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14. ABSTRACT
The original goals were to explore potential mechanisms underlying epileptogenesis in Tuberous Sclerosis Complex (TSC) disease, with a focus on altered astrocyte-neuronal interactions caused by astrocyte-specific TSC deficiency. Astrocytes may contribute to abnormal neuronal excitability through mechanisms including glutamate uptake, potassium buffering and other means that alter expression and function of synaptic receptors for glutamate, or by altering the number of synapses. We chose to use a mouse GFAP (mGFAP) promoter sequence directing expression of a Cre-recombinase in most astrocytes and a subpopulation of the adult stem cells in the subventricular zone. We unexpectedly found, however, that there are indeed recombinant neurons that are derived from mGFAPcre expressing progenitors and are deficient for TSC1. We have compared directly and in the same brain region the effect of neuronal-intrinsic mTOR activation on the morphology and physiological functions of wild-type and recombinant neurons, as well as the effects of Tsc1-deficient astrocytes on neuronal morphology and neuronal activity associated with seizures.

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Tuberous Sclerosis; Epilepsy; Astrocytes; Neurons; Astrocyte-Neuron Interactions

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2016 Progress report
GRANT10931149
PI: David Sulzer; Co-PI: James Goldman

Altered Astrocyte-Neuron Interactions and Epileptogenesis in Tuberous Sclerosis Complex Disorder

Introduction

This is our 2011 TSCRP Idea Development Award (07/12-6/15) final report. The original goals were to explore potential mechanisms underlying epileptogenesis in Tuberous Sclerosis Complex (TSC) disease, with a focus on altered astrocyte-neuronal interactions caused by astrocyte specific TSC deficiency. We had hypothesized that abnormal cells in non-tuber cortex might form an abnormally excitable network that underlies seizure generation in TSC. Epileptogenesis in non-tuber neural tissue in TS may thus arise by an imbalance of decreased inhibitory and increased excitatory synaptic transmission. Astrocytes may contribute to abnormal neuronal excitability through mechanisms including glutamate uptake, potassium buffering and other means that alter expression and function of synaptic receptors for glutamate, or by altering the number of synapses. To address a specific role for astrocytes in regulating synaptic function during development, we chose to use a mouse GFAP (mGFAP) promoter sequence directing expression of a Cre-recombinase in most astrocytes and a subpopulation of the adult stem cells in the subventricular zone. Previous studies have reported that there is no targeting of postnatal or adult neural stem cells or their progeny in the hippocampus or other brain regions, rendering these mice particularly useful for selective targeting of astrocytes. We unexpectedly found, however, that there are indeed recombinant neurons that are derived from mGFAPcre expressing progenitors and are deficient for TSC1. This provides an important new aspect of analysis: in our novel neuroglial TSC1 deficient mouse model, we are able to compare directly and in the same brain region the effect of neuronal-intrinsic mTOR activation of synaptic activities on wild-type and recombinant neurons, as well as the effects of Tsc1-deficient astrocytes on neuronal morphology and neuronal activity associated with seizures.

Keywords: Tuberous Sclerosis, Epilepsy, Neurons, Astrocytes, GFAP, mouse models

Accomplishments:
Year 1:

We established the mouse GFAP-Cre mediated Tsc1 conditional knockout (TSC1^{mGFAPCre} CKO) mouse model, and found that:

a) TSC1 ^{mGFAPCre}CKO mice develop spontaneous clinical seizures at the age of 2.5 months;

b) TSC1^{mGFAPCre}CKO mice show astrogliosis, activated mTOR signaling, and enlarged cellular size of astrocytes and a few enlarged pyramidal neurons in layer II-III cortex and in hippocampus CA1-3 regions;

c) Astrocyte glutamate transport and potassium buffering functions remained intact in TSC1^{mGFAPCre}CKO mice at 1 month and 2 months of age, prior to the onset of clinical seizures. Astrocytes do show increased GFAP and S100b immunoreactivities at 1 month, and this increase becomes more pronounced with age. However, astrocytes do not show decreases in glutamate transporter currents, and GLT-1 and GLAST immunoreactivities until 3.5 months of the age. A slight decrease in astrocyte uptake of synaptically evoked glutamate appears at the age of after the occurrence of clinical seizures;

d) TSC1^{mGFAPCre}CKO display synaptic damage induced by spontaneous seizures at the age of 3months;

e) Increased spine density on pyramidal neuron dendrites at the age of one month, prior to the onset of spontaneous seizures (Figure 1).

