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

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14. ABSTRACT
This report is on the second year of our 2011 Tuberous Sclerosis Complex Research Program Idea Development Award (07/12-6/15). The original goals are to explore the potential mechanism for epileptogenesis in Tuberous Sclerosis Complex (TSC) disease, with a focus on altered astrocyte-neuronal interactions caused by astrocyte-specific TSC deficiency. Our hypothesis is 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 could also regulate neuronal excitability by glutamate uptake and other means that alter expression and function of synaptic receptors for glutamate, or by altering the number of synapses. A deficiency of GABAergic interneurons, a possible downstream consequence of altered mTOR activity in astrocytes, may further contribute to the early onset and severity of seizures in TS.

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

INTRODUCTION

This report is on the second year of our 2011 Tuberous Sclerosis Complex Research Program Idea Development Award (07/12-6/15). The original goals are to explore the potential mechanism for epileptogenesis in Tuberous Sclerosis Complex (TSC) disease, with a focus on altered astrocyte-neuronal interactions caused by astrocyte-specific TSC deficiency. Our hypothesis is 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 could also regulate neuronal excitability by glutamate uptake and other means that alter expression and function of synaptic receptors for glutamate, or by altering the number of synapses. A deficiency of GABAergic interneurons, a possible downstream consequence of altered mTOR activity in astrocytes, may further contribute to the early onset and severity of seizures in TS.

RESEARCH PROGRESS

To address a specific role of 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 shown 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. Our first year preliminary findings in the mouse GFAP-cre mediated Tsc1 conditional knockout ($TSC1^{mGFAPCre}$ CKO) mice include: 1) $TSC1^{mGFAPCre}$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 cell size for astrocytes and a few enlarged neurons; C) glutamate transport and potassium buffering functions are intact in $TSC1^{mGFAPCre}$CKO astrocytes in young mice; D) $TSC1^{mGFAPCre}$CKO mice exhibit abnormal excitatory synaptic transmission; E) synaptic damage induced by spontaneous seizures F) increased spine density on pyramidal neuron dendrites occurs before the onset of spontaneous seizures.

During this second year of the award, we have fully characterized neuronal and astrocyte phenotypes in our $TSC1^{mGFAPCre}$CKO mice at ages before and after the occurrence of clinical seizures. Unexpectedly, by using a dtomato-expressing cre reporter Ai9 mouse line (Figure1A), we identified dtomato-positive recombinant pyramidal neurons in superficial cortical layers and in hippocampal pyramidal layers (Figure 1B), indicative of a radial glial origin of these late-born excitatory neurons. In $TSC1^{mGFAPCre}$CKO mice. Most of these recombinant neurons expressed high levels of pS6 at the age of 1 month, suggesting hyperactive mTOR in response to the loss of Tsc1 alleles (Figure 1B). With age, some recombinant neurons continued to maintain high levels of pS6 and displayed increased soma sizes and dendritic trees. Interestingly, other recombinant neurons show reduced pS6 levels with normal soma size and morphology, suggesting that these neurons have a negative feedback mechanism to counteract the effect of the TSC1 deletion.

We examined the electrophysiological properties of glutamatergic synapses in CA1 pyramidal neurons. Our preliminary findings during the first year of the award strongly implied changes in both amplitude and frequency of miniature EPSC in CA1 pyramidal neurons of TSC1CKO mice, indicative of increased excitatory synaptic transmission. In the second year of the award, we further characterized the synaptic transmission of CA3-CA1 synapses by measuring miniature EPSCs, evoked EPSCs and paired pulse facilitation in wt and TSC1CKO mice at the age of 3.5months. Our findings include 1) no changes in astrocyte glutamate uptake and potassium buffering before the occurrence of clinical seizures (Figure 2); 2) a slight decrease in astrocyte synaptically evoked glutamate uptake after the occurrence of clinical seizures (Figure 3D,E); 3) increased frequency and amplitude of miniEPSCs/evoked EPSCs in CA1 pyramidal neurons, indicative of increased neuronal excitability (Figure 3A,B); 3) decreased paired pulse facilitation (PPF) in CA1 pyramidal neurons

(Figure3C), suggesting enhanced presynaptic neurotransmisster release; 4) by recording high frequency stimulation evoked EPSCs, we found an increased amplitude and increased decay time (Figure 4A,B) which represents the amount of glutamate released at the synapse, that in turn depends on the amount of extracellular glutamate molecules available for transport and the expression of transporter proteins. TSC1CKO mice however showed a decrease in evoked inward current, compared to the control mice. All these data suggest an increase in excitatory neural transmission in our TSC1^{mGFAPCre}CKO mice during epileptogenesis.

