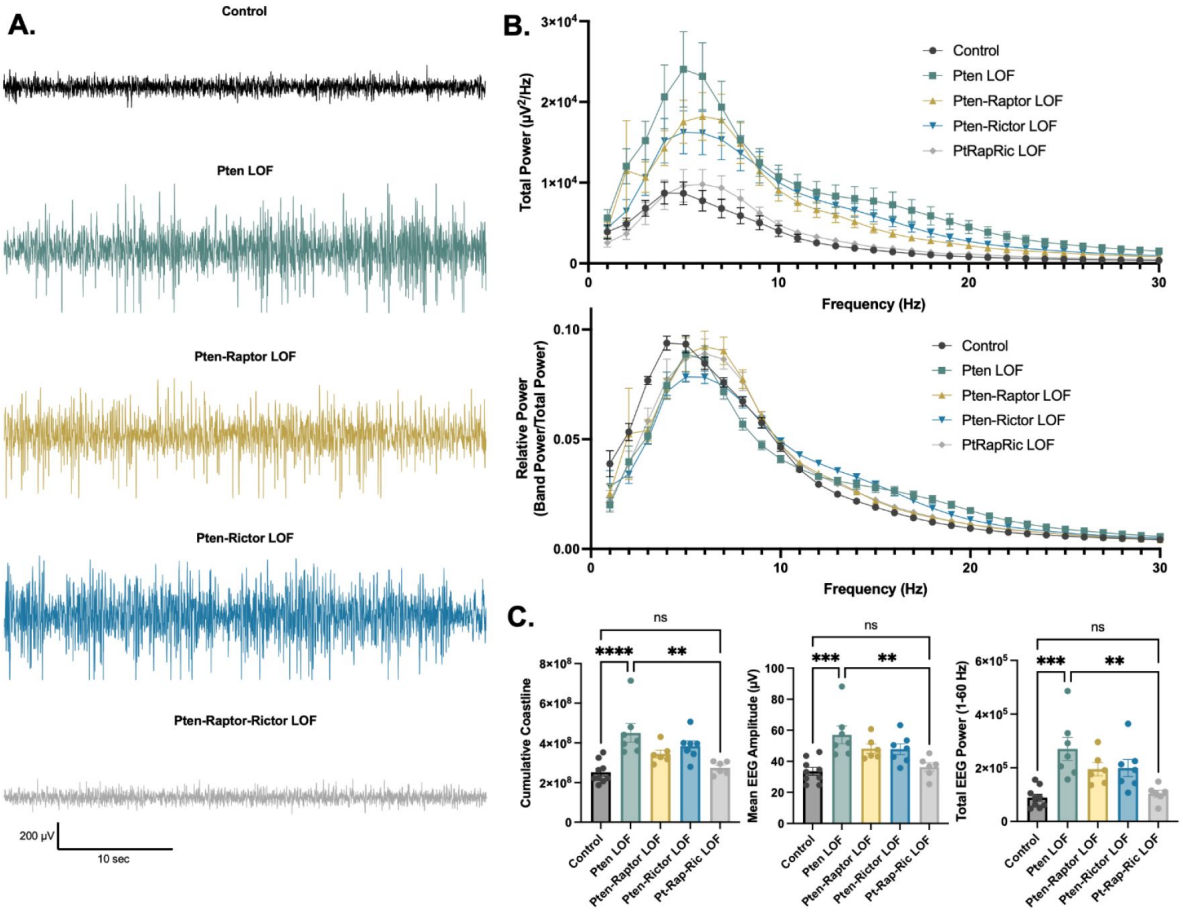

Figure 4.

Combined mTORC1 and mTORC2 inactivation, but neither alone, rescues Pten LOF-induced abnormalities in the interictal EEG.

A) Examples of typical EEG traces for each genotype. In EEG epochs that were not characterized as GTCS or SWD events, *Pten* LOF animals had higher levels of EEG activity as quantified by EEG line length, absolute mean amplitude, and total power. These changes were not significantly decreased by *Raptor* LOF and *Rictor* LOF but were normalized by combined *Raptor*/*Rictor* LOF. B) Total EEG power was increased by *Pten* LOF and attenuated but not normalized by either *Raptor* or *Rictor* LOF. Relative power was decreased in delta and increased higher frequencies by *Pten* LOF. *Pten-Raptor* LOF, *Pten-Rictor* LOF, and *Pten-Raptor-Rictor* LOF animals all showed a milder rightward shift of EEG power. C) Line length, mean amplitude, and power are increased in *Pten* LOF and normalized by *Pten-Raptor-Rictor* LOF. Error bars show mean ± s.e.m. ns indicates p>0.05, * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001 as assessed by one-way ANOVA with Tukey multiple comparisons correction. Two-way ANOVA p-values for EEG power are reported in **Tables 1** and 2.

