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TITLE: The Contribution of Rapamycin-Insensitive Processes to Neurological Symptoms in TSC

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14. ABSTRACT

Tuberous sclerosis complex (TSC) is a neurocutaneous syndrome that is characterized by benign tumors in multiple organs and associated with neurological symptoms such as epilepsy, autism spectrum disorder (ASD), intellectual disability, and other neuropsychiatric disorders. Together, these neurological disorders have been termed TSC-associated neuropsychiatric disorders (TAND), and there is growing recognition of the impact of these symptoms on the lives of patients with TSC. TSC is caused by mutations in either *TSC1* or *TSC2*, and these proteins form a complex (TSC1/2) that functions as a critical inhibitor of the kinase, mammalian target of rapamycin (mTOR). mTOR is important signaling hub that regulates cell growth and proliferation, and it becomes inappropriately active in TSC, leading to many of the symptoms. The mTOR inhibitors rapamycin and everolimus (termed rapalogs) have revolutionized the treatment of many manifestations of TSC, but the neurological symptoms have been more recalcitrant. Everolimus was shown to be effective as an adjunct for refractory epilepsy in TSC, but there was an average 50% reduction in seizure frequency with most patients continuing to have several seizures per day. In addition, two trials of everolimus for behavioral and neuropsychiatric disorders associated with TSC have shown no effect. Therefore, we hypothesize that rapalog-insensitive effects contribute to the development and expression of the neurological symptoms associated with TSC.

15. SUBJECT TERMS
Tuberous sclerosis, autism, mTOR inhibitors, epilepsy

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1. INTRODUCTION

Tuberous sclerosis complex (TSC) is a neurocutaneous syndrome that is associated with neurological symptoms such as epilepsy and several neuropsychiatric disorders, collectively termed TSC-associated neuropsychiatric disorders (TAND). TSC is caused by mutations in either *TSC1* or *TSC2*, and the proteins encoded by these genes form a complex (TSC1/2) that functions as a critical inhibitor of the kinase, mechanistic target of rapamycin (mTOR). mTOR is important signaling hub that regulates cell growth and proliferation, and it becomes inappropriately active in TSC, leading to many of the symptoms. mTOR inhibitors, known as rapamycin analogs (rapalogs) such as everolimus, have become the mainstays of treatment of several manifestations of TSC. However, these compounds have been found to be incompletely effective for TSC-associated epilepsy and no benefit has been demonstrated for TAND at this point. Therefore, there is urgent need to better understand the pathogenesis of TSC-associated epilepsy and TAND and to develop better treatments for both disorders. We have found that there are genes whose expression or ribosomal binding is dysregulated in TSC-deficient neurons and that these changes are not completely reversed by treatment with rapamycin. Several of these genes have roles in synaptic formation and function, suggesting that rapamycin-insensitive processes in TSC-deficient neurons can contribute to alterations in neural circuits. The objective of this study is to identify if and when important molecular and physiological changes in TSC-deficient neurons can be prevented with rapamycin and characterize rapamycin-insensitive pathways in neurons and their contribution to abnormalities in TSC. In the future, these results may be used as a rationale for trials of early mTOR inhibition in at-risk individuals. In addition, the data generated by this research will add novel molecular pathways for study into the pathogenesis of neuronal abnormalities in TSC, which we hope will lead to novel treatments to improve the lives of individuals affected with this disease.

2. KEYWORDS

Human iPSC, TSC1/2, mTOR, disease phenotyping, autism

3. ACCOMPLISHMENTS

Aim 1: To characterize rapamycin-insensitive molecular pathways in human neurons with mutations in *TSC2*. We will use induced pluripotent stem cells (iPSCs) generated from a patient with TSC due to a mutation in *TSC2*, as well as lines that have been derived from the patient line and either have the mutation in *TSC2* corrected or a mutation in the second allele of *TSC2*. We will differentiate these cells into neurons, and we will treat the cells with either rapamycin or vehicle. We will then perform RNA sequencing and translating ribosome affinity profiling (TRAP) to understand the transcriptional and translational changes that are resistant to rapamycin treatment and may represent novel drug targets for neuronal dysfunction in TSC.