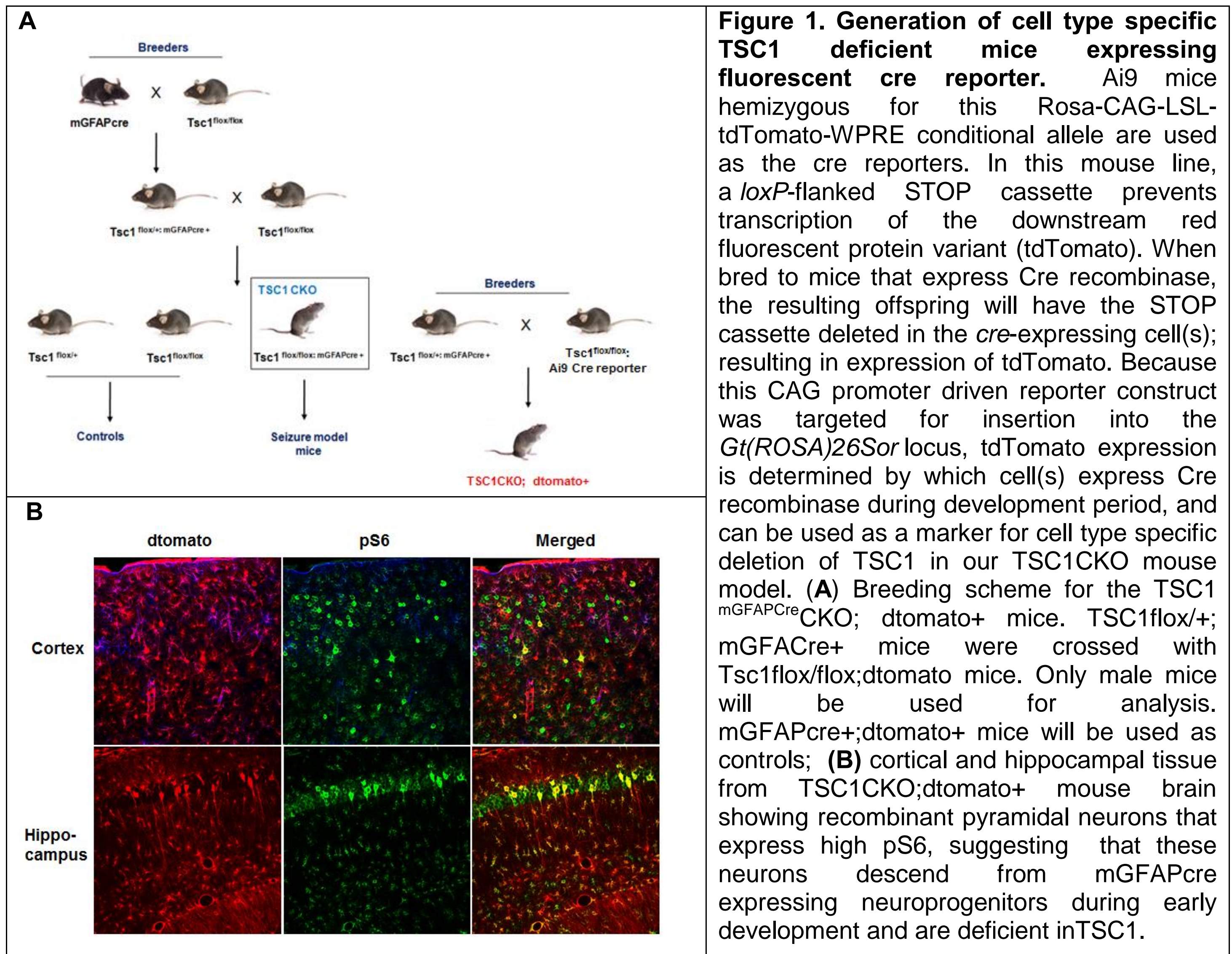

Figure 1. Generation of cell type specific TSC1 deficient mice expressing fluorescent cre reporter. Ai9 mice hemizygous for this Rosa-CAG-LSL-tdTomato-WPRE conditional allele are used as the cre reporters. In this mouse line, a *loxP*-flanked STOP cassette prevents transcription of the downstream red fluorescent protein variant (tdTomato). When bred to mice that express Cre recombinase, the resulting offspring will have the STOP cassette deleted in the *cre*-expressing cell(s); resulting in expression of tdTomato. Because this CAG promoter driven reporter construct was targeted for insertion into the *Gt(ROSA)26Sor* locus, tdTomato expression is determined by which cell(s) express Cre recombinase during development period, and can be used as a marker for cell type specific deletion of TSC1 in our TSC1CKO mouse model. (**A**) Breeding scheme for the TSC1^{mGFAPCre}CKO; dtomato+ mice. TSC1flox/+; mGFACre+ mice were crossed with Tsc1flox/flox;dtomato mice. Only male mice will be used for analysis. mGFAPcre+;dtomato+ mice will be used as controls; **(B)** cortical and hippocampal tissue from TSC1CKO;dtomato+ mouse brain showing recombinant pyramidal neurons that express high pS6, suggesting that these neurons descend from mGFAPcre expressing neuroprogenitors during early development and are deficient inTSC1.