efficacy of the targets [12]. For the subset of mTORopathies in which mTORC1 but not mTORC2 signaling is hyperactive, selective inhibition of mTORC1 and its downstream effectors rescues many animal phenotypes, including seizures [33,34]. Studies using rapamycin as an mTORC1 inhibitor also came to this conclusion in Pten LOF models, but potential inhibition of mTORC2 and the finding that genetic inhibition of mTORC1 via *Raptor* deletion did not stop seizures, whereas inhibition of mTORC2 did [21], challenged this view. Here, we tested whether mTORC1 or mTORC2 inhibition alone were sufficient to block disease phenotypes in a model of somatic Pten LOF. Neither was, which agrees with previous findings that seizures persist in both Pten-Raptor LOF and Pten-Rictor LOF animals in separate model systems [21,22]. This data could be interpreted as suggesting that epilepsy downstream of Pten LOF can proceed via mTORC-independent mechanisms, such as elevated ß-catenin or its protein phosphatase activity [25,35]. However, we found that simultaneous mTORC1 and mTORC2 inhibition corrected all of the pathological features of our model except for a rightward shift in relative EEG power (**Fig. 4B**). This suggests that epilepsy can be caused by hyperactivity of either mTORC1 or mTORC2-dependent signaling downstream of Pten LOF, but not alternative pathways. Future studies should determine whether this is also true for other gene variants that hyperactivate both mTORC1 and mTORC2, as well in other models that have fully penetrant generalized seizures.

Epilepsy in Pten-Rictor LOF mice likely emerges via mechanisms similar to those in mTOR pathway variants such as TSC that hyperactivate mTORC1 but not mTORC2, although additional effects of mTORC2 inactivation may contribute [22]. Epilepsy in Pten-Raptor LOF occurs in the absence of macrocephaly, a hallmark of mTORC1 hyperactivity, possibly due to mTORC2 hyperactivity's effect on synaptic transmission [20]. Roles for both complexes in emergent neural network function are corroborated by findings that behavior can be altered by either mTORC1 or mTORC2 inactivation [36]. Rapamycin derivatives are somewhat selective for mTORC1 [37,38] and are currently being tested in the clinic to treat mTORopathies and PTEN-related disorders [18]. Even more selective targeting of mTORC1 than can be achieved through rapamycin and its derivatives has been proposed as a way to effectively treat disease with fewer side effects [39]. Our data suggest that this may only be true for mTORopathies that only hyperactive mTORC1. For others, including *PTEN*, *PIK3CA*, and *AKT*, dual inhibitors or inhibitors of PI3K, which are being tested preclinically [40-42], are likely to be required.

Materials and Methods

Animals

Raptor (Jackson Labs #013188) and *Rictor* (Jackson Labs #020649) homozygous floxed mice were crossed to a *Pten* homozygous floxed line ([43] Jackson Labs #006440). Experimental animals were the offspring of two animals homozygous floxed for *Pten* and heterozygous floxed for *Raptor* and/or *Rictor*. Intracranial viral injections were done at P0 or P1. Animals that were homozygous for the floxed *Pten* allele and either homozygous or wild-type at the *Raptor* and/or *Rictor* allele were injected with an AAV9 viral vector expressing an eGFP-Cre fusion driven by the *hSyn* promoter (Addgene #105540). Control animals were of the same genotype but were injected with an AAV9 virus expressing eGFP under control of the *hSyn* promoter, but not Cre (Addgene #105539). Pup injections were conducted with hypothermic anesthesia. A Nanoject was used to deliver 100 nL of Cre or control virus to three sites spanning the left hemisphere of the cortex. Pups were quickly rewarmed and returned to their home cage. Additional control animals with no floxed genes on a C57B6/J background (Jackson Labs #000644) were injected with the eGFP-Cre expressing AAV9 to ensure that Cre expression did not have effects independent of the $Pten^{fl/fl}$ genotype (**Supplementary Figure 1**).

Surgeries and EEG Recordings

Animals were aged to six weeks and then implanted with a wireless EEG monitoring headcap. Animals were anesthetized with 4% isofluorane and arranged on a stereotaxic surgical apparatus with ear bars. Anesthesia was maintained with 1.5-2.5% isofluorane. The skull surface was exposed and holes were made with a 23g needle at six locations on the skull surface. 3/32" screws (Antrin Miniature Specialties) were inserted into the holes. Screws were secured with VetBond then covered with surgical cement. The screws were attached to a six-pin Millimax strip which was secured to the skull with additional cement. Animals were administered 5 mg/kg ketoprofen post-surgery and allowed to recover for at least 5 days before EEG recording. Surgical protocol was based on guidance provided by Pinnacle Technologies (*https://www.pinnaclet.com*).

