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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, epilepsy

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 to date

For aim 1, we have first begun by performing two important foundational studies. The first is the generation and characterization of a second isogenic allelic series of iPSCs with mutations in *TSC2*. Growing evidence demonstrates that cell line background is an important source of confounding in experiment using iPSCs, and therefore, we felt it necessary to have a second isogenic series of *TSC2* iPSCs (Anderson et al., Stem Cell Reports, 2021). iPSCs were first derived from a patient with a point mutation in *TSC2* using standard methods of generation and quality control. CRISPR editing was then used to either correct the patient mutation, leading to a control cell line with two functional copies of *TSC2*, or induce the patient mutation in the second *TSC2* allele, resulting in a cell line that had no functional copies of *TSC2*. To characterize these cells, we first wanted to evaluate mTOR activity in neurons derived from these cell lines. We infected

these cells with a vector that contains the transcription factor Neurogenin-2 (NGN2) under a tetracycline inducible promoter, and after selection, we activated NGN2 using doxycycline and differentiated cells from each of these lines into cortical neurons. These cells were dissociated at day 6 of differentiation and re-plated onto plates specific for high content imaging. After several weeks of differentiation, we fixed and stained these cells for the neuronal marker MAP2, as well as phosphorylated-ribosomal protein S6 (phospho-S6), which is a known measure of mTOR activity. We observed a clear increase in cell size and phospho-S6 levels in the *TSC2*-/- neurons as expected (Figure 1), in agreement with our prior work (Winden et al., J Neurosci, 2019).