Our data do not support the theory that a contribution of astrocyte glutamate uptake and potassium buffering dysfunction lead to epileptogenesis in our neuroglial TSC-deficient mouse models: glutamate and potassium uptake remain intact in TSC1 deficient astrocytes in our TSC1^{mGFAPCre}CKO mice before the onset of clinical seizures. Astrocytes do show increased GFAP and S100b immunoreactivities by 1 month of the age, 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 age (our previous findings during the first year of the award). These data suggest that the astrocyte pathology evolves slowly in our mouse model. It remains difficult for us to answer to the important question of how and if astrocytes participate in the evolution of neuronal hyperexcitability and seizures, but it is possible that the major changes in astrocytes do not manifest themselves until after the onset of seizures or concomitant with the onset of seizures, suggesting that the seizures produce further pathology in astrocytes. This resulting astrocyte pathology may indeed contribute to the ongoing seizure activity.

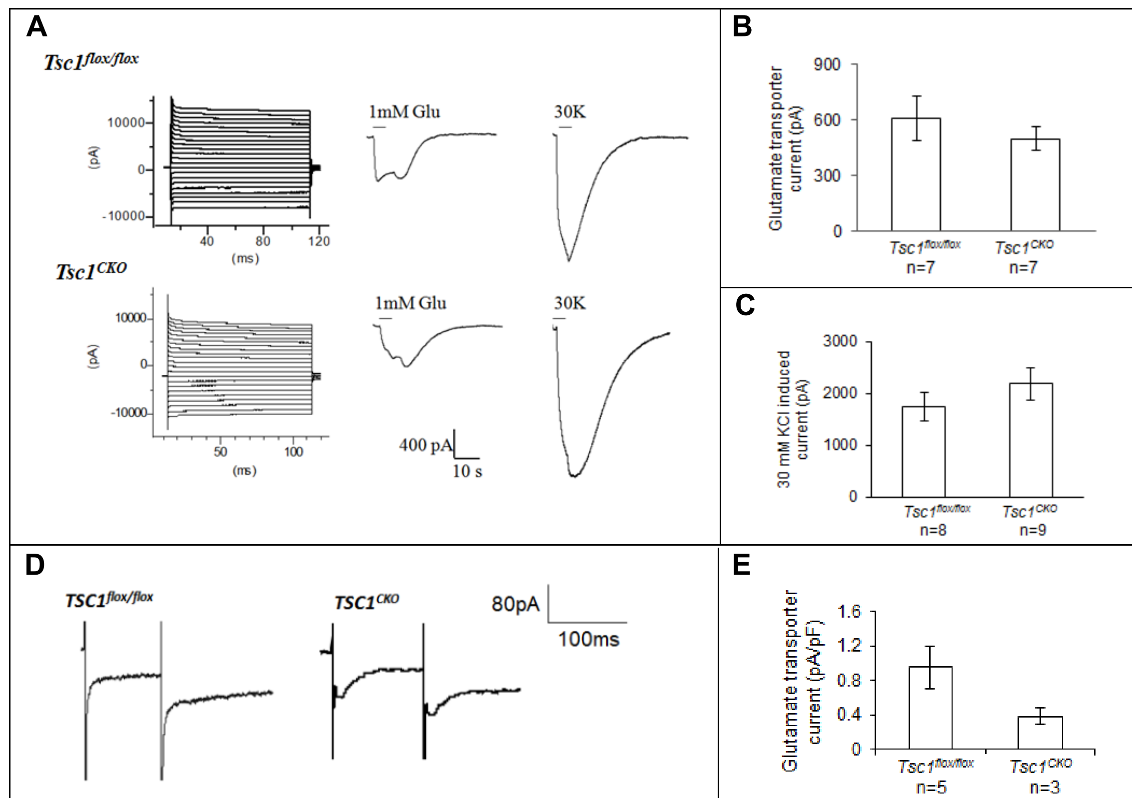

Figure 2. Astrocyte glutamate transporter dependent current.

