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TITLE: Investigative Studies into mTORC1-Dependent Dendritic Branch Potentiation in TSC

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CONTRACTING ORGANIZATION: Wake Forest University Health Sciences
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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Many patients with TSC have long-term memory impairment. Yet, the underlying mechanism leading to cognitive deficits is not well understood. Memories are stored at clusters of synapses, within a dendritic branch, and requires the protein composition of that branch to be remodeled to strengthen communication. In the proposed studies, we will specifically test the ***hypothesis*** that in preclinical models of TSC branch potentiation is reduced. Indeed, calcium imaging of neurons isolated from WT, TSC1 KO, and TSC1 heterozyotic (Het) mice reveals a dose-dependent loss of branch potentiation. We are investigating the underlying molecular mechanism leading to deficits in branch-specific potentiation in TSC. We found that several mRNAs that code for ion channels are predicted to be regulated by the microRNA miR-129-5p. These ion channels are overexpressed and mislocalized in TSC null dendrites and include Kv1.1, CaV2.2, and α2δ1. Unexpectedly, blocking N-type calcium channels (CaV2.2) with its specific antagonist ω-conotoxin restores branch-specific potentiation in TSC1 null neurons. In addition, we have further characterized the expression of Kv1.1 in TSC1 null neurons. Together these studies are the **first** to show that TSC neurons lack branch-specific potentiation and have aberrant intrinsic properties, suggesting that TSC may be considered a channelopathy. Over the next year we will further characterize the heterozygotic condition that segregates into two populations – one similar to WT and one similar to KO. **Note**: We have continued to feel the effects of Covid 19 on the supply chain.

15. SUBJECT TERMS

TSC Associated Neuropsychiatric Disorder (TAND); Kv1.1; CaV2.2; N-type voltage-gated calcium channels (N-VGCC); branch specific potentiation, dendrite, RNA, microRNAs.

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1. **INTRODUCTION:**

TSC-associated neuropsychiatric disorders (TAND) have been reported to be the most difficult and life altering challenge for adults, children, families, and caretakers. Lifetime prevalence of TAND is high, on the order of 90%. TAND includes, but is not limited to impaired cognition, developmental delays, autism spectrum disorder (ASD), attention deficit disorder (ADD), anxiety, and depression [2]. The heterogeneous nature of TAND among patients (i.e. cognitive deficits and anxiety but not ADD) makes it difficult to treat. Herein, we leverage the robust literature and our own work regarding the molecular basis of learning and memory to address two CDMRP high priority areas: (1) Gaining a deeper knowledge of TSC signaling pathways and the cellular consequences of TSC deficiency and (2) understanding phenotypic heterogeneity in Tuberous Sclerosis Complex (TSC). The proposed studies will provide the first example describing an underlying **molecular mechanism** for branch potentiation, a cellular model for memory that we hypothesize is disrupted in both TSC1 knockout mice. Further, this mechanism displays heterogeneity within the clinically relevant heterozygous condition which, unexpectedly, results in low activity of mammalian target of rapamycin complex 1 (mTORC1) at the synapse. Importantly, **these studies are innovative as our preliminary data challenges the current dogma that overactive synaptic mTORC1 is at the core of cognitive problems in TSC.**

2. **KEYWORDS:**

TSC-associated neuropsychiatric disorders (TAND), calcium imaging, protein synthesis, ion channels, dendrites, branch-specific potentiation, cellular models of learning and memory, RNA-binding proteins, HuD, Kv1.1, N-type calcium channels, miR-129-5p

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

STATEMENT OF WORK as of June 01, 2022
START DATE June 01, 2021

Site 1:	Wake Forest University Health Sciences (WFUHS) Medical Center Blvd. Winston-Salem, NC 27157 PI: Kimberly Raab-Graham, PhD

○

Research-Specific Tasks:

Specific Aim 1: Specific Aim 1: Test the hypothesis that Kv1.1 expression is branch-specific in TSC deficient neurons.		**% complete**
Major Task 1: Establish *Kv1.1, HuD, and mTORC1 activity across genotypes*		
Establish Animal Protocol	1-3	100%
Subtask 1: Isolate Synaptoneurosomes from *Tsc1*^{tm1Djk}/J CRE rAAV under a synapsin promoter will be stereotaxically injected bilaterally in the hippocampus to generate TSC1-null hippocampal neurons, as we previously reported. Notably, tdTomato or GFP rAAV have been and will be injected to generate a control/wild-type (WT) group. We will also use *tsc1*$^{+/-}$ (HET), *tsc1*$^{-/-}$ (KO), *tsc2*$^{+/-}$ (HET), and *tsc2*$^{-/-}$ (KO) mice generated by crossing *tsc1 or 2* - floxed animals with mice that express Cre-recombinase driven by a CaMKIIα promoter. 6-8 animals/sex = 12 animals per genotype or injection will be required to obtain a power=0.8 [Piface application].	3-15	100%
Subtask 2: Establish cultured neurons: To maintain cultures we will prepare cultured neurons on a weekly basis from C57BL6 and/or *tsc1*$^{fl/fl}$, tsc1$^{fl/cre}$ P0-2 mice. Thus, we will require 6 breeding pairs per genotype to ensure 1 litter/week for 52 weeks/year for the duration of the award (3 years). This breeding scheme allows us to rotate in new breeders as needed. Note: We were unable to recover our TSC2 breeders after the pandemic. We have fully characterized both an AAV mediated TSC1 KO and a genetic TSC1 KO.	3-36	90%
Subtask 3: Cultured neurons immunofluorescence.	6-18	85%
Subtask 4: Voltage/*Calcium* (alternative) Imaging. We added electrophysiology to this task.	9-24	80%
Subtask 5: Protein Synthesis Assays	24-36	60%
Subtask 6: TORCAR detection	24-30	25%
Subtask 7: Knockdown of HuD	18-36	50%
Milestone #1: Co-author manuscript on branch specific expression of Kv1.1 in TSC null neurons	24-36	60%
Major Task 2: Test the hypothesis that TSC deficient neurons lack branch-selective potentiation.		
Subtask 1: ChR2 stimulation of hippocampal neurons (During troubleshooting of ChR2 expression we have stimulated with high Kcl before we invest in expensive caged glutamate (subtask 3)).	6-18	75%
Subtask 2: Knockdown of HuD and Kv1.1; We have determined that CaV2.2 expression disrupts branch potentiation. Therefore, we are using CaV2.2 blockers as an alternative to this subtask.	12-30	10%
Subtask 3 (alternative) caged glutamate stimulation of hippocampal neurons. Our depolarization method of KCl has been very informative in the aftermath of the pandemic and slow supply chain. We are completing our studies using this method.	18-36	90%
Milestone #2: Co-author manuscript on branch potentiation in TSC	30-36	60%
Milestone #3: Co-author a Review on Ion channels in TSC *(What we did during "stay at home" order due to Covid-19*	30-36	90%

What was accomplished under these goals?

1) Major Activities: The major activities of third year of funding included:
(1) Re-establishing preclinical TSC mouse colonies (due to COVID-19), (2) confirming the loss of branch-selective potentiation in TSC1 deficient neurons and gaining insight into the mechanism which based on last year's findings included a complete characterization of CaV1.2 and Kv1.1 in TSC1 null neurons.

2) Specific Objectives:

Specific Aim 1: Through state of the art and novel assays, we will test the hypothesis that Kv1.1 expression is branch-specific in TSC deficient neurons.

Specific Aim 2: We will test the hypothesis that TSC deficient neurons lack branch-selective potentiation.

3) Significant Results:

TSC mouse colonies: Over the two past years we have been building up our colonies that we were forced to reduce to breeding only during the "stay at home" order in North Carolina mandated during the pandemic. Unfortunately, we lost the TSC2 fl/fl colony and the Channel rhodopsin (ChR)-TSC1 mice.

Due to the slow supply chain and the complications with mouse models, we have pushed on by utilizing our alternative strategy of 10-30 mM potassium to depolarize neurons to measure branch-specific potentiation (see results below). In addition, we have characterized the TSC1 null neurons by electrophysiology to verify our results to add an extra layer of rigor to our studies since we had to go with our alternative strategy. Collectively, through these experimental strategies, we have found that many ion channel's expression and function, including Kv1.1, CaV1.2, CaV2.2, a2d1, a2d2, and CaV2.1, are disrupted in TSC.

Rationale for proposed studies:
Dendritic branch-selective potentiation is considered a mechanism for information storage in the brain (Losonczy et al., 2008). Dendritic expression of a potassium channel serves as a "shock absorber" thus titrating the impact of excitatory synaptic input to potentiate dendritic branches in a site-specific manner. Our previous findings suggest that mTORC1 activity represses the local translation of the voltage-gated potassium channel Kv1.1 in neuronal dendrites under normal conditions. Such a mechanism would facilitate an increase in synaptic efficacy and learning and memory. Unexpected, at synapses isolated from the hippocampus of TSC1 KO and TSC2 het mice, preclinical models of TSC, we found that Kv1.1 protein levels were elevated compared to WT mice (Egido-Betancourt and Taylor et al., *in preparation*). This finding motivated new questions: (1) How is Kv1.1 distributed throughout TSC-deficient neurons (TSC1 and 2 KO and TSC 1 and 2 hets) relative to WT neurons? (2) What is the mechanism that promotes the increase in Kv1.1 expression at synapses? (3) How does elevated Kv1.1 levels affect site-specific branch potentiation in TSC (Specific Aim 2)?

