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14. ABSTRACT

This study is designed to identify mTOR-downstream molecules or pathways that account for synaptic and cognitive deficits in TSC, with the goal of identifying targets for more specific treatment. We had focused on macroautophagy (autophagy hereafter), a homeostatic catabolic degradation process downstream of mTOR, which is inhibited by hyperactive mTOR in Tsc1/2 deficient mouse brain. During the first project year, we had found significant cognitive impairment in Tsc2+/- mice and Atg7CKO autophagy deficient mice at the age of 3months. These mice however did not show cognitive deficits at 1month of age. Prior to the occurrence of cognitive impairment, Atg7CKO mice exhibited an increase in NMDA:AMPA ratio, increased frequency of miniature EPSCs and increased dendritic spine density, all indicating a blockade in postnatal synapse maturation. Atg7CKO mice moreover showed impaired CA3-CA1 long-term potentiation (LTP) and long term depression (LTD), both of which are well-known electrophysiological surrogates of hippocampus dependent learning and memory. Our findings therefore suggest that Autophagy is essential for synapse maturation and the development of normal synaptic plasticity and cognitive functions. We will continue to examine whether autophagy deficiency may underlie cognitive impairment in Tsc1/2 mutant mice during the next reporting period.

15. SUBJECT TERMS

Tuberous Sclerosis, cognitive impairment, autophagy, neuron, synapse maturation, mTOR

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
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1. INTRODUCTION:

Cognitive impairments, including long term and working memory deficits, are neuropsychiatric features of a majority of TSC patients. Recent studies using *Tsc1* or *Tsc2* heterozygous mutant murine models showed that mTOR disinhibition causes hippocampus dependent cognitive dysfunction. Whereas inhibiting mTOR activities can rescue cognitive impairments, recent clinical studies indicated that the effect of mTOR inhibitors is transient and the efficacy may be limited by their side effects. As such, unraveling the downstream substrates of overactive mTOR will be critical for developing more targeted and effective therapies for the neurocognitive symptoms in TSC. This project is designed to identify molecules or pathways downstream of mTOR that account for synaptic and cognitive deficits in TSC, with the goal of uncovering novel targets for more specific treatment while limiting side effects of mTOR inhibitors. We will focus on macroautophagy (autophagy hereafter), a homeostatic catabolic degradation process downstream of mTOR, which is inhibited by hyperactive mTOR in Tsc1/2 deficient mouse brain. We have found that impaired mTOR-autophagy suppresses postnatal synaptic pruning, a process necessary for the maturation of functional synaptic connections and neural circuits and required for multiple forms of learning and memory, and we will thus study whether impaired autophagy may underlie cognitive impairments in TSC mice by disrupting synapse maturation.

2. KEYWORDS:

Tuberous Sclerosis, cognitive impairment, autophagy, neuron, synapse maturation, mTOR

3. ACCOMPLISHMENTS:

The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?

Aim 1: To determine the role of impaired mTOR-autophagy in cognitive dysfunction in *Tsc1 or Tsc2 (Tsc1/2)* deficient mice (**Time frame**: months 1-24).

Aim 2: To identify synaptic mechanisms of impaired mTOR-autophagy for cognitive dysfunction in *Tsc1/2* deficient mice (**Time frame**: months 1-24).

Aim3 3: To identify molecular mechanisms of impaired autophagy for synaptic dysfunction in *Tsc1/2* deficient mice (**Time frame**: months 13-36).

What was accomplished under these goals?

Task 1 (Aim1). To determine the role of impaired mTOR-autophagy in cognitive dysfunction in *Tsc1 or Tsc2 (Tsc1/2)* deficient mice

Hypothesis: Autophagy deficiency downstream of overactive mTOR is implicated in cognitive impairment in *Tsc1/2* deficient mouse models.

Research Plan: We planned to assess learning and memory cognitive functions using Morris water maze and fear conditioning tests in control and autophagy gene Atg7 conditional knockout (CKO) Tsc2 mutant (Tsc2+/-) and mGFAPCre mediated Tsc1 knockout ($Tsc1^{mGFAPCre}$CKO) mice. Mice were tested at the age of 1 and 3 months to determine the progression of cognitive impairment. Behaviors were compared among control, *Tsc2+/-*, and rapamycin treated *Tsc2+/- mice and Tsc2+/-:*$Atg7^{CamKCre+}$CKO double mutant (Tsc2-Atg7 DKO) mice, with the goal to isolate the role of mTOR-regulated autophagy in cognitive dysfunction in *Tsc2+/-* mice, and among $Tsc1^{flox/flox}$ control, $Tsc1^{mGFAPCre}$CKO, and rapamycin treated $Tsc1^{mGFAPCre}CKO$ and Tsc1-Atg7 double knockout (Tsc1-Atg7 DKO) mice to identify the role of mTOR-autophagy in cognitive deficits in *Tsc1* deficient mice.