After recovery from surgery, animals were fitted with wireless three-channel EEG preamplifiers (Pinnacle Technologies) and EEG signal was recorded at 256 Hz with simultaneous video recording. EEG recording session length was dependent on battery life. A total of at least 150 hours of EEG data was collected for each experimental animal.

Histology and imaging

EEG-implanted animals were euthanized by isofluorane overdose followed by transcardiac perfusion with 4% paraformaldehyde. Brains were postfixed in 4% paraformaldehyde for 24 hours and then transferred to 30% sucrose for at least 48 hours. 40 µm coronal slices were cut and preserved in a cryoprotectant solution (50% PBS, 30% ethylene glycol, 20% glycerol). Prior to staining, slices were washed three times with PBS. Eight cross-sectional unstained slices were mounted with DAPI Fluoromount-G (SouthernBiotech) and used to assess cortical thickness, GFP expression, and cell density. For other analyses, three sections per animal at approximately Bregma -1.6, -2,1, and -2.6 were used. A fluorescent Nissl stain (Invitrogen N21482, 1:50) was used to assess soma size.

After washing, slices were placed in blocking solution (10% normal goat serum, 0.1% Triton X-100, and PBS) for 1 hour. Slices were incubated in the following primary antibodies for 3 hours at room temperature: phospho-S6 Ribosomal Protein Ser240/244 (rabbit monoclonal, 1:1000 dilution, Cell Signaling Technology, catalog #5364, RRID: AB_10694233), phospho-AKT Ser473 (rabbit monoclonal, 1:1000 dilution, Cell Signaling Technology, catalog #4060), and NeuN (guinea pig polyclonal, 1:1000 dilution, Synaptic Systems, catalog #266004). Following primary antibody application, slices were washed three times in PBS and the incubated in AlexaFluor secondary antibodies goat anti-guinea pig 647 and goat anti-rabbit 594 (Invitrogen) for 1 hour at room temperature.

Slides stained for Nissl substance, NeuN/pS6, and NeuN/pAkt were imaged at 10x (2048×2048) with 5 µm z-step on a Nikon C2 confocal microscope (UVM Microscopy Imaging Core). One image of the left (GFP-expressing) cortical hemisphere in each of three region-matched slices per animal was collected for analysis. Widefield images (2560×2160) of unstained DAPI-mounted cross-sectional slices were taken with a Zyla sCMOS camera (Andor) mounted on an upright microscope (IX73; Olympus) at 5x and 20x resolution. Pixel width was manually calculated using an 0.1 mm hemocytometer.

EEG analysis

EEG files were converted from Pinnacle's proprietary format to European Data Format (EDF) files and imported to Matlab. A filtering program was used to flag traces in which seizures were likely to be occurring based on amplitude and line length between data points. For Control and Pten-Raptor-Rictor LOF animals, all 10 second epochs in which signal amplitude exceeded 400 µV at two time points at least 1 second apart were manually reviewed by a rater blinded to genotype. This method was not suitable for the Pten LOF, Pten-Raptor LOF, and Pten-Rictor LOF animals because of the density of high-amplitude interictal activity they displayed. For these animals, at least 100 of

the highest-amplitude traces were manually reviewed and then traces with persistent abnormally low amplitude, often indicating postictal suppression, were reviewed as well. Flagged traces were displayed for a rater to mark the beginning and end of each seizure. Spike-and-wave discharge events were manually marked in 8 evenly spaced one-hour epochs in each of two 24-hour recording sessions per animal and verified on video not to be caused by movement such as chewing or scratching.

Baseline EEG measures were taken from a representative sample of EEG files for each animal. 692 evenly spaced five-second epochs were sampled over 24 hours, repeated for two recording sessions per animal. Line length was defined as the sum of the linear distances between adjacent data points during the five-second analysis epoch. Mean amplitude was defined as the mean of the absolute value of data points in the five-second analysis epoch. Power spectral density was calculated with the pwelch function in Matlab. All three EEG channels were analyzed and the mean of all channels was used for statistical analysis.

Image Analysis

Image analysis was conducted using ImageJ/Fiji. Soma size was measured by dividing Nissl stain images into a 10 mm^2 grid. The somas of all GFP-expressing cells fully within three randomly selected grid squares in Layer II/III were manually traced. pS6 and pAkt expression were measured by drawing 200 μm wide columns spanning all cortical layers. Background was subtracted from images with a 30 μm rolling ball algorithm (Fiji/ImageJ). The mean pixel value of the column was recorded and values were averaged by animal.

Statistical analysis

Prism 9 (GraphPad Prism) was used to conduct statistical analyses and create graphs. Group differences were assessed using one-way ANOVA with Tukey post-hoc analysis in GraphPad9, except where noted.