Aim 2: To determine the contribution of rapamycin-insensitive processes to neural circuit abnormalities in TSC. We will use the same iPSCs described above, and we will transduce these cells with vectors enabling fully optical stimulation and recording. We will differentiate these cells into neurons, and we will treat the cells with either rapamycin or vehicle. At various points during neuronal maturation, we will interrogate the network activity and connectivity to understand the contribution of rapamycin-insensitive processes to the development of abnormal neural networks.

Aim 3: To determine whether there are developmental windows during which rapamycin treatment can prevent the emergence of rapamycin-insensitive processes that affect development of human neuronal networks. We will use the same series of iPSCs described above, and we will generate forebrain organoids that recapitulate several important stages in brain development. We will treat these organoids with rapamycin or vehicle during various windows of differentiation and maturation. We will then assess the development of networks within organoids using calcium imaging, and we will evaluate dysregulation of molecular processes using single cell sequencing.

Studies and results

In our prior progress report, we had described the development of a second isogenic series of iPSCs derived from a patient with TSC (“24 line”). The patient had a heterozygous pathogenic missense variant in TSC2 (TSC2+/-), and the null cell line was engineered to have the same variant but be homozygous (TSC2-/-). The control line had a correction of the pathogenic variant and had two functional alleles of TSC2 (TSC2+/+). We transduced each cell line with a bidirectional lentivirus vector that encodes the human transcription factor Neurogenin-2 (NGN2) in one direction under a doxycycline promoter and the tetracycline responsive transactivator in the other under a constitutive promoter. Importantly, both directions also contained antibiotic resistance genes, allowing for selection in the induced and baseline states. We then differentiated these stem cells into neurons by inducing expression of NGN2. After 14 days of

differentiation, we fixed the neurons and performed immunocytochemistry for phosphorylated ribosomal protein S6 (pS6) and MAP2. We demonstrated that the TSC2-/- neurons showed increased pS6, in agreement with mTOR activation (Figure 1). In addition, we found that TSC2-/- neurons showed increased cell size, which consistent with previously published work (Figure 1).