(A) Representative recording of glutamate transporter activity (left) and potassium induced current (right) in control and TSC1CKO hippocampal slices.

(B&C) Compared with control astrocytes, TSC1CKO astrocytes show intact glutamate uptake (B) and potassium buffering(C).

(D) Synaptically evoked glutamate transporter current by stimulation of Schaffer collateral (0.2 ms, 5 mA) in 14-week old mice.

(E) Glutamate transporter capacity is computed by glutamate transporter current (pA)/ cell capacitance (Cm, pF). Compared to control mice, TSC1CKO astrocytes showed a decrease in evoked glutamate uptake (P=0.06).

We also tested whether a failure in pruning excessive excitatory synapses may contribute to the pathogenesis of epilepsy in TS by producing neuronal over-excitability, and that this is in part due to loss of astrocyte's normal role in normal GABA interneuron development, and dysregulation of normal synaptic pruning. We examined excitatory synaptic density by DiOlistic labeling and immunohistochemistry at the age of 1 month, before the occurrence of clinical seizures. Our data showed 1) increased spine density (Figure 5A) and 2) increased pre and post-synaptic protein puncta in TSC1^{mGFAPCre}CKO CA1 brain (Figure 5B). We are currently recording neuronal activity at this age prior to the occurrence of clinical seizures.

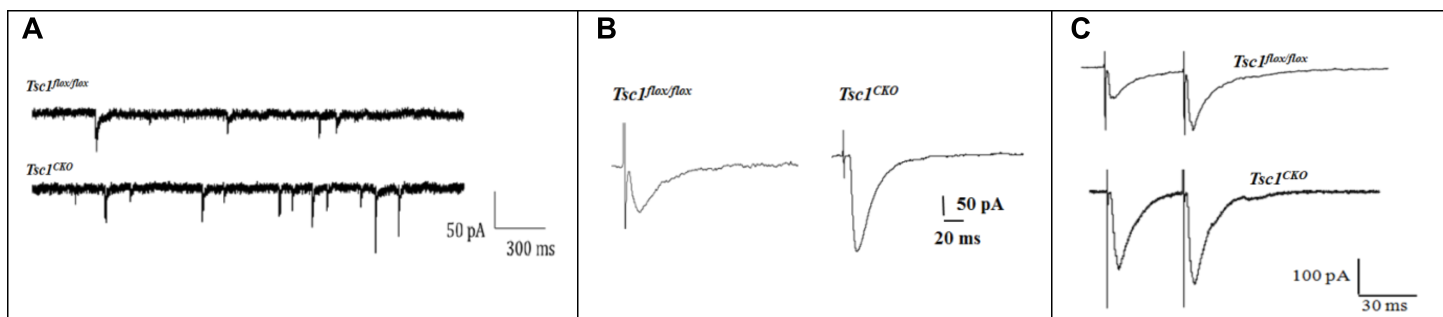

Figure 3. Neuronal activity in TSC1^{mGFAPCre}CKO mice. (A) Representative traces showing miniature EPSC (mEPSC) activity in control and *Tsc1*^{CKO} CA1 pyramidal neurons. These preliminary results imply changes in both frequency and amplitude of miniature EPSCs in CA1 pyramidal neurons of *Tsc1*^{CKO} 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, which classically indicates an elevanted presynaptic release probability.