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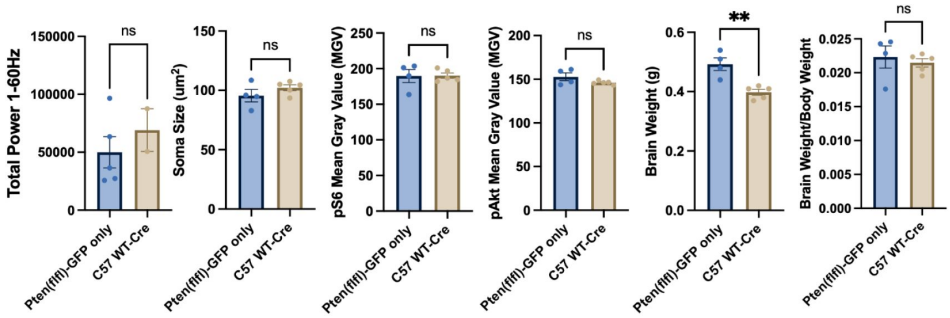
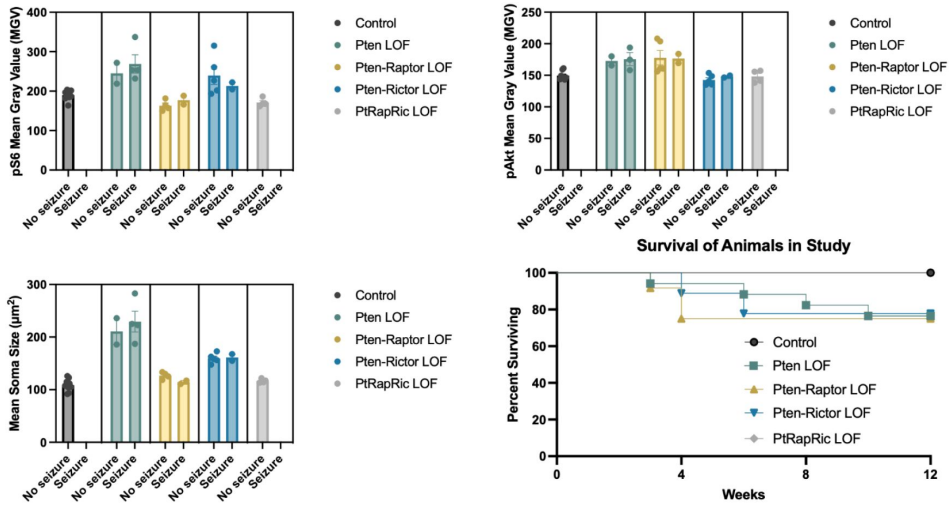


Supplementary Figure 1.

Cre virus exposure does not significantly impact cortical morphology or baseline EEG.

C57B6/J mice with no floxed genes were injected with the Cre virus as an additional control group. These mice did not have GS or SWDs. They were also not different from Pten$^{fl/fl}$ injected with the Control virus, except for significantly different brain weights. The brain weight/body weight ratio did not differ between the groups.



Supplementary Figure 2.

Focal cortical Pten LOF, Pten-Raptor LOF, and Pten-Rictor LOF cause a spectrum of outcomes.

A subset of animals in the *Pten* LOF, *Pten-Raptor* LOF, and *Pten-Rictor* LOF groups displayed spontaneous epileptiform activity, but not generalized seizures. When compared side by side, animals within each genotype that did and did not display generalized seizures showed similar mTOR pathway activity levels and soma sizes. Survival plot shows survival of animals in the study by genotype. Some animals in the *Pten* LOF, *Pten-Raptor* LOF, and *Pten-Rictor* LOF groups were found dead during the study, but no deaths were observed in Control or *Pten-Raptor-Rictor* LOF groups. Mortality often occurred prior to EEG recordings, so we could not ascertain whether early mortality was associated with generalized seizures.

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Article and author information

Erin R. Cullen
Department of Neurological Sciences, Larner College of Medicine, University of Vermont, Burlington VT, 05405, USA

Isabelle Mittelstadt
Department of Neurological Sciences, Larner College of Medicine, University of Vermont, Burlington VT, 05405, USA

Matthew C. Weston
Department of Neurological Sciences, Larner College of Medicine, University of Vermont, Burlington VT, 05405, USA, Fralin Biomedical Research Institute at VTC, Center for Neurobiology Research, Roanoke VA, 24016, USA, School of Neuroscience, Virginia Polytechnic and State University, Blacksburg VA, 24060, USA
For correspondence: mcweston7c@vt.edu
ORCID iD: 0000-0001-5558-7070

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Reviewer #1 (Public Review):

Hyperactivation of mTOR signaling causes epilepsy. It has long been assumed that this occurs through overactivation of mTORC1, since treatment with the mTORC1 inhibitor rapamycin suppresses seizures in multiple animal models. However, the recent finding that genetic inhibition of mTORC1 via Raptor deletion did not stop seizures while inhibition of mTORC2 did, challenged this view (Chen et al, Nat Med, 2019). In the present study, the authors tested whether mTORC1 or mTORC2 inhibition alone was sufficient to block the disease phenotypes in a model of somatic Pten loss-of-function (a negative regulator of mTOR). They found that

inactivation of either mTORC1 or mTORC2 alone normalized brain pathology but did not prevent seizures, whereas dual inactivation of mTORC1 and mTORC2 prevented seizures. As the functions of mTORC1 versus mTORC2 in epilepsy remain unclear, this study provides important insight into the roles of mTORC1 and mTORC2 in epilepsy caused by Pten loss and adds to the emerging body of evidence supporting a role for both complexes in the disease development.