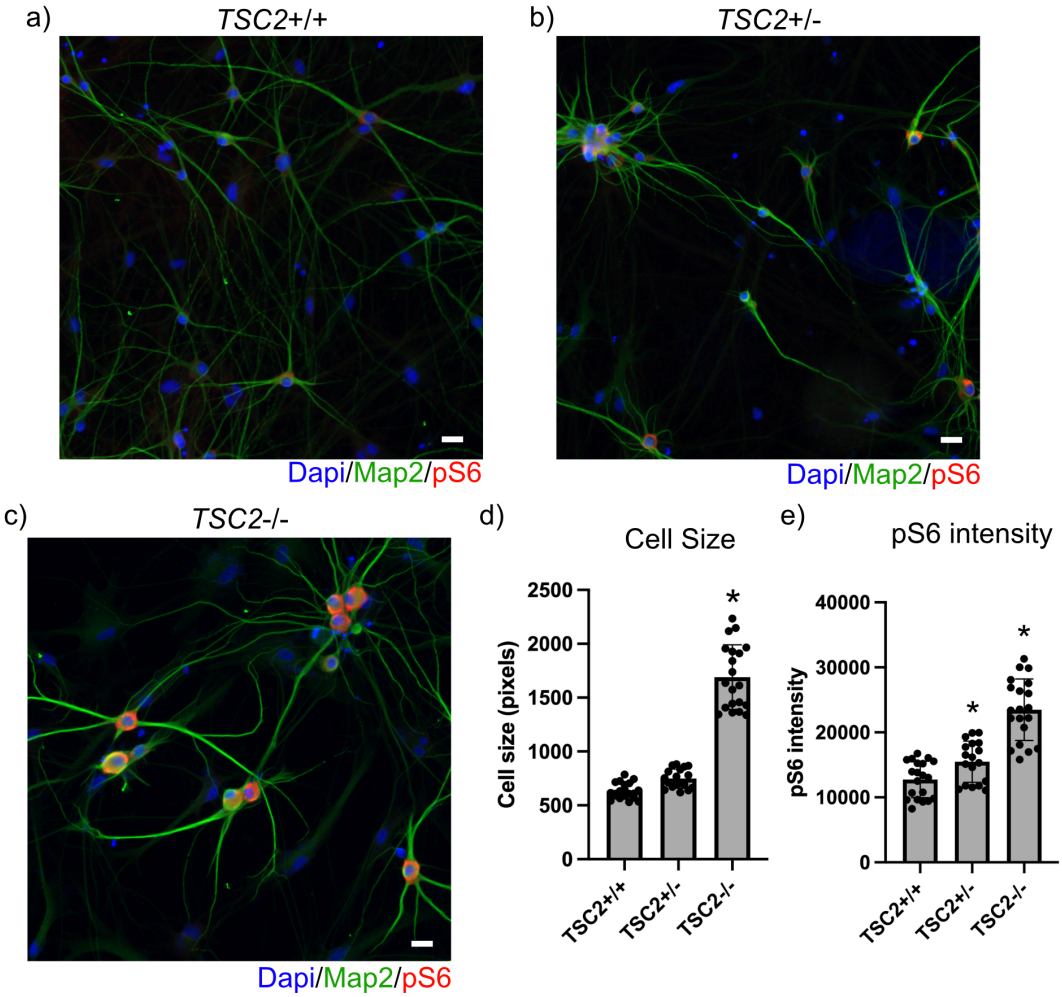

Figure 1: Isogenic series made from iPSCs from a patient with TSC show expected changes in mTOR activation and spontaneous activity. iPSCs from all genotypes from the 24 line were infected with the NGN2 expression vector and differentiated into cortical neurons. a) TSC2+/+, b) TSC2+/-, and c) TSC2-/- neurons were fixed at day 51 and immunostained for MAP2 and pS6. Manual quantification of d) size and e) pS6 expression from isolated neurons. N=20 wells per genotype, $p<0.05$, one-way ANOVA with Dunnett's multiple comparisons test.

We then investigated the electrophysiological phenotypes of these neurons using multielectrode arrays (MEA). We dissociated developing neurons of all three genotypes six days after differentiation, and we plated these neurons with wildtype astrocytes on the MEA. We then serially measured neuronal activity throughout differentiation, and we confirmed that the TSC2-/- neurons were spontaneously hyperactive (Figure 2). These data demonstrate that this second isogenic series of iPSCs recapitulates known TSC phenotypes.

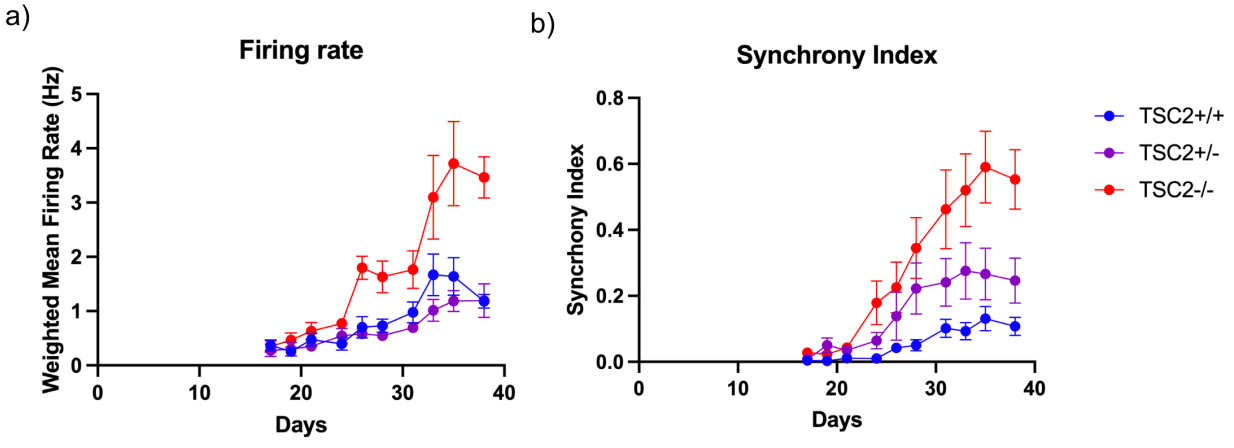

Figure 2: Confirmation of electrophysiological phenotypes in iPSC-derived neurons. a) Multielectrode array recordings for iPSC-derived neurons from the 24 line isogenic series. Representative recording of cortical neurons differentiated using induced NGN2 expression from all genotypes from the 24 isogenic series. The y-axis shows the mean firing rate corrected for the number of active electrodes, and the x-axis shows the day of differentiation. b) This plot shows the Synchrony index on the y-axis and the day of differentiation on the x-axis.

