Establishment of Tsc1$^{flox/flox}$-GFAP-Cre (Tsc1^{GFAP}CKO) mice for evaluating potential new antiepileptic therapies for tuberous sclerosis complex

Steven C. Leiser[1], Afshin Ghavami[1], Mei Kwan[1], Jose Beltran[1], Dekun Song[1], David M. Devilbiss[1], Michael Wong[2], Nick Rensing[2], Steve L. Roberds[3], and Dani Brunner[3]

[1]PsychoGenics Inc, Paramus, NJ; [2]Washington Univ. Sch. of Med., St. Louis, MO; [3]Tuberous Sclerosis Alliance, Silver Spring, MD

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Abstract

In this two part study, we evaluated incidence of seizures using EEG and changes in mRNA biomarkers using quantitative PCR (qPCR) in the *Tsc1*^{GFAP}CKO mice. First, we evaluated effects of rapamycin on incidence of seizures and mortality given that previous reports have indicated these mice are prone to postnatal epilepsy and developmental delays rescued by treatment with the mTOR inhibitor. Mice were continuously recorded from P35-P49. The Vehicle-treated group (n=10) exhibited on week 5 and 6, an average of 3.5 ± 1.1 and 2.33 ± 1.1 seizures respectively. In contrast, the Rapamycin-treated group that started at P35 (n=8) exhibited a reduction in seizures for weeks 5 (1.75 ± 0.65) and 6 (0.38 ± 0.26) nearly reaching significance at week 6 (p=0.06, Tukey-Kramer). Moreover, the Rapamycin-treated group that started at P21 (n=10) showed no seizures during week 5 and 6 (p<0.05, Tukey-Kramer). In summary, whereas Tsc1 genotype mice treated with vehicle daily from PND_{21-48} suffered robust electrographic seizures and a significant mortality over this period, mice treated with 3 mg/kg rapamycin from PND_{21-48} showed zero seizures and zero mortality over the same period. A third group that received vehicle from PND_{21-35} and rapamycin 3 mg/kg from PND_{36-48} showed reduced number of seizures and zero mortality. Thus, we confirm the protective effects of the mTOR inhibitor rapamycin against seizures and premature death in these mice. Secondly, in a separate cohort, we used qPCR to evaluate changes in mRNA levels of markers involved in axon formation, synapse function, glutamate transport, mTOR activation, cell adhesion, angiogenesis, cell regulation, inflammation and unfolded protein response (UPR) activation in the brain at 5 weeks of age. Expression levels of mRNA between control (CRE$^{-/-}$; Tsc1$^{+/-}$) and CRE$^{+/-}$; Tsc1$^{+/-}$ mice were similar for all transcripts reported in this study. Lack of changes in transcript abundance in heterozygous mice might be explained by unchanged relative expression levels for the Tsc1 mRNA in the brain. In contrast, Tsc1^{GFAP}CKO mice (CRE$^{+/-}$; Tsc1$^{-/-}$) showed genotype-dependent changes in some of the transcripts analyzed. In some cases affected transcripts in homozygous mice showed significant variation among cohorts that cannot simply be explained by differential effectiveness of CRE-mediated knockout since, except one animal, all homozygous mice showed similarly reduced expression levels of Tsc1 mRNA. mRNA levels might covary with seizure frequency or age of seizure onset which needs to be assessed in future studies. Overall, our findings suggest a clear utility of using these mice to screen potential anti-epileptic therapeutics.

Methods

Tsc1^{GFAP}CKO mice were bred at PsychoGenics using breeding pairs obtained from Prof. Michael Wong's Laboratory (Washington University, St. Louis, MO).

For EEG study, *Tsc1*^{GFAP}CKO mice (Cre^{+}; *Tsc1*$^{flox/flox}$) mice were balanced among 4 treatment groups: (i) Vehicle, P21-P48, (ii) Rapamycin (3mg/kg, IP) P21-P48, (iii) Vehicle P21-P34, (iv) Rapamycin (3mg/kg, IP) P35-P48. EEG headmounts were implanted between P23-P27 and continuously recorded from P35-P49. EEG recordings were analyzed during week 5 and 6 for number of seizures as described by Dr. Michael Wong (i.e. Zhang et al., PLoS ONE 8(2): e57445, 2013).