Strengths:
The animal models and the experimental design employed in this study allow for a direct comparison between the effects of mTORC1, mTORC2, and mTORC1/mTORC2 inactivation (i.e., same animal background, same strategy and timing of gene inactivation, same brain region, etc.). Additionally, the conclusions on brain epileptic activity are supported by analysis of multiple EEG parameters, including seizure frequencies, sharp wave discharges, interictal spiking, and total power analyses.

Weaknesses:
The sample size of the study is small and does not allow for the assessment of whether mTORC1 or mTORC2 inactivation reduces seizure frequency or incidence. This is a limitation of the study.

The authors describe that they inactivated mTORC1 and mTORC2 in a new model of somatic Pten loss-of-function in the cortex. This is slightly misleading since Cre expression was found both in the cortex and the underlying hippocampus, as shown in Figure 1. Throughout the manuscript, they provide supporting histological data from the cortex. However, since Pten loss-of-function in the hippocampus can lead to hippocampal overgrowth and seizures, data showing the impact of the genetic rescue in the hippocampus would further strengthen the claim that neither mTORC1 nor mTORC2 inactivation prevents seizures.

Some of the methods for the EEG seizure analysis are unclear. The authors describe that for control and Pten-Raptor-Rictor LOF animals, all 10-second epochs in which signal amplitude exceeded 400 μV at two time-points at least 1 second apart were manually reviewed, whereas, for the Pten LOF, Pten-Raptor LOF, and Pten-Rictor LOF animals, at least 100 of the highest-amplitude traces were manually reviewed. Does this mean that not all flagged epochs were reviewed? This could potentially lead to missed seizures. Additionally, the inclusion of how many consecutive hours were recorded among the ~150 hours of recording per animal would help readers with the interpretation of the data.

Finally, it is surprising that mTORC2 inactivation completely rescued cortical thickness since such pathological phenotypes are thought to be conserved down the mTORC1 pathway. Additional comments on these findings in the Discussion would be interesting and useful to the readers.

Reviewer #2 (Public Review):

Summary:
The study by Cullen et al presents intriguing data regarding the contribution of mTOR complex 1 (mTORC1) versus mTORC2 or both in Pten-null-induced macrocephaly and epileptiform activity. The role of mTORC2 in mTORopathies, and in particular Pten loss-off-function (LOF)-induced pathology and seizures, is understudied and controversial. In addition, recent data provided evidence against the role of mTORC1 in PtenLOF-induced seizures. To address these controversies and the contribution of these mTOR complexes in PtenLOF-induced pathology and seizures, the authors injected a AAV9-Cre into the cortex of conditional single, double, and triple transgenic mice at postnatal day 0 to remove Pten, Pten+Raptor or Rictor, and Pten+raptor+rictor. Raptor and Rictor are essentially binding partners of mTORC1 and mTORC2, respectively. One major finding is that despite preventing mild macrocephaly and increased cell size, Raptor knockout (KO, decreased mTORC1 activity)

did not prevent the occurrence of seizures and the rate of SWD event, and aggravated seizure duration. Similarly, Rictor KO (decreased mTORC2 activity) partially prevented mild macrocephaly and increased cell size but did not prevent the occurrence of seizures and did not affect seizure duration. However, Rictor KO reduced the rate of SWD events. Finally, the pathology and seizure/SWD activity were fully prevented in the double KO. These data suggest the contribution of both increased mTORC1 and mTORC2 in the pathology and epileptic activity of Pten LOF mice, emphasizing the importance of blocking both complexes for seizure treatment. Whether these data apply to other mTORopathies due to Tsc1, Tsc2, mTOR, AKT or other gene variants remains to be examined.

Strengths:
The strengths are as follows: 1) they address an important and controversial question that has clinical application, 2) the study uses a reliable and relatively easy method to KO specific genes in cortical neurons, based on AAV9 injections in pups. 2) they perform careful video-EEG analyses correlated with some aspects of cellular pathology.