The next key step in this project was determining whether there was a time period during which the abnormal activity becomes rapamycin independent. We had previously demonstrated that starting rapamycin treatment at seven days after differentiation and continuing treatment through development was sufficient to prevent spontaneous hyperactivity in TSC2-/- iPSC-derived neurons (Winden et al., J Neurosci, 2019). Therefore, we performed MEA recording from all three TSC2 genotypes of iPSC-derived neurons. At 35 days after differentiation when network activity has typically plateaued, we treated neurons from all three genotypes with different doses of rapamycin. Strikingly, we did not observe any significant difference in neuronal activity after 24 or 48 hours of treatment for any dose of rapamycin (Figure 3). These data demonstrate that by day 35 of differentiation, neuronal activity in iPSC-derived neurons has become independent of rapamycin.

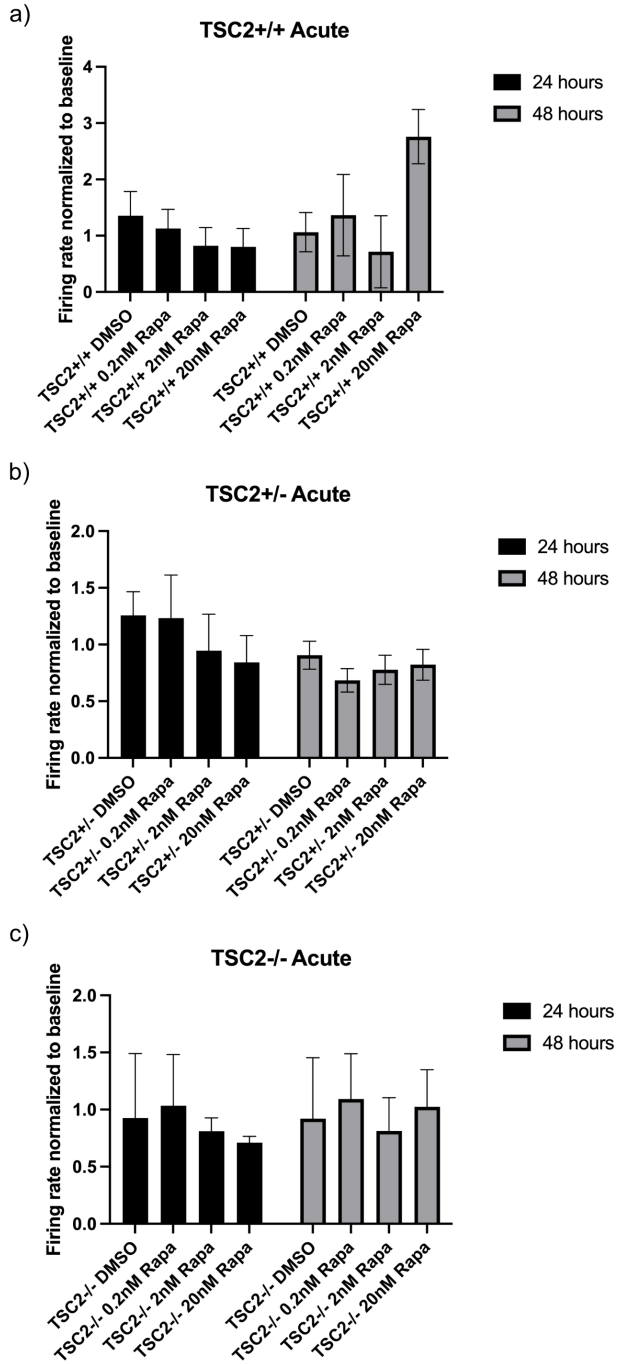

Figure 3: Acute treatment of iPSC-derived neurons with rapamycin. a) TSC2+/+, b) TSC2+/-, and c) TSC2-/- iPSC-derived neurons 35 days after differentiation were treated with the indicated doses of rapamycin for 24 or 48 hours. MEA recordings were performed at baseline prior to treatment and at 24 hours and 48 hours. The barplot shows the weighted mean firing rate for that treatment normalized to its baseline activity. There was no significant change in activity at either 24 or 48 hours of treatment, suggesting that spontaneous activity in iPSC-derived neurons at this timepoint is insensitive to rapamycin.

For aim 1, we had previously shown data optimizing methods for immunoprecipitation of ribosomes from iPSC-derived neurons and isolation of ribosomal bound mRNA. Based on the data above, we are setting up differentiations for all three genotypes of neurons, and we will perform a chronic treatment of rapamycin beginning seven days after differentiation and an acute treatment for 24 hours on day 34. We will plan to collect neurons for immunoprecipitation, RNA isolation, and RNA sequencing on day 35.

For aim 2, we are in the process of repeating the acute rapamycin treatment on MEA above. In addition, we are performing calcium imaging to analyze the effect of acute treatment on the activity of iPSC-derived neurons at a single cell level. Finally, to directly measure connectivity of the network, we have developed a novel lentiviral vector that contains both a channelrhodopsin (CheRiff) and calcium indicator (jRCaMP1b), but the two genes are oriented in opposite directions so that only one gene can be expressed at a given time. The control of which gene is expressed is mediated by Cre recombination, whereby unrecombined cells express only jRCaMP1b, and cells after recombination express only CheRiff. We are currently troubleshooting use of this vector, but we expect this tool to be capable of significantly aiding in these experiments.

For aim 3, we have not yet begun treating organoids with rapamycin, but we have other projects focused on creating organoids from *TSC2* iPSCs. Therefore, we are confident that we can start differentiation and treatment of cerebral organoids within the next year for this project.