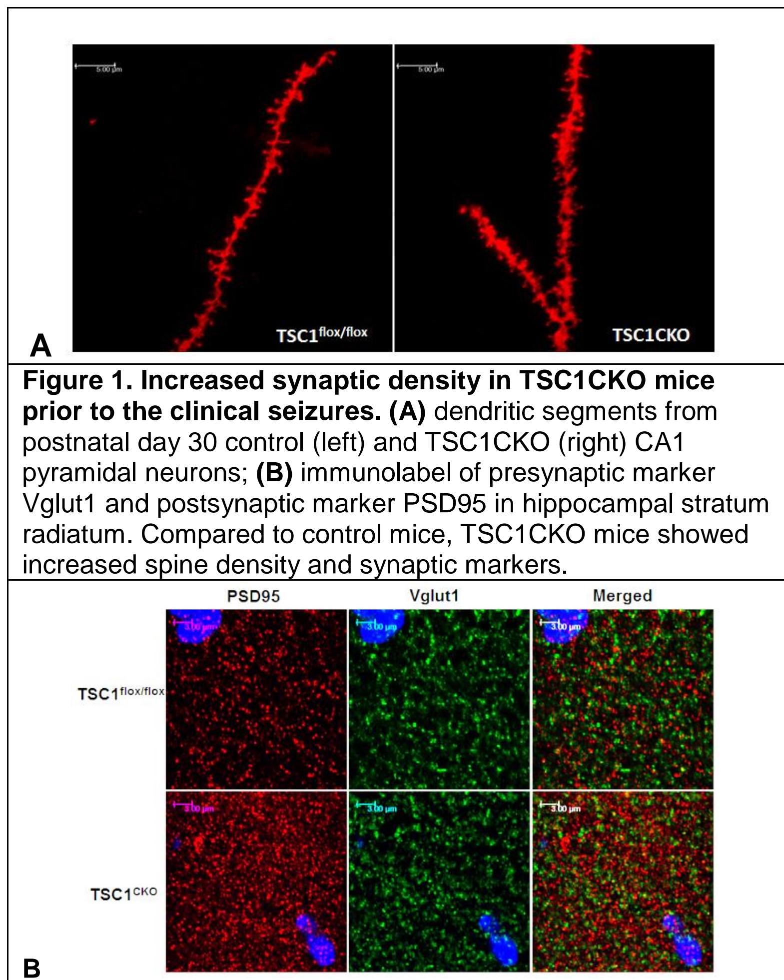


Figure 1. Increased synaptic density in TSC1CKO mice prior to the clinical seizures. (A) dendritic segments from postnatal day 30 control (left) and TSC1CKO (right) CA1 pyramidal neurons; **(B)** immunolabel of presynaptic marker Vglut1 and postsynaptic marker PSD95 in hippocampal stratum radiatum. Compared to control mice, TSC1CKO mice showed increased spine density and synaptic markers.

These data suggest that the astrocyte pathology evolves slowly in our mouse model. It is possible that the major changes in astrocytes do not manifest until during or after the onset of seizures, suggesting that the seizures produce the major pathology in astrocytes. Nevertheless, the consequent astrocyte pathology may itself contribute to the ongoing seizure activity.

