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14. ABSTRACT
We seek to understand four basic questions related to epileptogenesis: 1) What is the role of mTOR dysregulation in epileptogenesis in the developing brain? 2) What are the molecular mechanisms downstream of mTOR hyperactivation that trigger epileptogenesis in developing brains? 3) What are the long-term pharmacological treatments to prevent the development and/or progression of seizures? 4) What are biomarkers for epileptogenesis and the prognostic biomarkers for disease progression? We have established a neuroglial tuberous sclerosis complex 1(TSC1)-deficient mouse model in which spontaneous seizures begin at 2.5 months of the age and progress into 7-8months. This time course allows the analysis of molecular changes before and after the onset of spontaneous electrographic and behavioral seizures and provide reliable biomarkers for epileptogenesis and the progression of epilepsy. We have also developed a neuronal-specific mouse model, deleting Tsc1 by driving cre from the CAMKII promoter. We propose gene expression profiling, electrophysiological, biochemical, immunohistochemical and behavioral studies.

15. SUBJECT TERMS
Tuberous Sclerosis, Epilepsy, mTOR

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INTRODUCTION

Tuberous Sclerosis Complex (TSC) is a multisystem genetic disorder caused by germline mutations in the TSC1 or TSC2 genes, which form a regulatory complex responsible for limiting the activity of an important intracellular regulator of cell growth and metabolism known as mammalian target of rapamycin complex 1 (mTORC1. The pathological hallmark of TSC brains are cortical tubers, characterized by disorganized cortical lamination, aberrant dendritic arbors and axonal projections, astrocyte gliosis and abnormal cell morphology (ie, dysplastic and heterotopic neurons and giant cells). mTOR hyperactivity contributes to developmental brain malformations associated with seizures and cognitive and psychiatric deficits. In our studies, we want to understand the molecular changes that occur in neurons and astrocytes as a result of a deletion of TSC1, using a mouse model. These findings will help us understand molecular mechanisms underlying cellular changes in TSC and underlying epilepsy. The knowledge of these mechanisms will aid in a rational development of therapies.

KEYWORDS

Tuberous Sclerosis, Epilepsy, Neurons, Astrocytes, EEG, mTOR, mRNA translation,

ACCOMPLISHMENTS

The major goals of the project:

Specific Aim 1: To Determine the effects of TSC1 deletion in the Tsc1$^{CamkII\text{-}Cre}$CKO and Tsc1$^{mGFAP\text{-}Cre}$CKO mice mice on epileptogenesis and progress of seizures. Inactivation of the Tsc1 or Tsc2 gene in different subtypes of brain cells, including neurons, glia or progenitor cells, all cause spontaneous seizures in animal models, suggesting that both neuronal and astroglial cells are active elements in the epileptic brain. In this aim, we hypothesize that TSC1 deletion in astrocytes and a subset of late-onset forebrain pyramidal neurons underlie the changes in preclinical epileptic EEG activities which progress into clinical behavioral seizures. To test this hypothesis, we will monitor spontaneous electrographic and behavioral seizures by continuous video/EEG recording in free-moving mice.

Specific Aim 2. To analyze the molecular changes downstream of TSC deficiency in astrocytes and neurons that are related to the initiation and progression of seizures. The initiation of the first seizure and the progression of the disease after the onset of seizures are two distinct processes. The identification of molecular changes for these two processes may provide biomarkers for epilepsy prevention and disease modification/mitigation. We hypothesize that overactivation of mTOR controls the translation of a specific repertoire of neuronal and astrocytic genes, which cooperatively regulate the initiation and progression of epilepsy. Translational ribosomal profiling will be used to investigate gene expression in the both *Tsc1$^{CamkII\text{-}Cre}$CKO* and *Tsc1$^{mGFAP\text{-}Cre}$CKO* mice, before and after the occurrence of spontaneous seizures. The gene expression signatures in these mice will be compared with previous report of transcriptional changes in response to chemical induced epilepsies and to find common developmental changes in pathways responsible for epileptogenesis and for the progression of epilepsy.

Specific Aim 3. Determine the effects of mTOR inhibitor on the initiation and progression of seizures and on the molecular changes that accompany the initiation and progression of seizures. Preclinical studies demonstrate that mTOR inhibitors can effectively treat seizures in mice with established epilepsy related to mTOR pathway hyperactivation. However, the downstream mechanisms responsible for these effects have not been proven. In this aim, we will test the efficacy of potential treatments with mTORC1 inhibitors and identify molecule players that respond to or do not respond to mTORC1 inhibitors. We will examine the effects of

rapamycin on the molecular changes associated with the EEG/seizure activity, focusing on those genes and pathways we have found to be significantly altered in studies of Specific Aim 2.