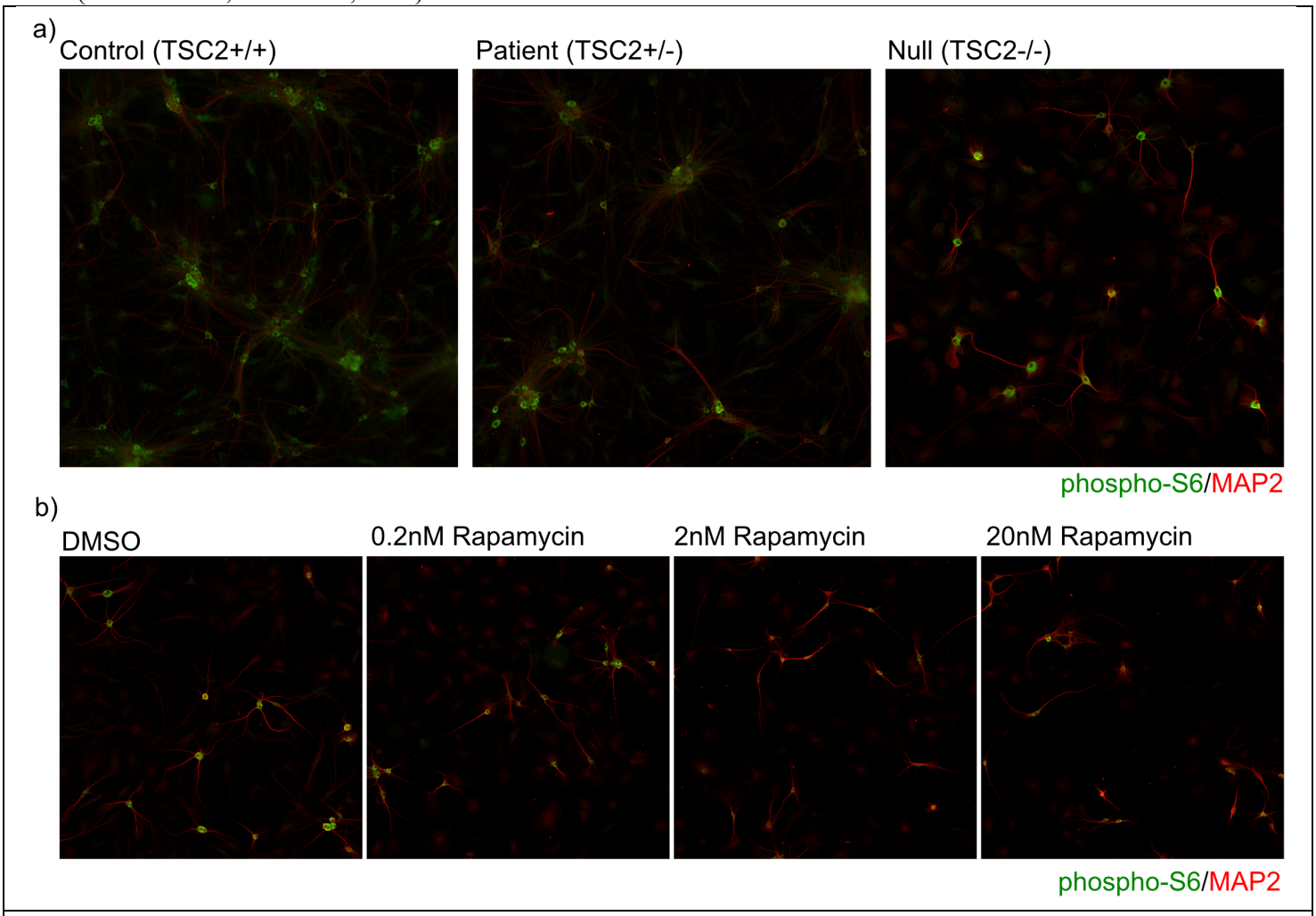

Figure 1: Characterization of a second isogenic series of TSC2-deficient iPSCs and their response to rapamycin. a) Immunocytochemistry of MAP2 and phosphorylated-ribosomal protein S6 (phospho-S6) in an allelic series of iPSC-derived neurons. There is a clear increase in size and intensity of phospho-S6 staining in the *TSC2*-/- neurons compared to either the *TSC2*+/+ or *TSC2*+/- neurons, consistent with known effects of mTOR hyperactivation due to loss of function of TSC2. b) Treatment of *TSC2*-/- neurons with rapamycin from day 6 of differentiation until fixation. There is a noticeable reduction of the intensity of phospho-S6 staining with rapamycin treatment at 0.2nM. There is further reduction of phospho-S6 staining intensity with 2nM and 20nM, although both of these conditions were fairly indistinguishable from each other.

The second key finding was examining the efficacy and toxicity of iPSC-derived neurons in response to rapamycin treatment. We evaluated three orders of magnitude of rapamycin dosing with the highest dose being 20nM, which we have previously demonstrated to sufficient to inhibit mTOR signaling. We observed that even at 0.2nM there appeared to be an effect on mTOR activity as measured by phospho-S6 and that 2nM and 20nM were not evidently distinguishable in regard to phospho-S6 levels (Figure 1).

We then endeavored to develop an assay to examine transcription and translation in iPSC-derived neurons. We developed a lentiviral vector that encodes a fusion protein of the constitutive ribosomal protein, RPL10A, and the tag enzyme HALO, which is able to covalently attach functionalized ligands to itself. We induced neuronal differentiation using doxycycline as above, and we then infected these neurons with the RPL10A-HALO virus at day 7. We then dissociated the iPSC-derived neurons and re-plated them with rat neurons 2-3 days later. After 21-24 days of differentiation of the human neurons, we performed translating ribosome affinity purification (TRAP) to isolate ribosomal-bound transcripts specifically within the human neurons. After isolation of purified mRNAs, we performed

quantitative PCR for the human and rat versions of different abundant transcripts. We observed that for two of three mRNAs tested there was significant enrichment of the human version of the transcript in ribosomal-bound fraction, consistent with our ability to specifically isolate transcripts bound to ribosomes. This was an important proof-of-principle experiment, although we did observe amplification of rat transcripts from the ribosomal-bound fraction, demonstrating that the pull-down of ribosomes was not completely pure. Given these data, we have continued to optimize these methods.