Year 2:

We recorded miniature EPSCs, evoked EPSCs and paired pulse facilitation in wt and TSC1CKO mice at CA3-CA1 synapses at the age of 1 month and 3.5 months. We found in the mutants:

1) an increased frequency and amplitude of mEPSCs / evoked EPSCs in CA1 pyramidal neurons, indicative of increased neuronal excitability (Figure 2);

2) decreased paired pulse facilitation (PPF) in CA1 pyramidal neurons, suggesting an enhanced probability of presynaptic neurotransmitter release (Figure 2);

3) by recording high frequency stimulation evoked EPSCs, we found increased amplitude and increased decay time: this value indicates the amount of glutamate released at the synapse, the amount of extracellular glutamate molecules available for transport, and the expression of transporter proteins.

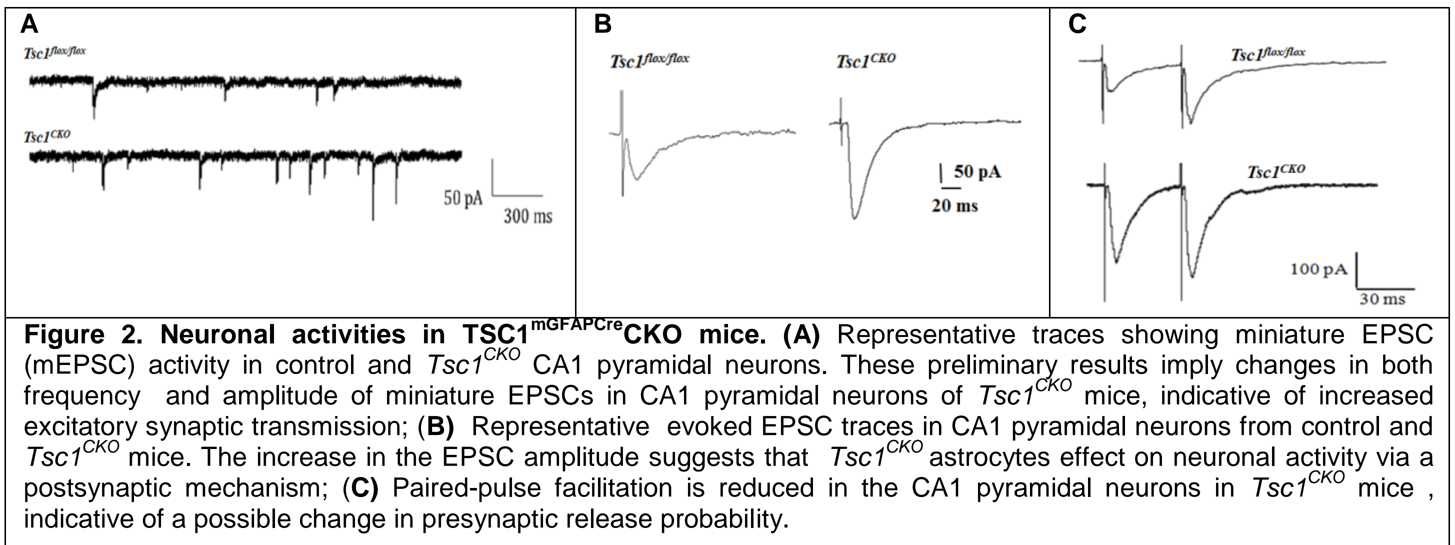


Figure 2. Neuronal activities in TSC1^{mGFAPCre}CKO mice. (A) Representative traces showing miniature EPSC (mEPSC) activity in control and *Tsc1^{CKO}* CA1 pyramidal neurons. These preliminary results imply changes in both frequency and amplitude of miniature EPSCs in CA1 pyramidal neurons of *Tsc1^{CKO}* mice, indicative of increased excitatory synaptic transmission; (**B**) Representative evoked EPSC traces in CA1 pyramidal neurons from control and *Tsc1^{CKO}* mice. The increase in the EPSC amplitude suggests that *Tsc1^{CKO}* astrocytes effect on neuronal activity via a postsynaptic mechanism; (**C**) Paired-pulse facilitation is reduced in the CA1 pyramidal neurons in *Tsc1^{CKO}* mice , indicative of a possible change in presynaptic release probability.