What we have accomplished under these goals:

In the first year of the grant, we developed, verified, and reproduced the critical molecular assays required for this work.
These included:
1. New Technology for Highly-Sensitive Ribosome Profiling in Tissue
2. Ligation-Free Ribosome Profiling in *Tsc1*$^{CamkII\text{-}Cre}$*CKO* Mice following Rapamycin Treatment
These techniques are in full implementation.

In the first year, we also bred colonies of the mice and obtained many brains required for the molecular studies. These mice have had the TSC1 gene deleted from excitatory neurons in the cortex. They include appropriate controls as well as mice treated with rapamycin, an mTOR inhibitor that allows the mice to survive without seizures.

In the second year of the grant, we performed RNASeq and Ribosome Profiling in the *Tsc1*$^{mGFAP\text{-}Cre}$*CKO* and *Tsc1*$^{CamkII\text{-}Cre}$*CKO* mice, including following Rapamycin Treatment. These mice contained the floxed Rpl22 exon and a substituted Rpl22 exon that contained a hemagglutinin tag. Thus, we were able to immunoprecipitate ribosomes using anti-HA antibodies from astrocytes and upper layer pyramidal neurons (*Tsc1*$^{mGFAP\text{-}Cre}$*CKO*) and pyramidal neurons (*Tsc1*$^{CamkII\text{-}Cre}$*CKO*). Thus, we are able, in the second mouse, to focus exclusively on pyramidal cells.

The RNASeq for 3 strains of mice (wildtype mice, *Tsc1*$^{CamkII\text{-}Cre}$*CKO* mice, and the *Tsc1*$^{CamkII\text{-}Cre}$*CKO* mice treated with rapamycin) was shown in the second year Progress Report. Rapamycin normalized the transcriptional signature, as would be expected of an mTOR inhibitor.

The Ribosomal Profiling of pyramidal neurons, giving the types and numbers of transcripts associated with ribosomes yields a measure of translational efficiency, reported in graphs in the second year Progress Report. We found that a number of transcripts were translated with a significantly different efficiency compared to that in control mice. Several of these translation efficiencies are decreased, although mTOR target genes are not. We were particularly interested to see that some of the translationally altered genes belong to known autism-associated gene groups, including oxytocin and vasopression, both of which show substantial translational downregulation. However, this decrease in translation efficiency may be compensatory, as TSC1(CKO) leads to very significant transcriptional upregulation of both hormones, a change that is normalized by rapamycin.

We performed a gene ontology analyses on these translational analyses, and found that among the genes that were translationally upregulated were genes that are functionally important for excitatory neurons, including genes that encoded synaptic proteins and neuronal transcription factors. Gene ontologies of genes that are translationally downregulated include many genes that encode proteins of metabolic pathways, including mitochondrial function. GO Analyses were shown in the second year Progress Report.

We performed physiological recordings from cortical pyramidal neurons, testing intrinsic properties, action potential thresholds, after-depolarization, spontaneous excitatory postsynaptic currents, NMDA Receptor mediated currents, and seizure activity in zero Magnesium concentration. The neurons showed a larger than normal response to NMDAR and AMPAR

agonists and a very short latency of seizure onset in zero magnesium, showing that these neurons were highly excitable. Recordings were shown in the second year Progress Report.

In the last (third year) of the grant, we completed our Ribosomal Profiling/RNASeq analysis of the impact of TSC1-cKO in principal neurons (Camk2a+ neurons) and further examined the effects of rapamycin treatment on these animals. The basic results are summarized in **Fig. 1** which shows volcano plots of the alternations in translation efficiency (TE) across three comparisons – the effect of TSC1-cKO on TE, the effect of rapamycin treatment on TE in WT, and the effect of rapamycin on TE in TSC1-cKO. Our expectation was that the majority of significant alterations in TE in TSC1-cKO mice would be increases because TSC1 is an inhibitor of mTOR signaling. Indeed, >75% of the translationally altered genes in our comparison of TSC1-cKO vs. WT are upregulated (44/58 genes, **Fig. 1A**). Another key finding is that, while rapamycin treatment has essentially no impact on TE in wildtype mice (**Fig. 1B**), there are numerous significant alterations (166 genes) in response to rapamycin-treatment of TSC1-cKO mice. As expected, the >72% of these result in translational downregulated, consistent with rapamycin's reversal of mTOR hyperactivation in these animals (**Fig. 1C**).