Overview: Over the last three years we have made several exciting discoveries regarding branch potentiation in preclinical models of TSC. We hypothesized that in the TSC deficient neurons there would be a **reduction** in branch-specific potentiation. We measure branch-specific potentiation by loading cultured hippocampal neurons with a membrane permeable calcium indicator and stimulating the neuron with high potassium (10-30 mM). First, we predict that

TSC1 deficient neurons will be hyperexcitable, as indicated by an increase in calcium signal (ΔF/F: ΔF=calcium signal after the addition of high K – calcium signal before the addition of high K; F= calcium signal before high K). Indeed, we found there is an increase in ΔF/F in dendrites in TSC KO neurons relative to wildtype (WT).

Next, we assessed branch-specific calcium signals. We calculated the branch variability index (BVI). Due to the cognitive problems reported in patients with TSC, we predicted that branch specific potentiation is reduced in TSC deficient neurons, as indicated by a lower BVI relative to WT neurons. Indeed, we reported the first year that BVI is reduced in a dose-dependent manner (WT>Het>KO). We have repeated these findings focusing on the difference between WT and KO Tsc1 neurons (Specific Aim 2) to delineate the molecular mechanism (Specific Aim 1).

We initially hypothesized that the loss of BVI in TSC deficient neurons is due to an increase in the voltage-gated potassium channel, Kv1.1 expression throughout the dendrites. However, the BVI remains the same between WT and KO neurons. These data together, suggest that Kv1.1 alone is not the cause of reduced BVI in KO neurons.

To identify the ion channel/proteins that reduce branch-specific potentiation in TSC null neurons we reasoned that the expression of another ion channel that opposes the increased expression/activity of Kv1.1 may be dysregulated in TSC null neurons. Last year we reported that blocking the N-type voltage-gated calcium channel (N-VGCC) with its specific antagonist restored branch specific potentiation. This year we have repeated these studies tripling our independent cultures and have indeed verified that (1) CaV2.2 is overexpressed in TSC1 null neurons, that it is mislocalized in dendrites in TSC1 null neurons, and that blocking CaV2.2 with its specific inhibitor restores branch specific potentiation.

Interestingly, N-VGCCs are typically presynaptic under neurotypical conditions. Last year we showed by immunostaining that CaV2.2, the protein that codes for N-VGCC, is mislocalized to the dendrites. This year we confirmed these results by calcium imaging. We depolarized both WT and TSC1 null neurons loaded with a calcium indicator with KCl and recorded the calcium signal at 10 and 50 um from the soma in the presence of blockers for L- and P/Q- calcium channels (Fig. 1; only 10 um shown). Notably, adding the N-channel blocker completely blocks the calcium signal both at 10 um and 50um (data not shown). These data demonstrate that N-VGCC is indeed functional in the dendrites.

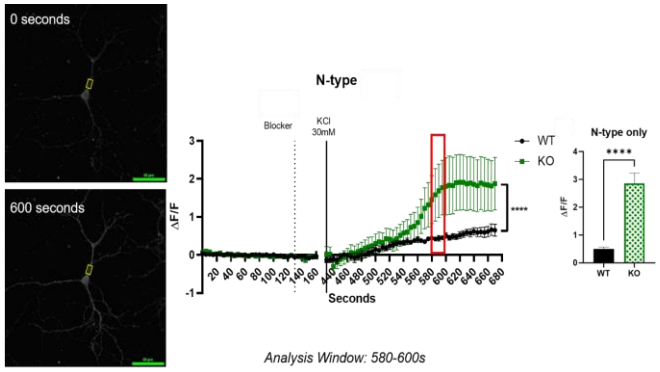

Figure 1. **Live calcium imaging of TSC1 null hippocampal neurons isolating N-VGCC activity.** Neurons imaged in the presence of nimodipine (10 µm; L-VGCC blocker) and Agatoxin (50 µm; P/Q-VGCC blocker). Neurons are imaged on the left, with the yellow box indicating the region of interest where the signal was detected. Middle graph reports ΔF/F overtime, green is KO and black is WT; red box is window quantified for bar graph on the right. Note, in agreement with previous immunostaining and Western blot results, N-VGCC dendritic activity is significantly higher than WT. Washing on conotoxin (50 nM) blocks N-VGCC mediated calcium activity in dendrites (data not shown).