Weaknesses:
The study has nevertheless a few weaknesses: 1) the conclusions are perhaps a bit overstated. The data do not show that increased mTORC1 or mTORC2 are sufficient to cause epilepsy. However the data clearly show that both increased mTORC1 and mTORC2 activity contribute to the pathology and seizure activity and as such are necessary for seizures to occur. 2) the data related to the EEG would benefit from having more mice. Adding more mice would have helped determine whether there was a decrease in seizure activity with the Rictor or Raptor KO. 3) it would have been interesting to examine the impact of mTORC2 and mTORC1 overexpression related to point #1 above.

Reviewer #3 (Public Review):

Summary: This study investigated the role of mTORC1 and 2 in a mouse model of developmental epilepsy which simulates epilepsy in cortical malformations. Given activation of genes such as PTEN activates TORC1, and this is considered to be excessive in cortical malformations, the authors asked whether inactivating mTORC1 and 2 would ameliorate the seizures and malformation in the mouse model. The work is highly significant because a new mouse model is used where Raptor and Rictor, which regulate mTORC1 and 2 respectively, were inactivated in one hemisphere of the cortex. The work is also significant because the deletion of both Raptor and Rictor improved the epilepsy and malformation. In the mouse model, the seizures were generalized or there were spike-wave discharges (SWD). They also examined the interictal EEG. The malformation was manifested by increased cortical thickness and soma size.

Strengths: The presentation and writing are strong. The quality of data is strong. The data support the conclusions for the most part. The results are significant: Generalized seizures and SWDs were reduced when both Torc1 and 2 were inactivated but not when one was inactivated.

Weaknesses: One of the limitations is that it is not clear whether the area of cortex where Raptor or Rictor were affected was the same in each animal. Also, it is not clear which cortical cells were measured for soma size. Another limitation is that the hippocampus was affected as well as the cortex. One does not know the role of cortex vs. hippocampus. Any discussion about that would be good to add. It would also be useful to know if Raptor and Rictor are in glia, blood vessels, etc.

Author Response

eLife assessment

This useful study addresses epilepsy caused by the loss of a molecule called Pten, resulting in hyperactivity of the mTOR pathway. The findings suggest that inhibiting two molecules called mTORC1 and mTORC2 can reduce epilepsy symptoms but there is much less effect when inhibited separately. The evidence supporting the conclusions is currently incomplete, but could be strengthened after additional experiments.

We thank the editors for this assessment and the reviewers for their comments. We will consider each of the recommendations we received and revise the manuscript accordingly.

Reviewer #1 (Public Review):

Hyperactivation of mTOR signaling causes epilepsy. It has long been assumed that this occurs through overactivation of mTORC1, since treatment with the mTORC1 inhibitor rapamycin suppresses seizures in multiple animal models. However, the recent finding that genetic inhibition of mTORC1 via Raptor deletion did not stop seizures while inhibition of mTORC2 did, challenged this view (Chen et al, Nat Med, 2019). In the present study, the authors tested whether mTORC1 or mTORC2 inhibition alone was sufficient to block the disease phenotypes in a model of somatic Pten loss-of-function (a negative regulator of mTOR). They found that inactivation of either mTORC1 or mTORC2 alone normalized brain pathology but did not prevent seizures, whereas dual inactivation of mTORC1 and mTORC2 prevented seizures. As the functions of mTORC1 versus mTORC2 in epilepsy remain unclear, this study provides important insight into the roles of mTORC1 and mTORC2 in epilepsy caused by Pten loss and adds to the emerging body of evidence supporting a role for both complexes in the disease development.

Strengths:

The animal models and the experimental design employed in this study allow for a direct comparison between the effects of mTORC1, mTORC2, and mTORC1/mTORC2 inactivation (i.e., same animal background, same strategy and timing of gene inactivation, same brain region, etc.). Additionally, the conclusions on brain epileptic activity are supported by analysis of multiple EEG parameters, including seizure frequencies, sharp wave discharges, interictal spiking, and total power analyses.

Weaknesses:

1. *The sample size of the study is small and does not allow for the assessment of whether mTORC1 or mTORC2 inactivation reduces seizure frequency or incidence. This is a limitation of the study.*

We agree that this is a minor limitation of the present study, however, for several reasons we decided not to pursue this question by increasing the number of animals. First, we performed a power analysis of the existing data. This analysis showed that we would need to use 89 animals per group to detect a significant difference (0.8 Power, p= 0.05, Mann-Whitney test) in the frequency of generalized seizures in the Pten-Raptor group and 31 animals per group in the Pten-Rictor group versus Pten alone. It is simply not feasible to perform EEG monitoring on this many animals. Second, even if we did do enough experiments to detect a reduction in seizure frequency, it is clear that neither Raptor nor Rictor deletion provides the kind normalization in brain activity that we seek in a targeted treatment. Both Pten-Raptor and Pten-Rictor animals still have very frequent spike-wave events (Fig. 3D) and highly abnormal interictal EEGs (Fig. 4), suggesting that even if generalized seizures were reduced, epileptic brain activity persists. This is in contrast to the triple KO animals, which have no increase in SWD above control level and very normal interictal EEG.