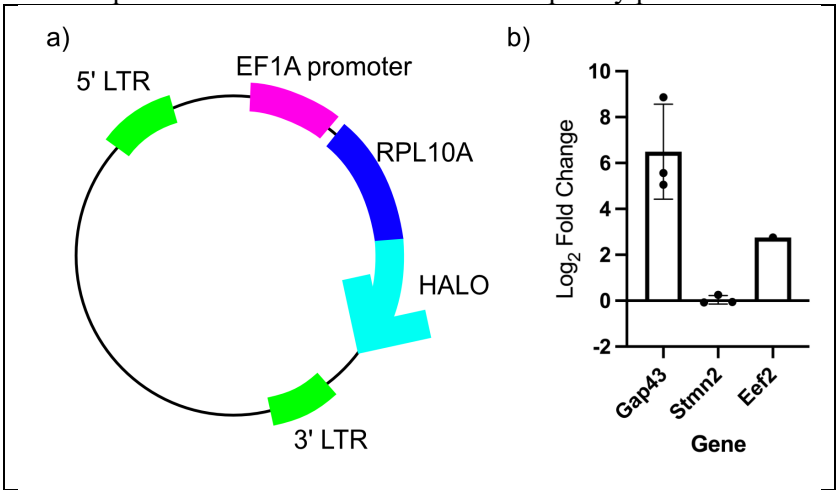

Figure 2: Isolation and analysis of ribosomes from iPSC-derived neurons. a) Map of the transfer plasmid for a lentiviral vector that we created, which expresses the constitutive ribosome protein fused to the HALO tag, which is an enzyme that is capable of covalently linking certain functionalized ligands to itself. The LTRs show the boundaries of the sequence that is integrated into the genome when infecting with a lentivirus. We infected iPSC-derived neurons at day 6 of differentiation and then mixed them with primary rat neurons. We then performed ribosomal purification using magnetic beads that are covered with HALO ligand. b) Quantitative PCR of certain high abundance neuronal targets comparing the mRNA isolated using TRAP to the overall mRNA using human- and rat-specific primers. For two out of three genes, we observed substantial enrichment of the human transcript, although we did observe amplification of rat-specific transcripts from the mRNA isolated from tagged ribosomes. These data suggest that the pull-down of ribosomal-bound mRNAs is still not completely clean and requires further optimization.

For aim 2, we have begun to examine the effect of rapamycin treatment on neuronal activity and connectivity using calcium imaging. We have differentiated control iPSCs into neurons using doxycycline treatment and then started treatment with either rapamycin at various doses (0.2nM, 2nM, or 20nM) or vehicle (DMSO) at day of differentiation 6. We allowed these neurons to mature and then infected them with a lentivirus that expresses the calcium indicator GCaMP6s under a human synapsin-1 promoter. We then examined spontaneous neuronal activity using live cell microscopy using standard methods (Afshar-Saber et al., Front Neurosci, 2018). We observed that rapamycin did not appear to significantly reduce the overall firing rate but may have decreased the connectivity within the network. We are currently in the process of redoing this experiment with all three *TSC2* genotypes.