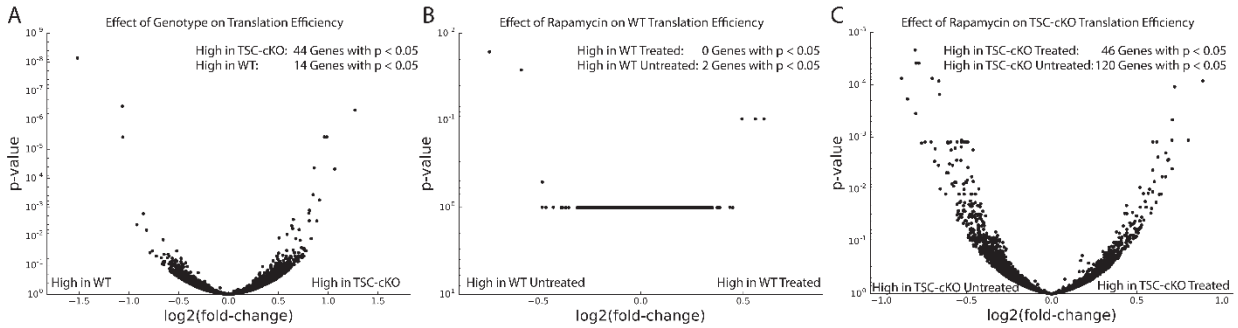


Figure 1. *Volcano plots showing translation efficiency alterations in A) TSC1-cKO vs. wildtype; B) rapamycin-treated wildtype vs. untreated; and C) rapamycin-treated TSC1-cKO vs. untreated mice.*

We next examined the impact of TSC1-cKO and rapamycin-treatment in the context of the canonical translational targets of mTOR signaling. mTOR is known to selectively control the translation of genes contain terminal oligopyrimidine (TOP) motifs in their 5'-UTRs. Many of these genes are involved in the translational machinery itself, including numerous ribosomal proteins and RNA binding proteins that mediate translation initiation and elongation. Importantly, because rapamycin is an allosteric inhibitor of mTORC1, we expected that the effects on TOP-motif containing genes would be relatively modest. Furthermore, we also expect relatively small effects sizes because we are examining homogenized brain tissue, and TSC1 is knocked-out selectively principal neurons. Nonetheless, they are detectable our

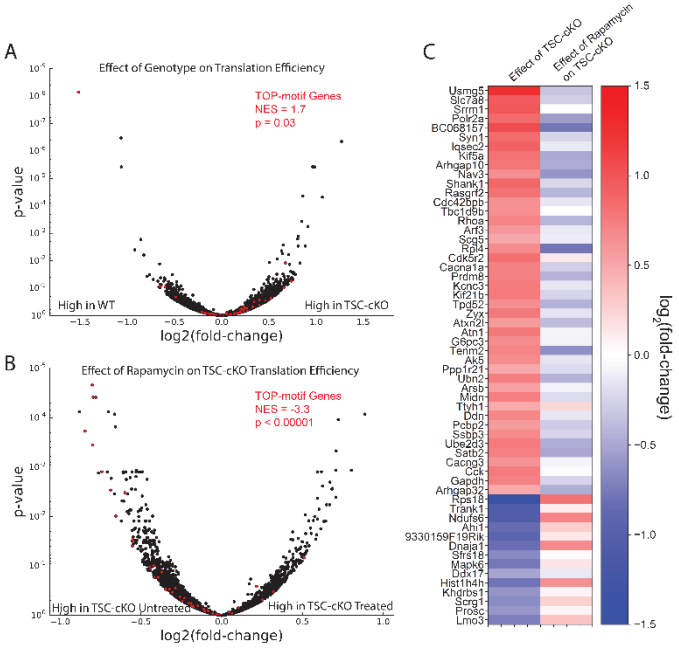


Figure 2. *A) Volcano plot showing alterations in TE for TSC1-cKO vs. wildtype with TOP-motif genes highlighted in red and enrichment statistics from GSEA. B) Same as A) for rapamycin-treated TSC1-cKO vs. untreated. C) Heatmap showing the inversion of the translationally altered gene signature from TSC1-cKO vs. widltype by rapamycin treatment.*