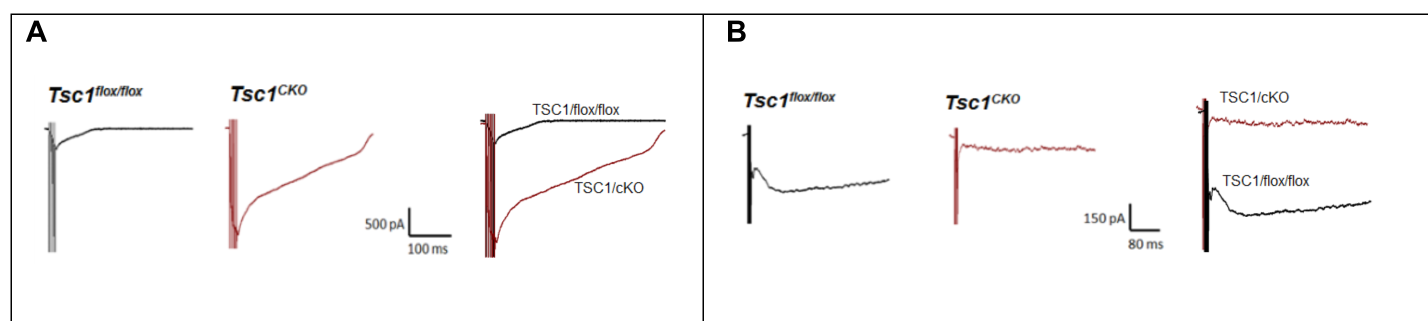

Figure 4. High frequency stimulation evoked inward currents in TSC1CKO neurons and astrocytes. (A) High frequency stimulation (HFS; 5ms interval. 4 stimulation. 1mA) induced glutamate transporter dependent inward current in CA1 pyramidal neurons; **(B)** HFS induced glutamate transporter dependent inward current in astrocytes; tail current induced by HFS of Schaffer collateral in astrocytes. Compared to controls, TSC1CKO mice showed a decrease in evoked inward current.

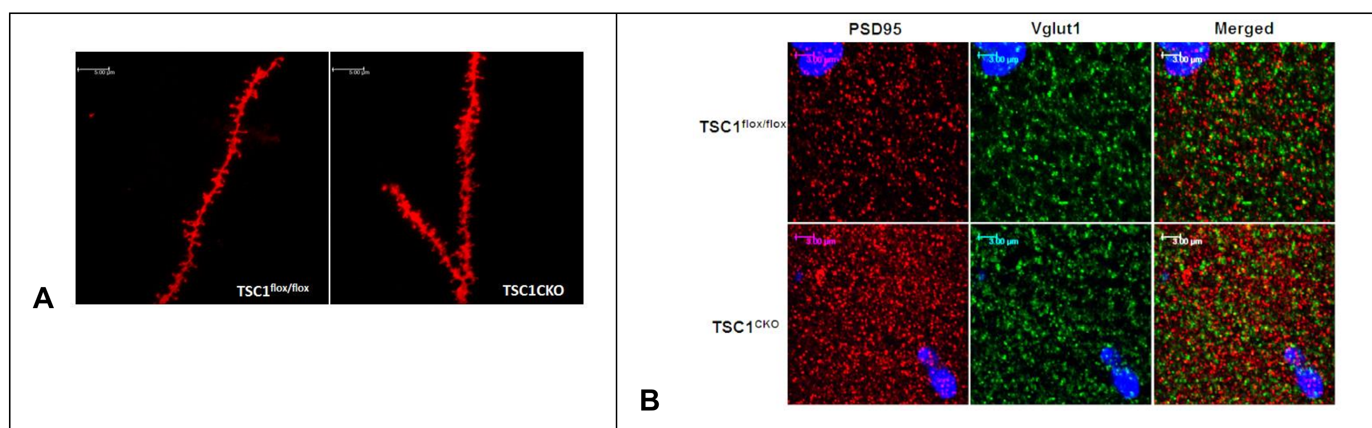

Figure 5. Increased synaptic density in TSC1CKO mice prior to clinical seizures. (A) Dendritic segments from postnatal day 30 control (left) and TSC1CKO (right) CA1 pyramidal neurons; **(B)** immunolabel of presynaptic marker Vglut1 and postsynaptic marker PSD95 in hippocampal stratum radiatum. Compared to control mice, TSC1CKO mice showed increased spine density and synaptic markers.

An alternative mechanism that may explain how epilepsy develops in our TSC1^{mGFAPCre}CKO mice is altered intrinsic neuronal hyperexcitability, especially in those recombinant neurons deficient for TSC1 and expressing high level of pS6. We are currently comparing the anatomic and physiological properties of mGFAPcre recombinant (dtomato+) and non-recombinant (dtomato-) neurons in the TSC1^{mGFAPCre}CKO; dtomato+