> 1. *The authors describe that they inactivated mTORC1 and mTORC2 in a new model of somatic Pten loss-of-function in the cortex. This is slightly misleading since Cre expression was found both in the cortex and the underlying hippocampus, as shown in Figure 1. Throughout the manuscript, they provide supporting histological data from the cortex. However, since Pten loss-of-function in the hippocampus can lead to hippocampal overgrowth and seizures, data showing the impact of the genetic rescue in the hippocampus would further strengthen the claim that neither mTORC1 nor mTORC2 inactivation prevents seizures.*

Thank you for pointing out this issue. Cre expression was observed in both the cortex and the dorsal hippocampus in most animals, and we agree that differences in cortical versus hippocampal mTOR signaling could have differential contributions to epilepsy. We focused our studies on the cortex because spike-and-wave discharge, the most frequent and fully penetrant EEG phenotype in our model, is associated with cortical dysfunction. We had also performed a preliminary analysis of the hippocampal Cre expression, which suggested that Cre expression in the hippocampus did not affect generalized seizure occurrence. We plan to include data on Cre expression in the hippocampus in the revised version of the manuscript.

> 1. *Some of the methods for the EEG seizure analysis are unclear. The authors describe that for control and Pten-Raptor-Rictor LOF animals, all 10-second epochs in which signal amplitude exceeded 400 μV at two time-points at least 1 second apart were manually reviewed, whereas, for the Pten LOF, Pten-Raptor LOF, and Pten-Rictor LOF animals, at least 100 of the highest-amplitude traces were manually reviewed. Does this mean that not all flagged epochs were reviewed? This could potentially lead to missed seizures.*

We reviewed at least 48 hours of data from each animal manually. All seizures that were identified during manual review were also identified by the automated detection program. It is possible but unlikely that there are missed seizures in the remaining data.

> 1. *Additionally, the inclusion of how many consecutive hours were recorded among the ~150 hours of recording per animal would help readers with the interpretation of the data.*

Thank you for this recommendation. We plan to include a table with more information about the EEG recordings in the revised version of the manuscript. The number of consecutive hours recorded varied because the wireless system depends on battery life, which was inconsistent, but each animal was recorded for at least 48 consecutive hours on at least two occasions.

> 1. *Finally, it is surprising that mTORC2 inactivation completely rescued cortical thickness since such pathological phenotypes are thought to be conserved down the mTORC1 pathway. Additional comments on these findings in the Discussion would be interesting and useful to the readers.*

Soma size was increased 120% by Pten inactivation and partially normalized to a 60% increase from Controls by mTORC2 inactivation (Fig. 2C). We and others have previously shown that mTORC2 inactivation in neurons reduces both soma size and dendritic outgrowth (PMIDs: 36526374, 32125271, 23569215). Thus, we do not find it completely surprising that mTORC2 inactivation reduces the cortical thickness increase caused by Pten loss. There may still be a slight increase in cortical thickness in Pten-Rictor animals, but it is statistically indistinguishable from Controls. We will elaborate on this in our revised submission.

Reviewer #2 (Public Review):

Summary:

The study by Cullen et al presents intriguing data regarding the contribution of mTOR complex 1 (mTORC1) versus mTORC2 or both in Pten-null-induced macrocephaly and epileptiform activity. The role of mTORC2 in mTORopathies, and in particular Pten loss-off-function (LOF)-induced pathology and seizures, is understudied and controversial. In addition, recent data provided evidence against the role of mTORC1 in PtenLOF-induced seizures. To address these controversies and the contribution of these mTOR complexes in PtenLOF-induced pathology and seizures, the authors injected a AAV9-Cre into the cortex of conditional single, double, and triple transgenic mice at postnatal day 0 to remove Pten, Pten+Raptor or Rictor, and Pten+raptor+rictor. Raptor and Rictor are essentially binding partners of mTORC1 and mTORC2, respectively. One major finding is that despite preventing mild macrocephaly and increased cell size, Raptor knockout (KO, decreased mTORC1 activity) did not prevent the occurrence of seizures and the rate of SWD event, and aggravated seizure duration. Similarly, Rictor KO (decreased mTORC2 activity) partially prevented mild macrocephaly and increased cell size but did not prevent the occurrence of seizures and did not affect seizure duration. However, Rictor KO reduced the rate of SWD events. Finally, the pathology and seizure/SWD activity were fully prevented in the double KO. These data suggest the contribution of both increased mTORC1 and mTORC2 in the pathology and epileptic activity of Pten LOF mice, emphasizing the importance of blocking both complexes for seizure treatment. Whether these data apply to other mTORopathies due to Tsc1, Tsc2, mTOR, AKT or other gene variants remains to be examined.