experiments. **Fig. 2A** shows a volcano plot for TE alterations in

TSC1-cKO mice relative to wildtype with the TOP-motif genes highlighted in red. Based on gene set enrichment analysis (GSEA), there is a modest but significant upregulation of TE for TOP-motif genes in the TSC1-cKO mice, consistent with mTOR hyperactivation in these animals. Upon treatment with rapamycin, there is a more dramatic decrease in TE for the TOP-motif genes (**Fig. 2B**). This may be because rapamycin will affect TOP-motif TE in all cell types in the brain, but the knock-out is principal neuron-specific. Finally, we show a heatmap in **Fig. 2C** of genes that are significantly up- or down-regulated in TSC1-cKO mice relative to wildtype in both the TSC1-cKO vs. wildtype comparison and the rapamycin-treated TSC1-cKO vs. untreated comparison. Nearly all of the up- and down-regulated alterations in TSC1-cKO mice are reversed by rapamycin treatment.

Next, we examined the cell type-specificity of the translational alterations in the above comparisons using the RiboTag system. In our animal model, we not only knock-out TSC1 selectively in Camk2a+ neurons, we also selectively activated expression of HA-tagged ribosomes in the same cells. This allows us to selectively immunoprecipitate (IP) polysomal RNA from Camk2a+ neurons and identify cell type-specific genes. To demonstrate the efficacy of this system, we used GSEA to compare the enrichment of neuron-, astrocyte-, microglia-, OPC-, and oligodendrocyte-specific genes among those selectively enriched by RiboTag IP (**Fig. 3**). As expected, only neuron-specific genes were significantly enriched by RiboTag IP of polysomal mRNA. Furthermore, all but the OPC-specific genes were significantly depleted.

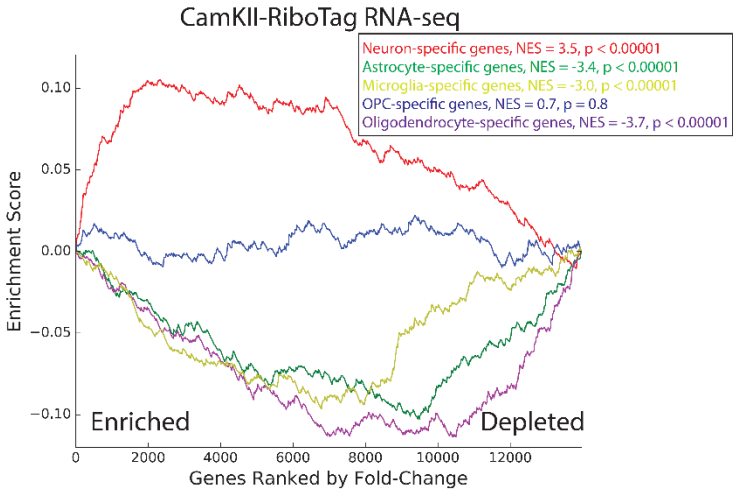


Figure 3. *GSEA of neural cell-type specific genes among the CamK2a-RiboTag enriched and depleted genes.*

We used these data to investigate the cell type-specificity of the TE alterations described above. Our expectation was that the genes altered by TSC-cKO would be principal neuron-specific, whereas the genes altered by rapamycin treatment would be significantly less specific because all cells have exposure to rapamycin. Remarkably, we found the exact opposite. Using GSEA, we determined the degree of enrichment or depletion of genes that were significant up- or down-regulated by TSC1-cKO and by

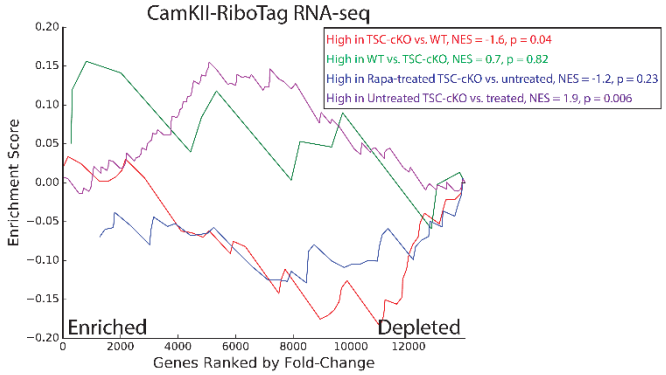


Figure 4. *GSEA of translationally altered gene sets from TSC1-cKO and rapamycin treatment of TSC1-cKO mice among the RiboTag enriched and depleted genes.*