Together, these independent findings confirm an increase in excitatory neural transmission in our TSC1^{mGFAPCre}CKO mice during epileptogenesis. As we failed to detect significant changes in astrocyte glutamate uptake and potassium buffering, we further examined why pyramidal neurons show enhanced excitability.

As mentioned above, we observed some “dysplastic neurons” with enlarged soma size and high levels of phospho-S6 in layer II-III cortex and in hippocampal pyramidal layers in the TSC1^{mGFAPCre}CKO mice. To determine the origin of these dysplastic neurons and whether behavioral seizures are due to enhanced intrinsic excitability of these neurons, we developed a novel TSC1^{mGFAPCre}CKO mouse model expressing a LoxP-stop-LoxP-dtomato cassette (TSC1^{mGFAPCre}CKO:Ai9-dtomato+), in which the expression of Cre recombinase can drive the expression of dtomato in Cre expression brain cells. Our findings in this new mouse model include:

a) A pool of dTomato-positive,Tsc1-deficient recombinant pyramidal neurons in the superficial isocortical layers (II-III) and in the hippocampal pyramidal layer, indicating a radial glial origin of these late-born excitatory neurons.

b) In TSC1^{mGFAPCre}CKO mice, most of these recombinant neurons expressed high levels of pS6 at the age of 1 month, suggesting overactive mTOR in response to the loss of TSC1 alleles. With age, some recombinant neurons maintained high levels of pS6 and displayed increased soma volume and increased dendritic trees. Interestingly, some recombinant neurons did not increase in size, but remained a normal size, compared to pyramidal neurons in the same layers that did not show Crew-recombination. This normal size suggests that these neurons possess a negative feedback mechanism that in part counteracts the effect of the TSC1 deletion.

c) We have compared dendritic spine density of mGFAPcre recombinant (dTomato+) and non-recombinant (dTomato-) neurons in both Tsc1wt:Ai9-dtomato+ an$^{d\ TSC1mGFAPCre}$CKO; Ai9-dtomato+ mice. Using DiOlistic labeling technique, we found that TSC1-deficient recombinant neurons exhibit more dendritic spines than wild type, non-recombinant neurons. The latter show a similar spine density to that of pyramidal neurons in a TSC1 wild type, mGFAPCre-expressing Ai9 dTomato mice. These data

suggest that the increased excitability in TSC1^{mGFAPCre}CKO mice might be due to Cre-expressing recombinant excitatory neurons and that the Cre and dTomato expression do not alter spine density.

d) Tsc1 deletion prior to the biogenesis of neurites would impact the complexity of the dendritic tree. We crossed the Tsc1flox/flox mice to the Ai9 Cre reporter mice to obtain Tsc1flox/flox;Ai9+ and Tsc1+/+;Ai9+ pups. We isolated neurons to establish in primary cultures, and transfected neurons from Tsc1flox/flox:Ai9+ or Tsc1+/+;Ai9+ mice pups with Cre expressing plasmids before plating into cultures. Cre recombinase will drive dTomato expression, which allows the imaging of dendritic spines in neurons in vitro, and removes the Tsc1 gene from Tsc1flox/flox neurons. Compared to Tsc1+/+; Cre-expressing control neurons, Tsc1flox/flox;Cre-expressing Tsc1 CKO neurons exhibit significantly more primary and secondary dendrites, causing them to appear "dysplastic". The increased dendritic complexity in TSC1 CKO recombinant, dysplastic neurons was confirmed in brain slices using luciferase yellow injection. Compared to non-recombinant neurons, dysplastic neurons showed a larger soma size and an increased basal dendritic tree complexity (Figure 3).