For mRNA and phosphoprotein analysis, n=5 per genotype per gender of Tsc1 mouse model was used in this study.

RNA and cDNA were prepared from whole brain tissue and underwent qPCR using Taqman probe assays purchased from ThermoFisher Scientific. Each sample was assayed in triplicates for each qPCR assay and normalized to housekeeping gene calnexin and presented as relative to control mouse expression.

Brain protein lysates were prepared from separate cohort of mice that had undergone focused microwave irradiation to preserve phosphoproteins. Western blotting techniques with commercially available antibodies were used to assess protein levels. Target signals were normalized to that of reference protein.

Results

EEG

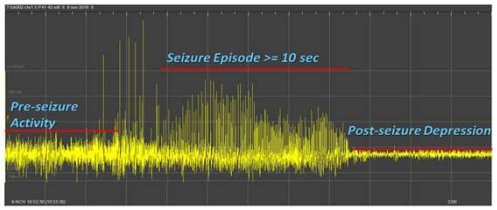

Figure 1: Stereotypical seizure in the frontal cortex of a Tsc1GFAPCKO mouse. Shown is a 2 minute segment of data (each vertical bar indicates 10sec). Electrographic seizures were identified by their characteristic pattern of discrete periods of rhythmic spike discharges that evolved in frequency and amplitude lasting at least 10 seconds, typically ending with repetitive burst discharges and voltage suppression. These were defined as having very stereotypical beginning, end, and evolution in the middle, starting with low amplitude fast activity and high frequency spikes (tonic), which gradually evolved into a slower, bursting (clonic) phase followed by severe voltage suppression; Most typical seizures last 30-40 seconds.

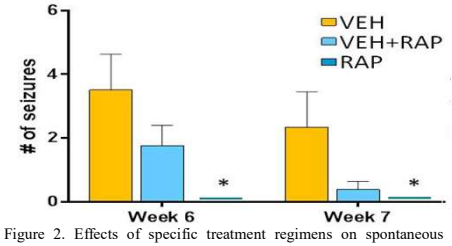

Figure 2. Effects of specific treatment regimens on spontaneous electrographic seizures (seizure count; left) and survival (right) in Tsc1 (mixed gender) mice. VEH group received vehicle from post-natal day P21-48; VEH+RAP group received vehicle from P21-34 and rapamycin (3 mg/kg/day) from P35-48; RAP group received rapamycin from P21-48. Data are presented as mean ± SEM (N=6-10/group) number of seizures per week (post-natal week 6 and 7). Significance (*p<0.05) was determined by Tukey-Kramer. There were no differences between males or females within treatment.

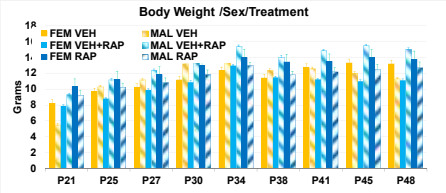

Figure 3. Effects of specific treatment regimens on body weight.

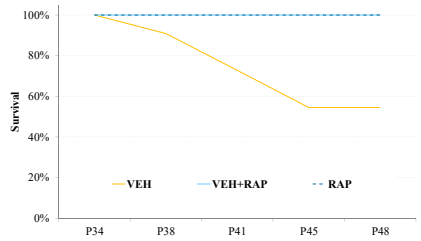

Figure 4: Nine out of 26 mice (~35%) that received vehicle treatment beginning day 21 perished by day 34 while no mortality was observed in the rapamycin treated groups (Chi square test; p=0.032). Survival rate was also examined across groups from the start of day 35 to day 48. By the end of week 7, VEH group sustained a mortality of 40% whereas 0% mortality was recorded in groups VEH+RAP and RAP (note overlapped lines; Chi square test; p=0.015).