mice. Interestingly, there was a modest but significant enrichment of the genes upregulated by TSC1-cKO among the RiboTag-depleted genes (**Fig. 4**). This result implies that there are significant non-cell autonomous effects of principal neuron-specific TSC1-cKO on brain cells. In contrast, the genes that there down-regulated by rapamycin treatment of TSC1-cKO mice were very significantly enriched among RiboTag-enriched genes, suggesting that many of the treatment effects are principal neuron-specific.

We have had a number of technical difficulties in establishing the video EEGs, including the interference from electromagnetic fields and vibration in our building. We are now improving the technique by using an enclosed Farady cage and vibration isolation. However, we have been using a microelectrode recording array to measure seizure-like EEG activity. We use this on freshly isolated slices of brain tissues, prepared as described in VII-1 (acute brain slice recordings).

In these experiments we used a microelectrode array on top of a fresh coronal slice to study the origins of seizures. Multi-electrode Array (MEA) recording on the acute mouse brain slices. 96 or 64 utah arrays was used to record local field potentials (LFP) filtered at 250Hz (low-pass Bessel filter) and unit spikes filtered at 500Hz (Bessel high-pass filter). The software such as the data acquisition (cereplex direct software suite v 7.0.4.0) and data analysis (Blackrock offline spike sorter (BOSS v1.03.00) and clampfit 10.7.0.3) were used.

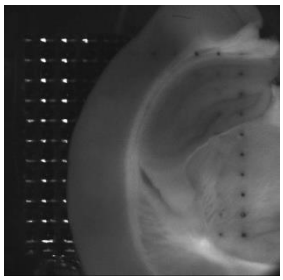
ImageJ was used for measuring the position of the electrode on the brain slices.

Note that the Tsc1 knockout neurons are in the upper cortical layers, and that the lower cortical layers are much less affected. 'when slices are bathed in a 0mM Mg++ solution, to induce seizures, we were able to record local field potentials from upper, mid, and lower cortical layers of the slice. As noted in **Figure 1**, in the control mouse (intact Tsc1, the seizure like events originate from deep in the cortex. In contrast, in the Tsc1 CKO mouse, there are such events in the upper cortical layers.

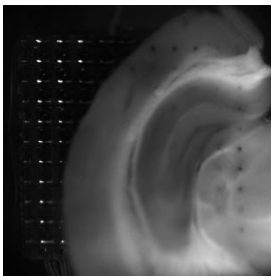
Left panel (wt): the seizure-like event onset in the wt cortex was later than the TSC1/cKO cortex (712 second vs 588 second from the beginning of the zero magnesium application). There is few LFP oscillation in the superficial layer (electrode #10, 100um from the pia). There were low voltage oscillation including positive (source) and negative (sink) voltage waves recorded in the deep cortical layers (electrode #11 and #12).

Right panel (TSC1/cKO): There were high frequent, and large amplitude negative voltage waves observed in the superficial layer (electrode #1). There were high frequent, mostly positive voltage waves in the deep layers (electrode #1 and #3)

Figure 1
Local field potential of onset of seizure-like event (SLE) recorded on acute brain slices from control and TSC1/cKO mouse (Zero magnesium)