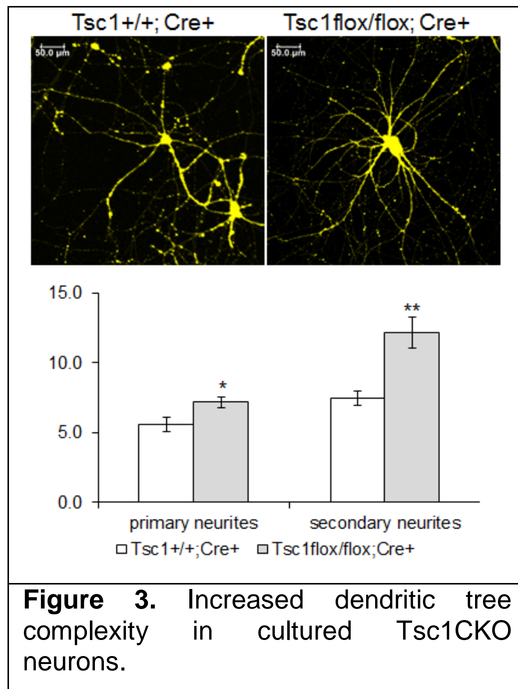


Figure 3. Increased dendritic tree complexity in cultured Tsc1CKO neurons.

Year 3:

We continued to characterize the physiological properties of 1) dtomato+ recombinant Tsc1KO neurons in TSC1^{mGFAPCre}CKO: Ai9-dtomato+ mice, 2) dtomato- non-recombinant neurons in the TSC1^{mGFAPCre}CKO: Ai9-dtomato+ mice, 3) dtomato+ pyramidal neurons in mGFAPCre+:Ai9-dtomato+ Tsc1 wild type control mice, and 4) dtomato- pyramidal neurons in mGFAPCre+:Ai9-dtomato+ control mice. All mice were examined at the age of 3.5months.

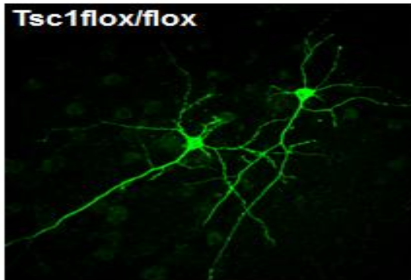
a) ***Intrinsic membrane properties.*** Compared to dtomato- neurons in TSC1^{mGFAPCre}CKO: Ai9-dtomato+ mice, dtomato+ and dtomato- pyramidal neurons in mGFAPCre+:Ai9-dtomato+ mice, TSC1KO dtomato+ pyramidal neurons in TSC1^{mGFAPCre}CKO: Ai9-dtomato+ mice exhibited increased membrane capacitance and decreased Membrane input resistance;

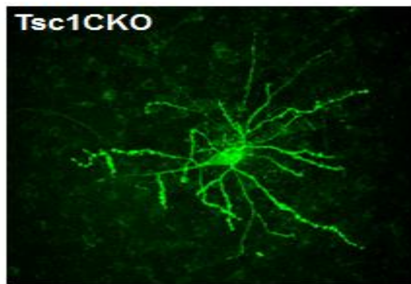
b) ***Spontaneous miniature excitatory postsynaptic currents (mEPSCs).*** mEPSCs were recorded in the presence of 1mM Tetrodotoxin (TTX) and 50mM picrotoxin. We found that the dtomato+ recombinant TSC1 knockout neurons show increased frequency of miniature EPSCs, increased amplitude of mEPSCs and a prolonged decay, compared to wild type, non-recombinant neurons in the TSC1^{mGFAPCre}CKO mice and to dtomato+ and dtomato- pyramidal neurons in TSC1 wild type, mGFAPCre expressing Ai9 dTomato mice .

c) ***Action potential (AP) and afterdepolarization (ADP)****.* Action potentials were evoked in neurons with brief (2 and 4 msec) depolarizing current pulses through the recording electrode. AP firing patterns were examined by injecting neurons with long depolarizing current (300 msec) (up to 400pA). TSC1KO dtomato+ pyramidal neurons in TSC1^{mGFAPCre}CKO: Ai9-dtomato+ mice exhibited lower action potential threshold, elevated or prolonged ADP, or fast AP firing pattern than all of the other three types of neurons (Figure 5).