Accomplishments: In years 1&2, we assessed hippocampus dependent associative (Fear conditioning test) and spatial (Morris water maze test) learning and memory in Tsc2 mutant mice, and found that while Tsc2+/- and Atg7$^{CamKCre+}$CKO mice both exhibited hippocampus-related cognitive impairment at 3 months of age, they do not exhibit cognitive deficits at the age of 1 month, the developmental time period when excitatory dendritic spine synapses are exuberant due to the insufficient synapse pruning (Tang et al., Neuron, 2014;83(5):1131-43. doi:10.1016/j.neuron.2014.07.040). We therefore consider that the increase in excitatory synapses due to autophagy deficiency contributes to cognitive impairment that occurs in a later life in TSC. To disentangle autophagy from other downstream effectors of mTOR, we assessed the effects of rapamycin on hippocampus dependent learning and memory. In vehicle treated mice, compared to *Atg7$^{flox/flox}$* controls, all mutant mice (Tsc2+/-, Atg7$^{CamKCre+}$CKO and Tsc2-Atg7 DKO) showed deficits in learning and memory. Rapamycin treatment significantly improved learning in the Tsc2+/- mice but not in the Atg7CKO or Tsc2-Atg7 DKO mice. Our data strongly support that autophagy deficiency underlies impaired hippocampus dependent cognitive function in the Tsc2+/- mice.

Neurocognitive function was assessed in a second neuroglia Tsc1 deficient (*Tsc1^{mGFAPCre}CKO*) mouse line. In years **1 &2**, we have focused on the breeding of the Tsc1$^{flox/flox}$ mice to the Atg7$^{flox/flox}$ mice to obtain TSC1$^{flox/flox}$:Atg7$^{flox/flox}$ double homozygous mice, then to the mouse GFAP promoter driven Cre (mGFAPCre) expressing mouse line to obtain TSC1$^{flox/+}$:Atg7$^{flox/+}$:mGFAPCre+ triple heterozygous mice. Male TSC1$^{flox/flox}$:Atg7$^{flox/flox}$ mice were crossed to female triple heterozygous mice to obtain Tsc1-Atg7 double knockout mice. Due to the involvement of multiple genetic loci, we failed to obtain sufficient double knockout mice for all planned experiments, and most of those double knockout mice were not viable by the age when all experiments were proposed.

In **Year 3**, we have examined neurocognitive behaviors in both *Tsc1^{mGFAPCre}CKO* and *Atg7^{mGFAPCre}CKO* mice, treated with or without rapamycin. We observed TSC-related neurological symptoms, including autism, cognitive impairment and epilepsy, in 5-6-week old adolescent male *TSC1*^{mGFAPCre}CKO mice. These KO mice however did not present motor defects or anxiety-like behaviors in open field (**Fig. 1A-E**). They instead spent more time self-grooming, suggesting autistic-like repetitive behaviors (**Fig. 1 F**). Social deficits were assessed using the traditional three-chamber social test as we described before in Tang et al., Neuron, 2014. While *TSC1^{mGFAPCre}CKO* mice showed a preference for interacting with a social target compared with nonsocial target (**Fig. 1G**, left), the preference index (the ratio of time sniffing mouse versus nonsocial target) was decreased (**Fig. 1G**, right). In the social novelty test, the *TSC1^{mGFAPCre}CKO* mice spent more time sniffing the familiar social targets (**Fig. 1H**, left). The preference index (the ratio of time sniffing a stranger mouse versus a familiar mouse; **Fig. 1H**, right) decreased, suggesting a reduced preference for social novelty. Cognitive function in the *TSC1^{mGFAPCre}CKO* mice was assessed using the novel object recognition test. Compared to control littermates, the *TSC1^{mGFAPCre}CKO* mice spent significant less time exploring the novel object, with no difference in time spent exploring the familiar object (**Fig 1 I**), indicative of cognitive impairment. We also assessed feared learning and memory in the *TSC1^{mGFAPCre}CKO* mice, and found significantly impaired contextual but not cued fear memory (**Fig. 1 J**). TSC1^{mGFAPCre}CKO mice grew normally for the first 6-8 weeks postnatally (**Fig. 1 K**), but began to exhibit noticeable spontaneous behavioral seizures between postnatal 2-2.5months (**Fig. 1 L**), which were especially evident after mechanical and/or emotional stress due to cage changes, transfer into new surroundings and handling. By 3.5months of age, ~60.2% mice show detectable behavioral seizures, and ~33% of these seized mice died unexpectedly (**Fig. 1M)**. ~50 percent of TSC1^{mGFAPCre}CKO mice may survive until 7 months, among which ~66.7% demonstrated seizures and 50% died. Seizure behaviors ranged from frequent jerking of head and body, chewing, and infrequently progression to a tonic-clonic seizure (extension of fore- and hindlimbs, falling on one side, rearing and bouncing in the cage) (**Fig.1M**). The episodes last for 0.5-2mins and were followed by complete physical inactivity. These mice were not tested for Morris Water Maze learning and memory due to their susceptibility to seizures upon stresses in the water maze. Atg7^{mGFAPCre}CKO autophagy deficient mice showed similar contextual fear memory deficits at 5-6 weeks of age (**Fig. 1N)**, but did not develop seizures. Rapamycin treatment can rescue cognitive dysfunction in the Tsc1^{mGFAPCre}CKO mice but not in the Atg7^{mGFAPCre}CKOmice. A limited number of Tsc1/Atg7 double knockout mice were obtained and evaluated for cognitive function, with (n=3) or without (n=2) rapamycin treatment, but the data are not interpretable due to the small sample size.