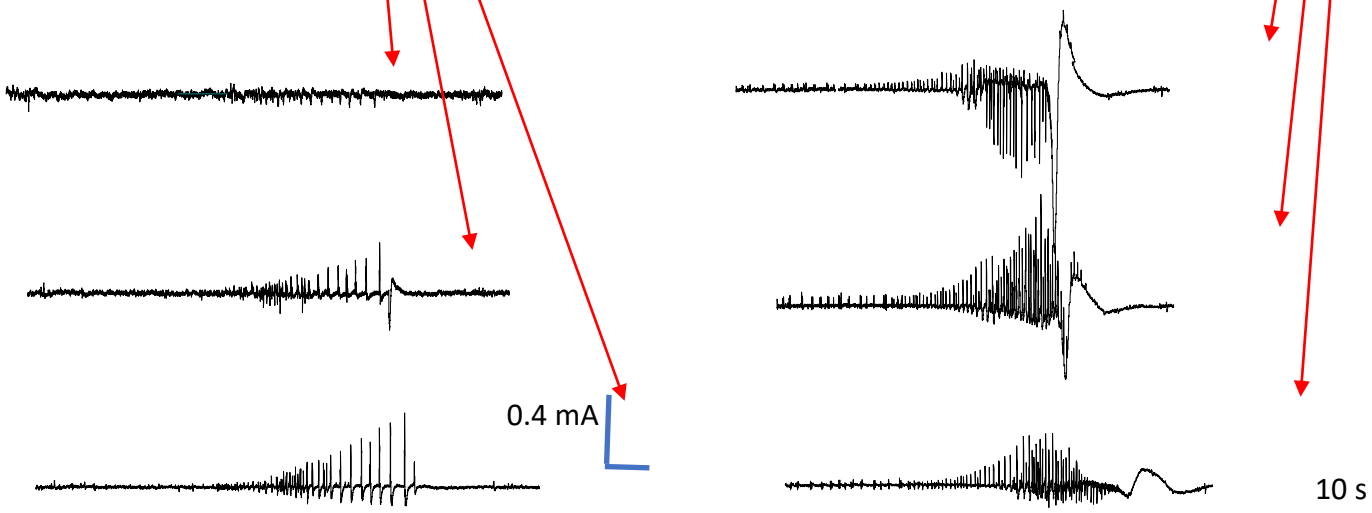


Depths of electrodes from surface:
100 μm
500 μm
900 μm



control 3-8-2019 slice4 (680th s to 780th s (100s)).

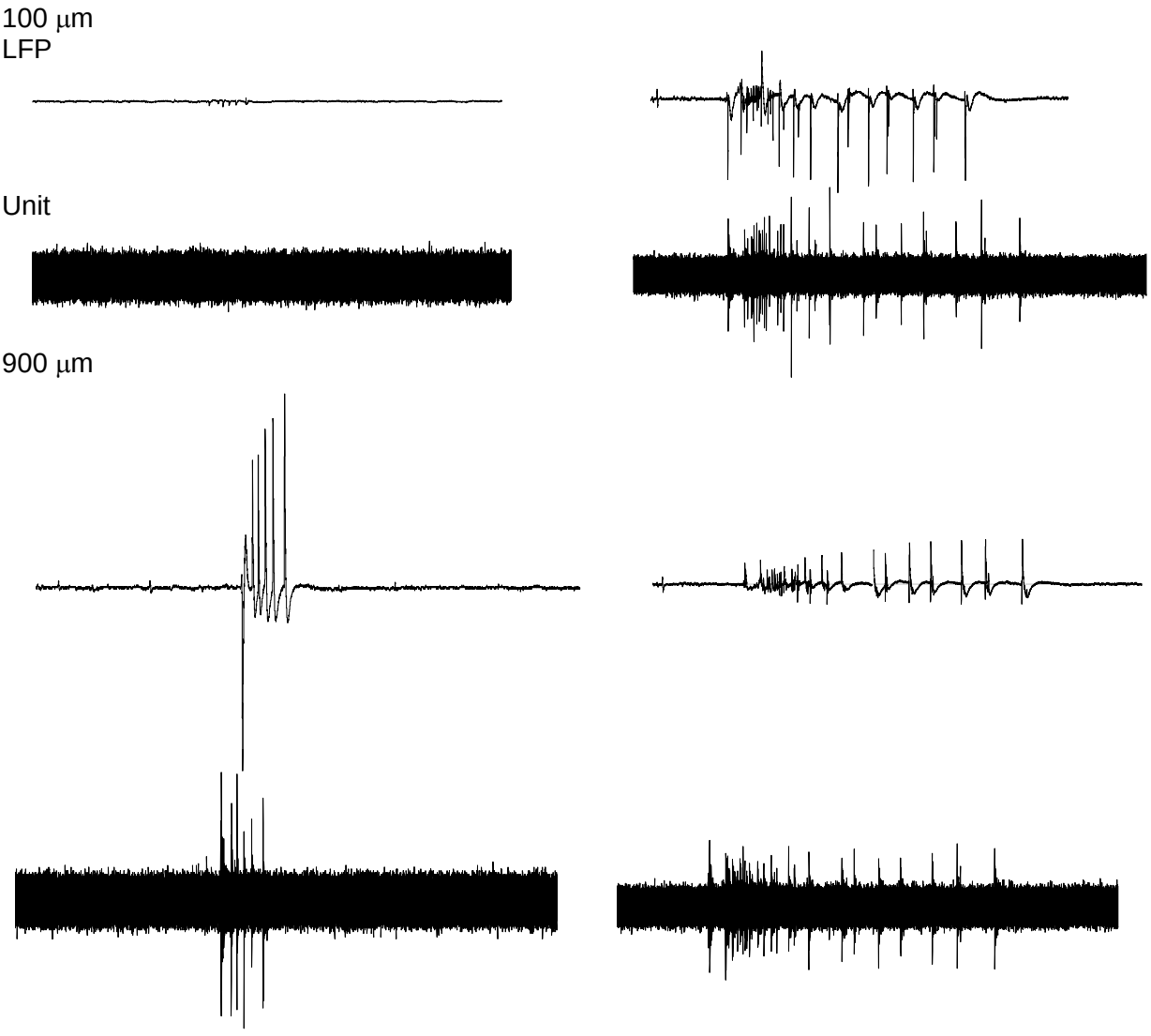
TSC1/cKO 3-1-2019 slice1 (560th s to 660th s)