d) ***Presence of spontaneous paroxysmal depolarization shift (PDS).*** PDS is a sudden, large depolarization of membrane potential, which lasts more than 100 msec and usually triggers spike firing (Johnston 1984), often observed with intracellular recording in focal or generalized convulsive epilepsy, characterized by a giant excitatory postsynaptic potential with a burst of action potentials riding on it and followed by a long period of hyperpolarization. PDS can be a network-driven synchronizing event and is an intracellular correlate for the interictal EEG spikes. We found that PDS can only be induced in dtomato+ neurons in TSC1^{mGFAPCre}CKO mice, but not in dtomato- neurons in TSC1^{mGFAPCre}CKO mice and both dtomato+ and dtomato- neurons from mGFAPCre+;Ai9-dtomato+ control mice.

e) ***Seizure-like activity induced by weak chemical/electrical stimulation***. Local pressure ejection of a weak NMDA receptor agonist, 1mM aspartic acid (4 sec, 10Kpa) induces transient NMDA receptor-dependent depolarization (plateau potential) and spike firing (Suzuki, 2008) in both dtomato+ and dtomato- neurons in mGFAPCre+:Ai9-dtomato+ control mouse cortex, and in dtomato- neurons in TSC1^{mGFAPCre}CKO: Ai9-dtomato+ mouse cortex. In contrast, dtomato+ dysplastic neurons in TSC1^{mGFAPCre}CKO: Ai9-dtomato+ mouse brain showed prolonged slow-depolarization after initial burst-firing induced by NMDA R agonist, followed by a sustained afterdischarge, indicative of long-lasting seizure-like activity (Figure 4). The aspartate-driven depolarization was blocked by an NMDA antagonist (Figure 6).

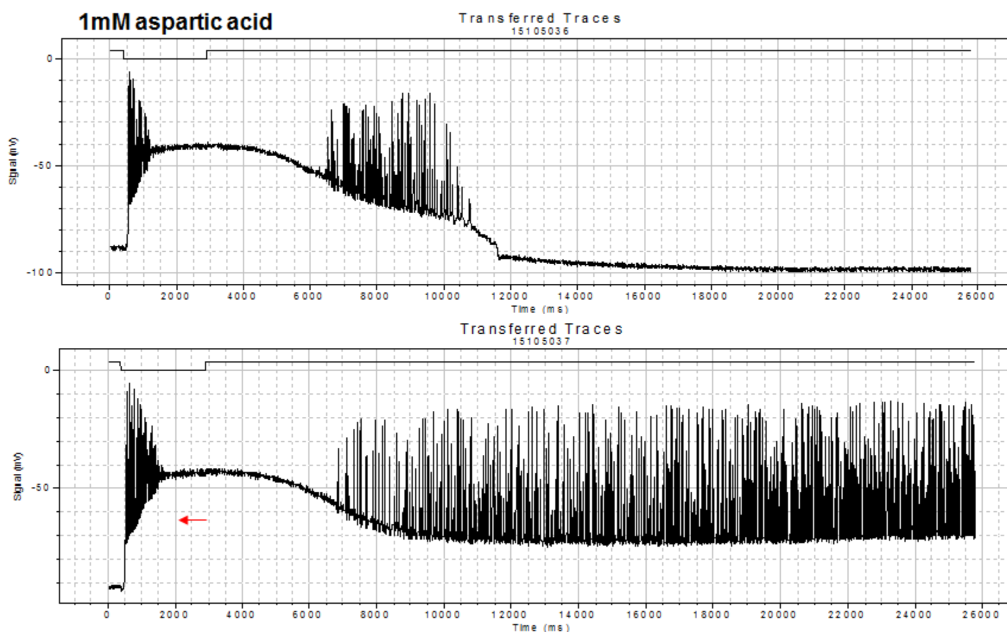




A

Figure 4. Prolonged discharge observed in the cortical pyramidal neurons in Tsc1mGFAPCreCKO mice. (A) Represented luciferase yellow injected normal size wild type (upper) and dysplastic Tsc1GFAPCreCKO (lower) neurons pyramidal neurons in cortical layers II-II. (B) Induction of electrographic seizures in dTomato negative (Upper) and positive (Lower) neurons in Tsc1GFAPCreCKO mice.