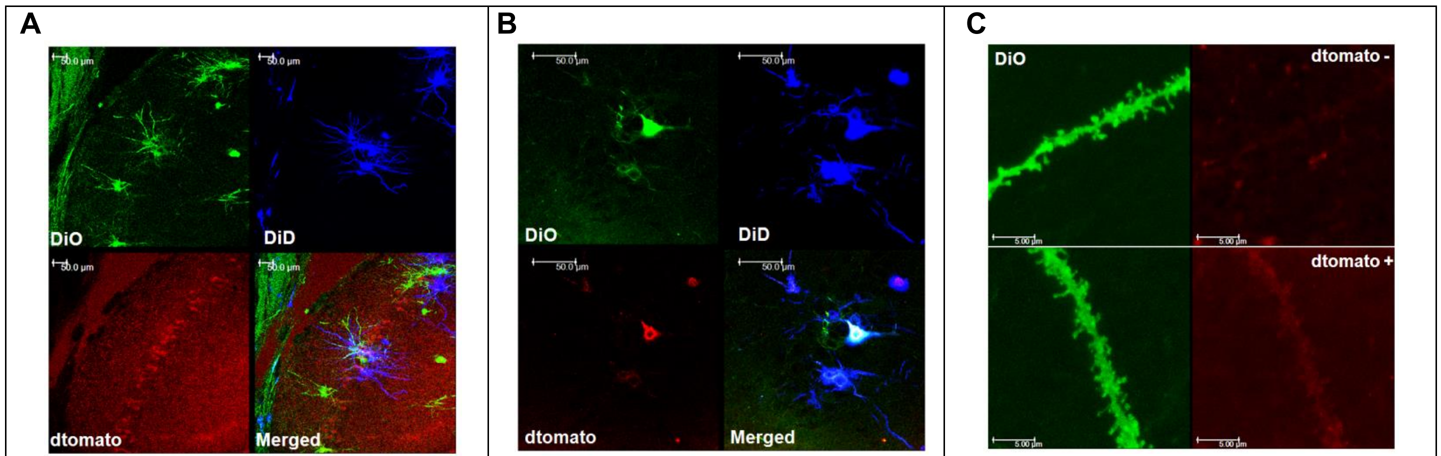

Figure 6. Diolistic label of dtomato+ recombinant and dtomato- wild type CA1 pyramidal neurons in TSC1CKO; dtomato+ mice. (A) low magnification images showing DiOlistically labeled dtomato+ and dtomato- neurons. DiO in green and DiD in blue. (B) high mag images showing DiO or DiD labeled dtomato+ neurons; (C) representative dendritic segments for dtomato- (upper) and dtomato+ (lower) CA1 pyramidal neurons in TSC1CKO mice.

mice,using DiOlistic labeling and patch clamping recording techniques. Our preliminary results in a very few samples (Figure 6) indicate that recombinant neurons expressing high levels of pS6 may exhibit higher dendritic spine density than dtomato negative neurons. We will confirm this in the 3rd year of the award. Both morphological and physiological properties of dtomato+ and dtomato- neurons will be compared between wt and TSC1^{mGFAPCre}CKO mice.

Given that all giant neurons are dtomato+ GFAPCre-recombinant, there is a positive correlation between neuronal size and mTOR activation in our mice. However, recombinant dentate granule cells remain a normal size at all times, and there is no detectable mossy fiber sprouting in the knockout mice, which has been reported to be correlated with epilepsy (Figure7). These data suggest that granule cells may have some other