Local field potentials and multi-unit activity (MUA) were recorded after onset of seizure-like activity in the wt and TSC1/cKo brain slices.

Left panel (wt): There were very low voltage oscillation (mostly positive waves) in the superficial layer (electrode #10, 100um from pia). No unit spiking was observed in the superficial layer. The large amplitude LFP and MUA were observed in the deep layer (electrode#12, 900um from pia)

Right panel (TSC1/cKO). Large negative voltage oscillations were observed in the superficial layers. At the meantime, large amplitude unit spikes were also observed at the location (electrode #2). The moderate positive and negative voltage oscillation as well as MUA was recorded in the deep layer.



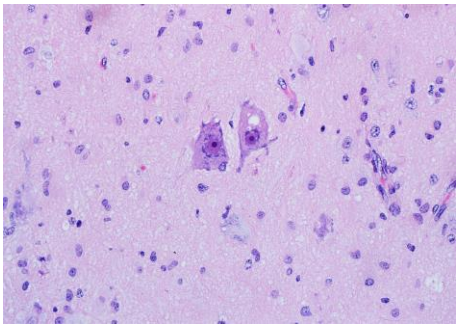
In another study, we have used the mouse model, in which we have extensively characterized the cytomegalic neurons, to compare these with cytomegalic neurons in the human TSC. Indeed, the large recombinant neurons in TSC1 CKO mice recapitulate aberrant features of cytomegalic neurons in human cortical tubers:

Similarities:

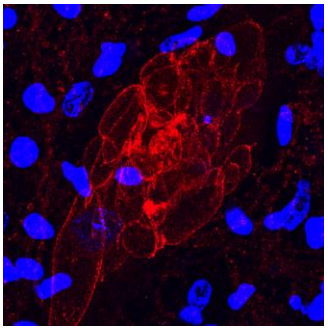
1. All are glutamatergic neurons
2. They display high levels of pS6 and p-4EB-P1 immunoreactivity, indicating high mTOR activity
3. They show prominent intracytoplasmic vacuolation

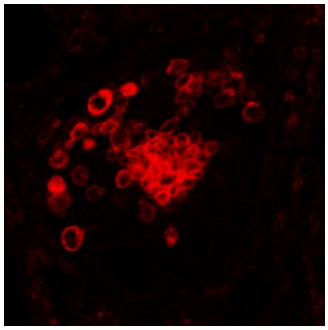
Vacuoles were immunostained for the membrane channel KCC2, suggesting problems with membrane trafficking in these neurons

Some of the antigens we found associated with cytomegalic neurons in the mouse were not found in human tubers – CD44, alphaB-crystallin, and ferritin.