B



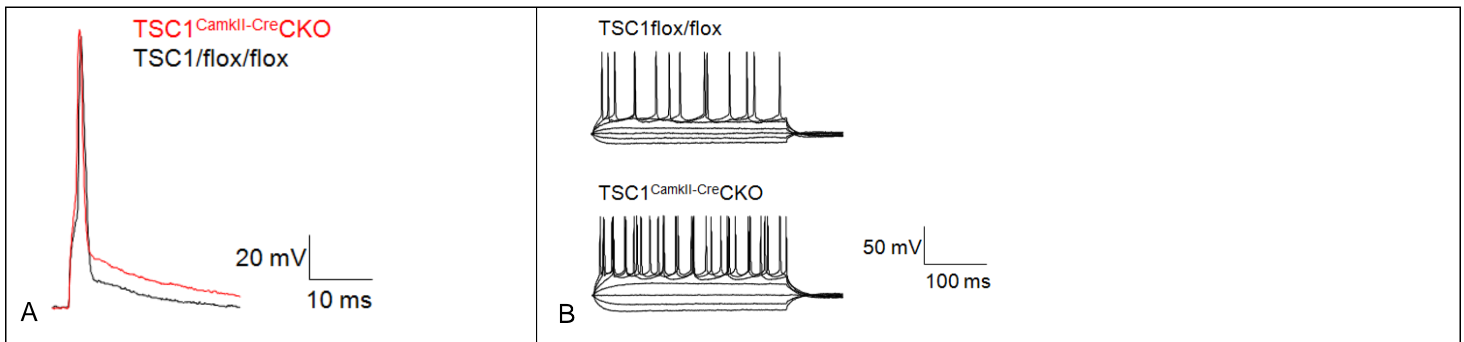


Figure 5. Cortical pyramidal neurons from TSC1$^{CamKII-cre}$CKO mouse show intrinsic membrane hyperexcitability. A. An enhanced afterdepolarization (ADP) after a single action potential (AP) induced with a short depolarizing current pulse (2 ms, 2 nA) was observed only in pyramidal neurons from TSC1$^{CamKII-cre}$CKO mouse, but not in pyramidal neuron from TSC fl/fl mouse. B. Example of voltage response to current injection through patch electrode (-200pA to 400 pA , 300ms). The number of action potential was greater in TSC1$^{CamKII-cre}$CKO pyramidal neurons than in control neurons in response to 200, 300 and 400pA current injection.

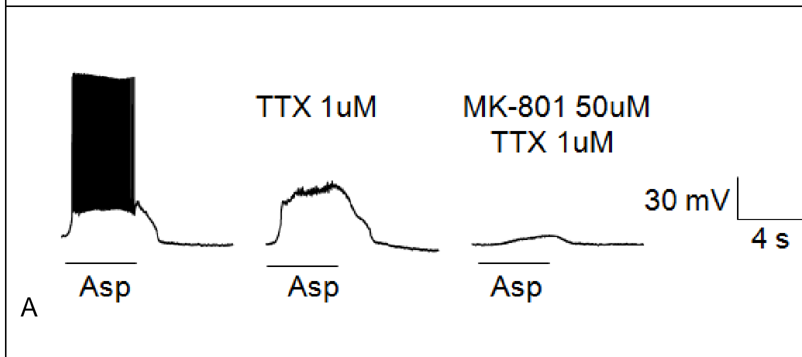


Figure 6. The NMDA receptor agonist aspartic acid (1 mM) induces a prolonged membrane depolarization in cortical pyramidal neuron from Tsc1$^{CamkII-Cre}$CKO mouse. A. Focally applying aspartic acid evoked action potential firing and membrane depolarization in cortical pyramidal neurons (PYN). TTX (1uM) blocked the induced action potential firing, but not membrane depolarization. The NMDA receptor antagonist (MK-801) almost completely blocked membrane depolarization.