What opportunities for training and professional development has the project provided?

There are two postdoctoral fellows and one research assistant working on this project and being mentored by Dr. Sahin. The trainees have all presented their work at the Sahin lab meetings. As results start to accumulate, we plan to present the results at national and international meetings.

How were the results disseminated to communities of interest?

Not disseminated yet.

What do you plan to do during the next reporting period to accomplish the goals?

As discussed above, we have optimized several techniques and identified optimal time windows for experiments. Therefore, we feel that we are in good position to make significant progress on the original aims of the proposal over the next year.

4. IMPACT:

What was the impact on other disciplines?
Nothing to Report.

What was the impact on technology transfer?
Nothing to Report.

What was the impact on society beyond science and technology?
Nothing to Report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change
Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to report.

Changes that had a significant impact on expenditures
Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report.

6. PRODUCTS:

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
Provide the following information on participants:

- what individuals have worked on the project?
- has there been a change in the other active support of the PD/PI(s) or senior/key personnel since the last reporting period?
- what other organizations have been involved as partners?

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort).
• Provide the name and identify the role the person played in the project. Indicate the nearest whole person month (Calendar, Academic, Summer) that the individual worked on the project. Show the most senior role in which the person worked on the project for any significant length of time. For example, if an undergraduate student graduated, entered graduate school, and continued to work on the project, show that person as a graduate student, preferably explaining the change in involvement.
Describe how this person contributed to the project and with what funding support. If information is unchanged from a previous submission, provide the name only and indicate "no change".

Personnel	Role	Cal months	
M. Sahin	PD/PI	0.6 cal	No change
Kellen Winden	Research fellow	1.2 cal	No change
Wardiya Afshar	Research fellow	9.6 cal	No change
Truc Pham	Research Assistant	4 cal	No change

Ryan Chen who worked on this project as a Northeastern University coop student has finished his coop program and returned to his undergraduate classes.

Truc Pham who was a research assistant in the lab, has been accepted to the MD-PhD training program at Washington University School of Medicine, St Louis and has left the lab. She will be replaced by another research assistant.

8. SPECIAL REPORTING REQUIREMENTS: None

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.