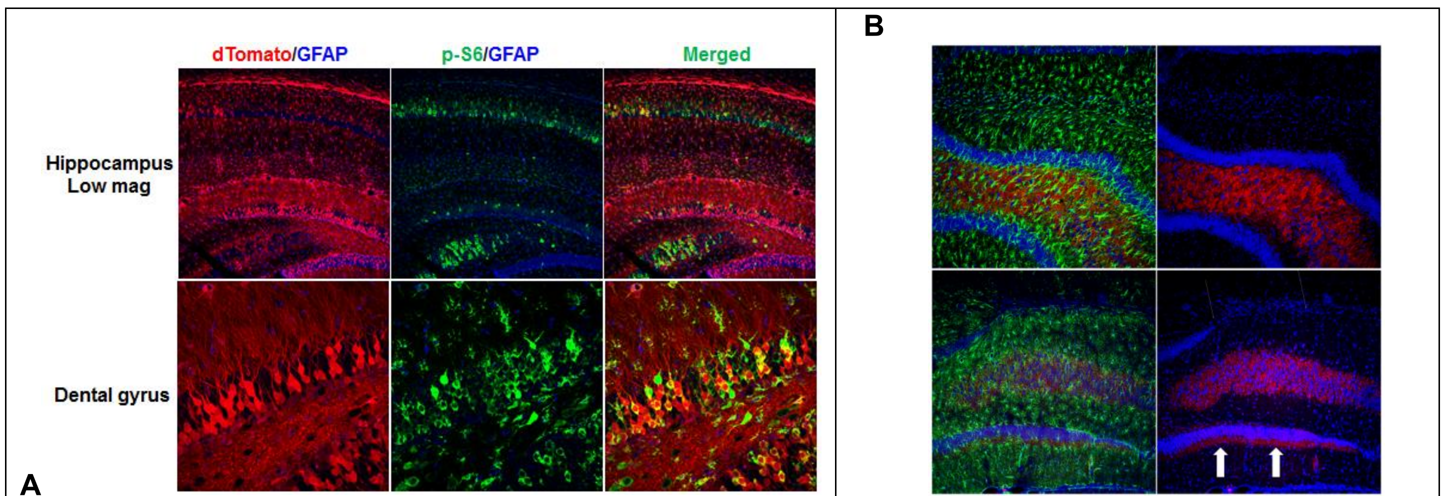

Figure 7. dtomato expression in dental gyral neurons in TSC1CKO mice. (A) recombinant dental gyrus granule cells (dtomato+, red). Note that only a small fraction of recombinant granule cells showed high level of pS6, and there is no difference in the soma size of dtomato+ and dtomato- neurons; (**B**) analysis of mossy fiber sprouting in TSC1CKO mice (upper), using an antibody against Zinc transporter 3 which is specifically expressed in axons of DG granular neuron. A pilocarpine induced seizure positive control brain (lower) showed mossy fiber sprouting in the inner part of molecular layer (thick arrows). TSC1CKO mice however did not display signs of axonal sprouting.

non-mTOR mechanisms to regulate cell size. It may be, however, that because of turnover of these neurons, that they do not have enough time to increase over their limited lifespan.

CONCLUSIONS:

The purpose of this study is to determine the specific role of astrocytes in epileptogenesis in tuberous sclerosis. We have used the mouse GFAP promoter driven cre mouse line, in which cre is reported to mainly express postnatally in astrocytes. We unexpectedly found recombinant neurons that are derived from GFAPcre expressing progenitors and are deficient for TSC1. Therefore, in our neuroglial TSC1 deficient mouse model, we are able to study the effect of neuronal intrinsic mTOR activation of synaptic activities (recombinant neurons), as well as the effects of Tsc1 deficient astrocytes on neuronal morphology and activities (non-recombinant neurons) that are associated with seizures.

The major findings we have during the first two years of this award include:

1) $TSC1^{mGFAPcre}$ CKO mice develop spontaneous seizures at the age of 2-3 months of age.
2) Tsc1 gene depletion results in astrocytic mTOR hyperactivation and reactive astrogliosis;
3) Postnatal deletion of *Tsc1* gene does not interfere with the overall capacity of astrocyte glutamate uptake and potassium buffering. However, synaptically invoked astrocyte glutamate uptake may be impaired due to the lack of TSC1 in astrocytes.
4) Astroglial TSC1 deletion leads to increased basal and evoked excitatory synaptic transmission during epileptogenesis;
5) Astroglial TSC1 deletion causes an increase in excitatory synapses prior to the occurrence of clinical seizures.

PLANS FOR YEAR 3 OF THE AWARD

During the third year of the award, we will assess the effect of astrocyte TSC deficiency on spine development in cortical neuronal co-cultures, using the Sulzer lab's established approaches. Cortical neurons will be co-cultured with wild type and TSC1CKO astrocytes. Dendrites and spine morphology will be assayed during maturation at 4, 7, 14 and 21 days in vitro (DIV) by immunostaining for the dendritic marker MAP2 and postsynaptic density protein PSD95 and imaged by confocal microscopy. Synaptic density and the number of functional synapses will be represented by the frequency of colocalization of the pre- and post-synaptic markers synaptophysin and PSD95. Inhibitory and excitatory synapse formation will be examined using antibodies against synaptophysin to label presynaptic terminals, vesicular glutamate transporter (VGluT) to distinguish excitatory terminals, vesicular GABA transporter (VGAT) for inhibitory terminals and the GABAAR-N2/N3 subunits to visualize postsynaptic receptors.

We will also assess in situ dendritic spines in sensory cortex layer V and hippocampal CA1 pyramidal neurons from wild type and TSC1 KO mice during the primary period of developmental pruning (postnatal day 21, 30 and 60) using DiOlistic labeling with a Helios Genegun system. Spine pruning will be represented by the difference in spine densities between P21 and P30 or P60, comparing wild type and TSC1 KO.