Year 4

We have asked for and received a one-year no cost extension to complete data analysis and to prepare the manuscript. During this year, we have continued our electrophysiological analysis of excitability of dtomato+ TSC1KO neurons in the TSC1^{mGFAPCre}CKO: Ai9-dtomato+ mice at different ages (1month, 2 months and 3.5months). We have increased the sample size for each measure to ensure that we have sufficient power to detect significant differences between groups.

At all ages, the Tsc1KO dtomato+ neurons in the TSC1KO mice exhibit increased frequency of miniature EPSCs, increased amplitude, prolonged decay, and lower threshold for action potentials, compared to dtomato- neurons in the TSC1KO mice, and to dtomato+ and dtomato- neurons in mGFAPCre+:Ai9-dtomato+ control mice. However, PDS and seizure like activities induced by weak chemical or electrical stimulation can only be evoked in recombinant TSC1KO neurons in TSC1^{mGFAPCre}CKO: Ai9-dtomato+ mice at the age of 3.5months, when these neurons become “dysplastic”.

We have characterized the temporal expression pattern of mouse GFAPcre during embryonic ages. Using Ai9 dtomato+ Cre reporter mice, we have examined the mouse GFAPCre mediated recombination at embryonic day 12 (E12), 15(E15), 18(E18), 19(E19) and 21 (E21). The Cre mediated dtomato expression begins on E18 in a small set of neural progenitors. Ongoing studies are to examine whether there is an age-dependent increase in mGFAPCre mediated neuronal recombination events.

We measured pre- and post-synaptic protein levels during development, at the ages of 1month, 2months and 3.5months. At the age of 1 month, prior to the onset of behavioral seizures, we found an increase in protein levels of pre- and postsynaptic proteins, including synapsin I, NR2B, GluR1 and GluR2, in the TSC1^{mGFAPCre}CKO mice. These data are consistent with our findings of increased dendritic spine synapses in

Tsc1KO neurons in the TSC1^{mGFAPCre}CKO mice. Our ongoing experiments are to determine the synaptic components specifically in the mGFAPCre expressing neurons in control and TSC1^{mGFAPCre}CKO mice.

In summary, we have established several TSC mouse models based on the mGFAP-Cre TSC1 CKO mouse. We have made a number of fundamental anatomic, electrophysiological and molecular observations. These include 1. the development of dysplastic, cytomegalic neurons, which display a number of the changes of hyperexcitable neurons in epilepsy; 2. increases in dendritic spine density produced by TSC1 CKO; 3. changes in astrocyte size, GFAP levels, and glutamate transporter currents, but these changes occur during or after the onset of seizures; 4. Normal, non-recombinant pyramidal neurons are surrounded by recombinant astrocytes, suggesting that TSC1-deficient astrocytes are not in themselves able to convert a pyramidal neuron to a dysplastic, hyperexcitable neuron. These novel observations are helping us to understand the pathology and clinical phenotype of TSC.

We are in the process of writing up a manuscript to report our findings. We have most of it written, but still require a few more electrophysiological recordings and protein assays to have enough observations for statistical significance testing.

Impact:

As noted above, our novel observations stand to add to our knowledge of the mechanisms of seizures and anatomic pathologies in TSC significantly. Specifically, our new neuronal-glial mouse model of TSC1 deficiency has allowed us to compare directly and in the same brain region the effect of neuronal-intrinsic mTOR activation on synaptic activities and morphology of TSC1 deficient neurons with those that contain normal levels of TSC1. This has allowed us to begin to define in detail the molecular changes that neurons udergo as a result of TSC1 deletion. We have also been able to distinguish the effects of Tsc1-deficient astrocytes on neuronal morphology and neuronal activity associated with seizures.
We will be able to learn in detail how the pathology of TSC is established and hopefully, based on molecular evaluations, suggest new strategies for therapies.

Changes/Problems: None

Products: None

Participants & Other Collaborating Organizations: None

Special Reporting Requirements: None

Appendices: None