Strengths:

The strengths are as follows: 1) they address an important and controversial question that has clinical application, 2) the study uses a reliable and relatively easy method to KO specific genes in cortical neurons, based on AAV9 injections in pups. 2) they perform careful video-EEG analyses correlated with some aspects of cellular pathology.

Weaknesses:

The study has nevertheless a few weaknesses: 1) the conclusions are perhaps a bit overstated. The data do not show that increased mTORC1 or mTORC2 are sufficient to cause epilepsy. However the data clearly show that both increased mTORC1 and mTORC2 activity contribute to the pathology and seizure activity and as such are necessary for seizures to occur.

We agree that our findings do not directly show that either mTORC1 or mTORC2 hyperactivity are sufficient to cause seizures, as we do not individually hyperactivate each complex in the absence of any other manipulation. We interpreted our findings in this model as suggesting that either is sufficient based on the result that there is no epileptic activity when both are inactivated, and thus assume that there is not a third, mTOR-independent, mechanism that is contributing to epilepsy in Pten, Pten-Raptor, and Pten-Rictor animals. In addition, the histological data show that Raptor and Rictor loss each normalize activity through mTORC1 and mTORC2 respectively, suggesting that one in the absence of the other is sufficient. However, we agree that there could be other potential mTOR-independent pathways downstream of Pten loss that contribute to epilepsy. We will revise the manuscript to reflect this.

1. *the data related to the EEG would benefit from having more mice. Adding more mice would have helped determine whether there was a decrease in seizure activity with the Rictor or Raptor KO.*

Please see response to Reviewer 1's first Weakness.

1. *it would have been interesting to examine the impact of mTORC2 and mTORC1 overexpression related to point #1 above.*

We are not sure that overexpression of individual components of mTORC1 or mTORC2 would result in their hyperactivation or lead to increases in downstream signaling. We believe that cleanly and directly hyperactivating mTORC1 or especially mTORC2 in vivo without affecting the other complex or other potential interacting pathways is a difficult task. Previous studies have used mTOR gain-of-function mutations as a means to selectively activate mTORC1 or pharmacological agents to selectively activate mTORC2, but it not clear to us that the former does not affect mTORC2 activity as well, or that the latter achieves activation of mTORC2 targets other than p-Akt 473, or that it is truly selective. We agree that these would be key experiments to further test the sufficiency hypothesis, but that the amount of work that would be required to perform them is more that what we can do in this Short Report.

Reviewer #3 (Public Review):

Summary: This study investigated the role of mTORC1 and 2 in a mouse model of developmental epilepsy which simulates epilepsy in cortical malformations. Given activation of genes such as PTEN activates TORC1, and this is considered to be excessive in cortical malformations, the authors asked whether inactivating mTORC1 and 2 would ameliorate the seizures and malformation in the mouse model. The work is highly significant because a new mouse model is used where Raptor and Rictor, which regulate mTORC1 and 2 respectively, were inactivated in one hemisphere of the cortex. The work is also significant because the deletion of both Raptor and Rictor improved the epilepsy and malformation. In the mouse model, the seizures were generalized or there were spike-wave discharges (SWD). They also examined the interictal EEG. The malformation was manifested by increased cortical thickness and soma size.

Strengths: The presentation and writing are strong. The quality of data is strong. The data support the conclusions for the most part. The results are significant: Generalized seizures and SWDs were reduced when both Torc1 and 2 were inactivated but not when one was inactivated.

Weaknesses: One of the limitations is that it is not clear whether the area of cortex where Raptor or Rictor were affected was the same in each animal.

We plan to include data further describing the location of knockout in each animal (in both the hippocampus and cortex) in the revised version of the paper. Initial analyses indicated that the affected area did not differ between groups.

Also, it is not clear which cortical cells were measured for soma size.

In the Methods it says "Soma size was measured by dividing Nissl stain images into a 10 mm2 grid. The somas of all GFP-expressing cells fully within three randomly selected grid squares in Layer II/III were manually traced." Earlier under "Histology and imaging" it says "Three sections per animal at approximately Bregma -1.6, -2,1, and -2.6 were used."

> *Another limitation is that the hippocampus was affected as well as the cortex. One does not know the role of cortex vs. hippocampus. Any discussion about that would be good to add.*

See response to Reviewer 1's second Weakness.

> *It would also be useful to know if Raptor and Rictor are in glia, blood vessels, etc.*

Raptor and Rictor are thought to be ubiquitously active in mammalian cells including glia and endothelial cells. Previous studies have shown that mTOR manipulation can affect astrocyte function and blood vessel organization, however, our study induced gene knockout using an AAV that expressed Cre under control of the hSyn promoter, which has previously been shown to be selective for neurons. Manual assessment of Cre expression compared with DAPI, NeuN, and GFAP stains suggested that only neurons were affected.