Phosphoproteins

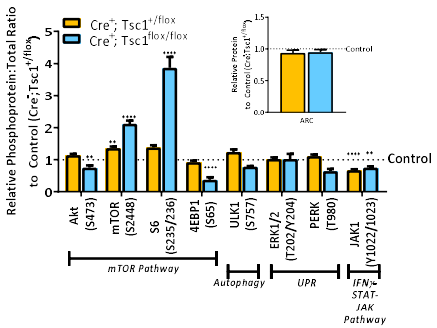

Figure 5: Ratio between phosphorylated and total proteins involved in autophagy, mTOR signaling, unfolded protein response (UPR) pathway, IFNγ-Jak-STAT signaling, and synaptic plasticity, in 7 weeks old control (Cre^{-}; *Tsc1*$^{+/flox}$), Cre^{+}; *Tsc1*$^{+/flox}$, and *Tsc1*^{GFAP} CKO (Cre^{+}; *Tsc1*$^{flox/flox}$) mice. One way ANOVA with Tukey's multiple comparisons test: **, p<0.01; ****, p<0.0001.

Transcripts

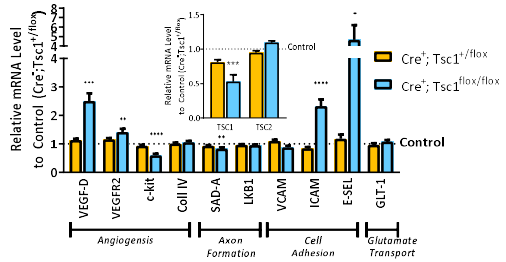

Figure 6. Expression profile of transcripts associated with angiogenesis, axon formation, cell adhesion, and glutamate transport, in brains of 7 weeks old control (Cre^{-};*Tsc1*$^{+/flox}$), Cre^{+};*Tsc1*$^{+/flox}$ and *Tsc1*^{GFAP} CKO (Cre^{+};*Tsc1*$^{flox/flox}$) mice. Inset shows *Tsc1* and *Tsc2* mRNA expression level in the same animals. Note that in whole-brain, deletion of both copies of *Tsc1* gene in astrocytes was required to observe a significant decrease in *Tsc1* mRNA expression. One way ANOVA with Tukey's multiple comparisons test: *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.

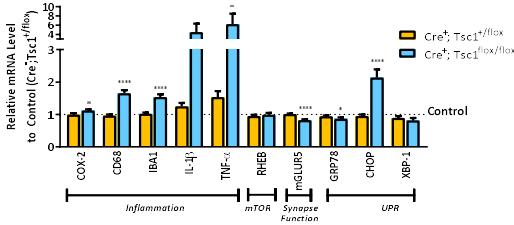

Figure 7. Transcript expression profile of markers associated with inflammation, mTOR activation and unfolded protein response activation in brains of 7 weeks old control (Cre^{-};*Tsc1*$^{+/flox}$), Cre^{+};*Tsc1*$^{+/flox}$ and *Tsc1*^{GFAP} CKO (Cre^{+};*Tsc1*$^{flox/flox}$) mice. One way ANOVA with Tukey's multiple comparisons test: *, p<0.05; ****, p<0.0001.

Summary

Whereas *Tsc1*^{GFAP}CKO mice treated with vehicle daily from P21-48 suffered robust electrographic seizures and a significant mortality, mice treated with 3 mg/kg rapamycin from P21-48 showed zero seizures and mortality over the same period. A third group that received vehicle from P21-35 and rapamycin 3 mg/kg from P36-48 showed reduced number of seizures and zero mortality. Thus, we confirmed the protective effects of the mTOR inhibitor rapamycin against seizures and premature death.

Dysregulation of markers associated with mTOR signaling, neuroinflammation, angiogenisis, cell adhesion have all been linked to Tuberous Sclerosis. Overall, our results show that transcripts and phosphoproteins linked to these pathways are also significantly affected in this mouse model. It would be interesting to evaluate whether mRNA and phosphoprotein levels might covary with seizure frequency or age of seizure onset.