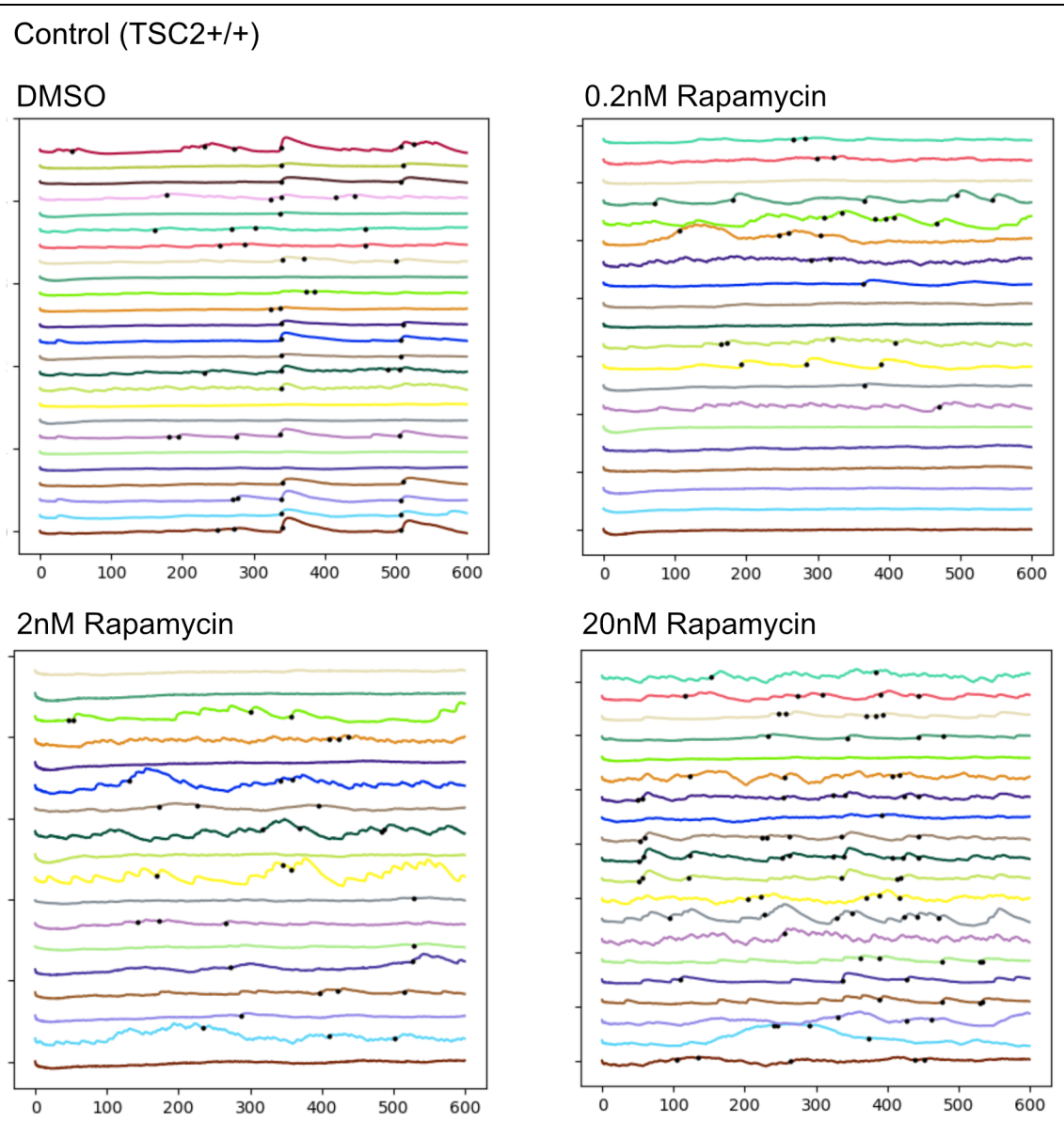

Figure 3: Calcium imaging in iPSC-derived neurons and the effect of rapamycin. Control (*TSC2*+/+) iPSCs were differentiated into neurons using doxycycline and re-plated for imaging. Neurons were infected with GCaMP6s under the human synapsin-1 promoter using lentivirus on day of differentiation 16 and then rapamycin at the indicated concentrations or DMSO was started on day 18. Calcium imaging was performed on day 44 using a frame rate on 10Hz. The images were then transformed into a 2D image using the maximal intensity projection and a convolutional neural network was used to automatically identify cell bodies. The fluorescence intensity was calculated for each cell, and the raw data were background subtracted and the ΔF/F was calculated, which is displayed on the plots above. The y-axis is scaled based on the maximum amplitude across all traces, and the x-axis represents the frame that corresponds to 10ms. Each trace shows an individual cell that is coded by a unique color. An automated algorithm was developed to identify calcium spikes, and the spike detections are denoted by black dots on the traces.

For aim 3, we have not yet begun treating organoids with rapamycin, but we have other projects focused on creating organoids from *TSC2* iPSCs ongoing in the lab. 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?

For Aim 1: We will differentiate our iPSC allelic series into neurons, and we will treat the cells with either rapamycin or vehicle. We will optimize our TRAP strategy as detailed above. 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.

For Aim 2: We will compare control neurons to TSC-deficient neurons in terms of network activity in cell culture. We will use the same iPSCs described above, and we will transduce these cells with vectors enabling fully optical stimulation and recording, as shown Figure 3. 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.

For Aim 3: We will start working on Aim 3 during year 2.

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:

Nothing to Report.

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".

Only change in participants is that our research assistant Lahin Lahani applied for Neuroscience Ph programs last year and was accepted at UCSF where she is now a first year graduate student.

Lahin was replaced by Truc Pham, who has been trained in all the cellular experiments proposed in this application. She is working 4/2 calendar months on this project. We also had an undergraduate student Ryan Chen from Northeastern University do his 'co-op' program working in our lab on this project (5.5 calendar months).

Current personnel report:

Personnel	Role	Cal months
M. Sahin	PD/PI	0.6 cal
Kellen Wilden	Research fellow	1.2 cal
Wardiya Afshar	Research fellow	9.6 cal
Truc Pham	Research Assistant	4.2 cal
Ryan Chen	Coop Student	5.5 cal

8. SPECIAL REPORTING REQUIREMENTS: None

9. APPENDICES:

n/a