Over the past year, we successfully developed a low density neuronal culture technique for live cell imaging for dendritic spine analysis in vitro. Using this technique, we will be able to study the effect of dendritic spine dynamics in primary neurons cocultured with wild type and TSC1 deficient astrocytes. Figure 8 shows an example of live imaging of dendritic spine dynamics in neurons (A) and pre- and postsynaptic markers in dendrites (B).

To assess pathophysiological consequences of increased spine density and enhanced excitatory synaptic transmission in TSC1 KO, we will examine seizure susceptibility in vitro and in vivo (video-EEG). To directly compare glutamatergic and GABAergic synaptic transmission, excitatory postsynaptic currents (EPSCs) and inhibitory postsynaptic currents (IPSCs) will be recorded by alternately clamping at AMPA or GABAA receptor channel reversal potential.

We will initiate Aim 3 “Analyze tuber, peri-tuber and non-tuber tissue from TS and control human brain tissue for mTOR, astrocyte function and synaptic formation” by requesting biopsied and autopsied tuber, perituber and non-tuber brain tissue from TS patients and age-matched control cortex to be obtained from the Maryland Brain Bank. Both fixed and frozen tissue will be subjected to biomarkers for astrocyte function and excitatory and inhibitory synaptic transmission. Neuronal morphology (dendrites and spines) will be analyzed using modified Golgi method.

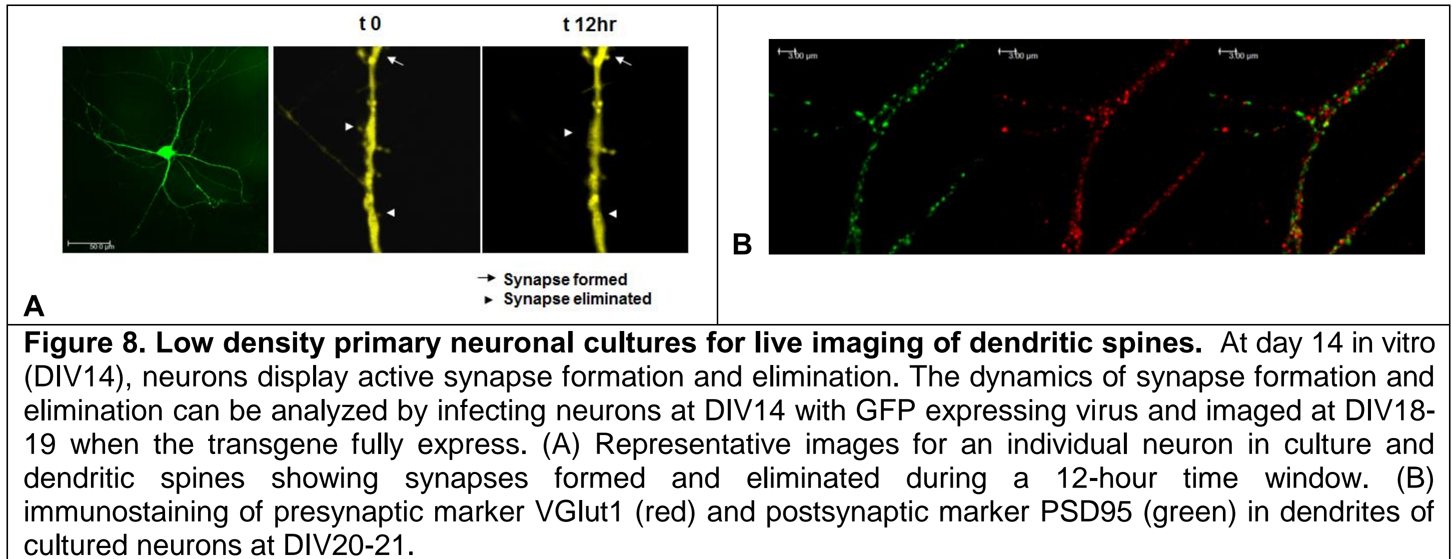

Figure 8. Low density primary neuronal cultures for live imaging of dendritic spines. At day 14 in vitro (DIV14), neurons display active synapse formation and elimination. The dynamics of synapse formation and elimination can be analyzed by infecting neurons at DIV14 with GFP expressing virus and imaged at DIV18-19 when the transgene fully express. (A) Representative images for an individual neuron in culture and dendritic spines showing synapses formed and eliminated during a 12-hour time window. (B) immunostaining of presynaptic marker VGlut1 (red) and postsynaptic marker PSD95 (green) in dendrites of cultured neurons at DIV20-21.

REPORTABLE OUTCOMES:

None