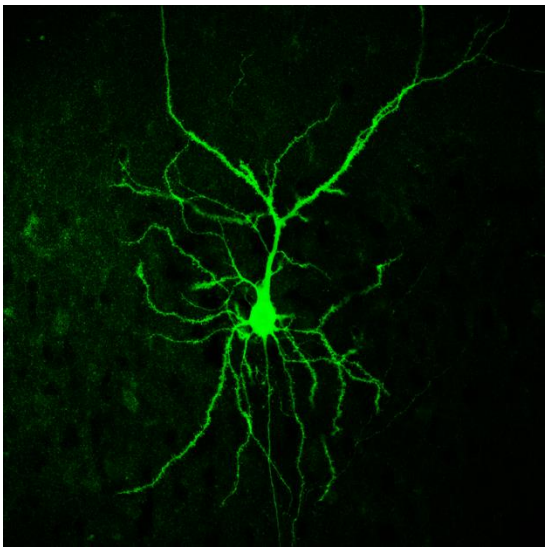
Vacuolated neuron in human TSC



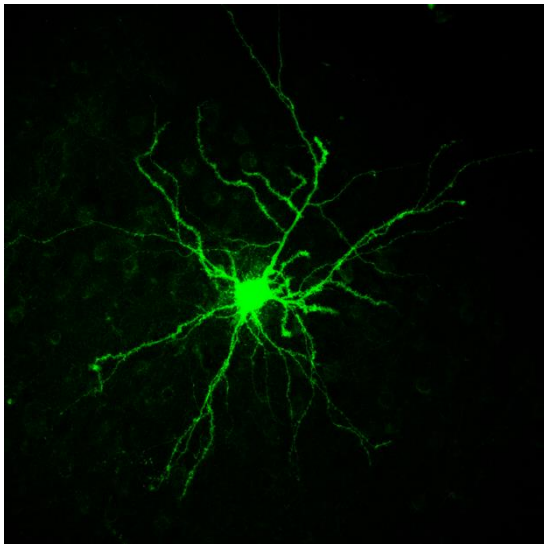


KCC2 immunofluorescence stain of vacuoles
Human Mouse

We also found highly abnormal dendritic organization in the mouse cytomegalic cells, including abnormally located dendrites on the cell body.



Normal mouse cortical pyramidal cell



Abnormal dendrites in TSC1 cKO neuron

We are currently working on three manuscripts.

1. This will detail the pathology and physiology of the *Tsc1*$^{mGFAP\text{-}Cre}$*CKO* and *Tsc1*$^{CamkII\text{-}Cre}$*CKO* mice. Both are interesting and useful to compare. In the *Tsc1*$^{mGFAP\text{-}Cre}$*CKO* mouse, astrocytes and some upper layer pyramidal neurons are recombined. Many of the mice develop seizures by 3 months of age. extremely large neurons, comparable to the "cytomegalic neurons" seen in human TSC (see below) are typical for these mice. These neurons are hyper-excitable, and by a number of measures, epileptogenic. Little change was seen in astrocytes, however, until after the development of seizures, indicating that astrocytes do not contribute to the initiation of seizures in this model. They do change, however, and after seizure onset, may contribute to the continuation and aggravation of seizures.

2. This will detail similarities and differences between the cytomegalic neurons of the mice and of human TSC. Similarities include dysmorphic forms, aberrant dendrite formation, intracytoplasmic vacuole formation (likely to be derived from Golgi), and apparent intracellular membrane trafficking problems. Some of the antigenic markers in both mouse and human cytomegalic neurons are not normally seen in neurons.

3. This will report the abnormal gene transcription and RNA translation in the mouse models. Here we will include neuronal and astrocytic changes due to mTOR activation and those changes that promote seizure activity.

Opportunities for training and professional development:

Nothing to report

Dissemination to communities of interest:

As noted above, several manuscripts will come out of our studies and will be submitted to peer-review journals.

IMPACT

These studies will have impact on several fields:

Impact on the development of principal discipline:

We have used a newly-generated technique ("ribotag") to illuminate basic issues of changes in translational efficiency in an mTOR-regulated disease, TSC.

We have found important transcriptional and translational changes and physiological alterations in excitatory neurons in a model of TSC. These studies have not been done

before and will lead to a far better understanding of the abnormalities in TSC neuronal function, particularly why neurons are so prone to seizure activity.

Impact on other disciplines:

As above, we have used a newly-generated technique ("ribotag") to illuminate basic issues of changes in translational efficiency in an mTOR-regulated disease, TSC. These studies will provide another example of the use of this technique, which is applicable to examining translational efficiencies in many cell and organ types.

Impact on technology transfer:

Nothing to report

Impact on society beyond science and technology:

Nothing to report

CHANGES/PROBLEMS:

Nothing to report

PRODUCTS:

Nothing to report

PARTICIPANTS:

Individuals who have worked on the project: No change.
Change in other support: Nothing to report.
Other organizations: Nothing to report