To further characterize Kv1.1 we recorded the intrinsic properties of the TSC null neurons. We found that the resting membrane potential was significantly hyperpolarized and the minimum amount of current required to reach the action potential threshold (rheobase) was significantly increased in TSC1 null neurons relative to WT neurons, with no change in threshold or input

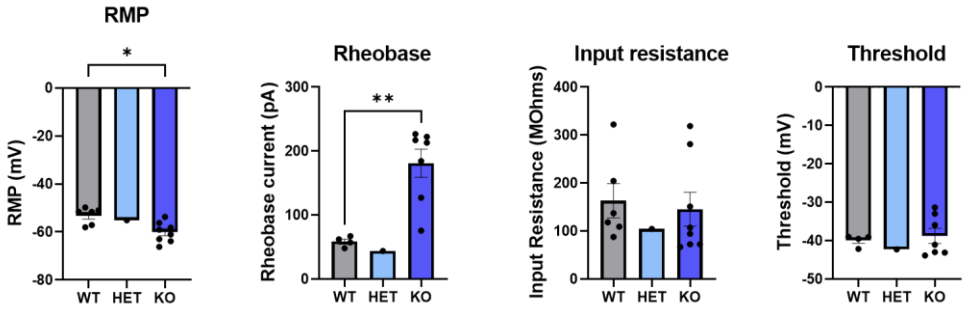

Figure 2. **Intrinsic properties of TSC1 null hippocampal neurons.** Basic intrinsic properties were recorded from hippocampal TSC1 null neurons and WT neurons. Passive properties were recorded without channel blockers. Ramp protocol was used to measure active properties. Current was injected continuously with increasing amount of current (0 to 300 pA) at a steady rate over 600 ms, a 30 s inter-trial interval, and repeated for 5 trials. Action Potential properties from the first action potential on each ramp trial was used for threshold and rheobase.

resistance (Fig. 2). Over the next few months we will verify that Kv1.1 channel activity contributes to these changes in intrinsic properties using a Kv1.1 channel specific blocker.

4) Other Achievements:

Nothing to Report.

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

The generated by these studies were used to train students in grantsmanship, experimental techniques, and served as a foundation for pre- and postdoctoral fellowship applications. For example, PhD candidate Hailey Edigo-Betancourt and I, as a team, we were nominated by the University to apply for the 2022 HHMI Gilliam Graduate Fellowship which we advanced to the Finalist stage (nominate by the University, within 123 finalists out of 292 applicants). Ms. Edigo-Betancourt used these data as preliminary data for a NIH D-SPAN application. This work will serve as the basis for her PhD dissertation, and in part for two other student's dissertations.

How were the results disseminated to communities of interest?

My lab participates in TSC Alliance March on the Hill. We discuss with representatives to Congress how our data generated by this award has open up new areas of research in other fields with mTOR dysfunction.

I was an invited Speaker for an NIA sponsored workshop: Synaptic Dysfunction: Intersection of Autism and Dementia. NIH/NIA workshop "What can autism tell us about Alzheimer's Disease" (online).

My students presented posters at local and international meetings:

Egido-Betancourt, H.X., Bach, E.C., **Raab-Graham, K.F.** Investigating the Role of P/Q- and N-type Voltage-Gated Calcium Ion Channels in a Preclinical Model of Tuberous Sclerosis
SACNAS National Diversity in STEM Conference
Poster Session: Thursday, October 27th, 2022, G41 10:00-12:30 p.m.

Woehr, A., Barth, S.H., **Raab-Graham, K.F.** "mTOR Hyperactivity Represses Inhibitory Synapse Formation in TSC." Wake Forest Undergraduate Poster Symposium. Winston-Salem, NC, 2022.

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

We will continue to characterize the TSC1 heterozyogotic mice. We have found that these mice separate into two groups – those with high Kv1.1 and HuD, similar to the KOs and those that resemble WT mice. We will examine branch-specific potentiation in the two populations of hets. During this project we found that it would be helpful to find a blood based biomarker to help segregate the two populations. We are collaborating with an expert in isolating neuronal derived exosomes to identify blood based biomarkers to stratify mice prior to euthanizing them. We have received a Neuroscience Clinical Trial and Innovation Center (NCTIC) WFUSOM CTSI Pilot award (**Raab-Graham**, Strowd, and Deep (co-PIs)) to do this both in preclinical models and TSC patients. In addition, we will finish characterizing Kv1.1 in TSC1 neurons. Finally, we will determine if miR-129 is dysregulated in TSC and is responsible for coordinating the expression of Kv1.1 and CaV2.2.

4. **IMPACT:**

What was the impact on the development of the principal discipline(s) of the project?

These studies have identified a potential molecular mechanism for disrupted branch specific potentiation, a form of information storage. The finding that blocking N-VGCC restores branch specific potentiation allows us in future studies to determine if channel blockers can restore cognitive deficits in preclinical models of TSC. In addition, identifying two populations in the heterozyogotic mouse models is significant as this is the genotype that is relevant to the patient population, and may provide insight into the heterogeneous nature of patient symptoms. Future work will (1) identify blood based biomarkers to predict severity of patient symptoms and (2) use behavioral tasks and EEG to measure cognition and seizure severity.

What was the impact on other disciplines?

Many of our findings in preclinical models of TSC we have found to be replicated in mouse models of Alzheimer's disease, suggesting common mechanisms. This is an emerging hot topic as National Institute of Aging (NIA) hosted a workshop that I was an invited speaker in called "What can autism tell us about Alzheimer's Disease."

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

These studies and our other DOD funded studies have served as an example to representatives of Congress how TSC serves as a linchpin disorder and that any discovery made in these preclinical models are broadly applicable.

5. **CHANGES/PROBLEMS:**

Changes in approach and reasons for change

We have not made any significant changes in approach. We have employed the use of high potassium, our alternative, to stimulate cultured neurons and have answered the question we set out to answer. We recently have included electrophysiology to complement our calcium imaging

to provide rigor and reproducibility to support our findings. We are in the process of preparing three manuscripts.

Actual or anticipated problems or delays and actions or plans to resolve them

Due to Covid 19, the loss of mouse colonies, and the supply chain delays our experiments have been slowed down. Despite these delays, we have made progress and have solid data supporting our objectives of this grant with exciting new target to treat cognition in TSC.

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

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals.

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. **PRODUCTS:**

Publications, conference papers, and presentations

March 8-9, 2022, Synaptic Dysfunction: Intersection of Autism and Dementia. NIH/NIA workshop "What can autism tell us about **Alzheimer's Disease**" (online).

Investigating the Role of P/Q- and N-type Voltage-Gated Calcium Ion Channels in a Preclinical Model of Tuberous Sclerosis
Egido-Betancourt, H.X., Bach, E.C., **Raab-Graham, K.F**
SACNAS National Diversity in STEM Conference
Poster Session: Thursday, October 27th, 2022, G41 10:00-12:30 p.m.

Woehr, A., Barth, S.H., **Raab-Graham, K.F.** "mTOR Hyperactivity Represses Inhibitory Synapse Formation in TSC." Wake Forest Undergraduate Poster Symposium. Winston-Salem, NC, 2022.

Website(s) or other Internet site(s)
Nothing to Report

Technologies or techniques
Nothing to Report

Inventions, patent applications, and/or licenses
Nothing to Report

Other Products
Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Kimberly Raab-Graham, PhD
Project Role	PI
Researcher Identifier	
Nearest Person month worked:	3
Contribution to the project	Oversees all aspects of the project including intellectual and experimental design, rigor, and reproducibility. In addition the PI oversees hiring, budgets, and manuscript preparation.
Funding Support:	This grant, R01 NS105005, R01 AA026551, P50 AA026117
Name:	Zhiyong Deng, Ph.D.
Project Role	Research Scientist
Researcher Identifier	
Nearest Person month worked:	4
Contribution to the project	Dr. Deng is an expert molecular biologist who has generated reagents and trained students and performed biochemistry and immunostaining.
Funding Support:	This grant and R01 NS105005

Name:	Hailey Egido-Betancourt
Project Role	Graduate student
Researcher Identifier	
Nearest Person month worked:	6
Contribution to the project	Ms. Egido-Betancourt performed the calcium imaging, Western blotting, and immunostaining, and electrophysiology. Ms. Egido-Betancourt also maintains mouse colonies and cultured neurons.
Funding Support:	R01 NS105005-03S1 (Raab-Graham, PI) and this grant.
Name:	Sam Barth
Project Role	Graduate student
Researcher Identifier	

Nearest Person month worked:	2
Contribution to the project	Mr. Barth helped maintain and grow up colonies, he optimized the protein synthesis assay, and he cultured neurons.
Funding Support:	F31 NRSA (Raab-Graham, mentor) and T32 DA041349 (Jones PI, Raab-Graham Mentor)

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. **SPECIAL REPORTING REQUIREMENTS**

Nothing to Report

9. **APPENDICES:**

Nothing to Report