Taken together, our data strongly support that autophagy deficiency underlies impaired hippocampus dependent cognitive function in both Tsc2+/- and Tsc1^{mGFAPCre}CKO mouse models of TSC.

Task 2 (Aim2). To identify synaptic mechanisms of impaired mTOR-autophagy for cognitive dysfunction in *Tsc1/2* deficient mice

Hypothesis: Impaired neuronal autophagy interferes with hippocampal long-term potentiation (LTP) and long-term depression (LTD) (Task 2a), and that impaired neuronal autophagy disrupts hippocampal CA3-CA1 synapse maturation (Task 2b).

Research Plan: To determine the effect of impaired autophagy on hippocampal synaptic plasticity and to Characterize developmental synapse maturation in autophagy deficient mice

Accomplishments: In **Year 3,** we characterized the temporal and spatial expression of mGFAPCre throughout embryonic and postnatal development. mGFAP-Cre mice were bred with the Ai9 Cre reporter mouse line (*Jax 007905*) to map in vivo Cre activity. These reporter mice express the *dtomato* reporter gene constitutively only after Cre-mediated recombination occurs. There was no detectable reporter expression in brains from *Ai9* mice without the *cre* transgene. The temporal expression of mGFAP-Cre was determined in brains from embryos at E12, E15, E17-18, E20, and from pups at postnatal day 4 (P4), 6 (P6) and day 14(P14). We found that tdTomato+ cells began to appear in the ventricular zone between cortex and hippocampus at E18, immediately after endogenous mouse GFAP immunoreactivity became detectable in radial glia at E17. Many tdTomato+ cells were radial glia, based on their shape and immunohistochemical profile, with some newborn neurons positive for neuronal marker TuJ1. The majority of cortical and hippocampal astrocytes showed significant Cre recombination starting from P6, which coincided with the temporal expression of endogenous mouse GFAP promoter in astrocytes. A subset of cortical and hippocampal neurons was also positive for tdTomato, indicative of Cre-mediated recombination. The recombinant neurons were mainly excitatory projection neurons, identified by the pyramidal shape of their somata, *their* prominent apical dendrites and the expression of *glutamatergic* markers. These data are not shown in this report but will be submitted for publication during next reporting period.

Given the mixed cell types that experience Cre mediated recombination in mGFAPCre mice, it is unlikely to determine the contribution of neuronal autophagy to synapse function and plasticity. As such, we chose to use the neuronal specific *Atg7$^{CamKII\text{-}Cre}$CKO* mice to study the mechanisms of neuronal autophagy in regulating synaptic function during development. As we presented in **Years 1-2** progress reports, we have characterized CA3-CA1 synaptic plasticity in P35 *Atg7$^{CamKII\text{-}Cre}$CKO* mice, and found that long-term potentiation (LTP), an activity dependent synaptic plasticity widely proposed as a cellular model for learning and memory, was impaired in Atg7$^{CamKCre+}$CKO mice, and that the development of long term depression (LTD) upon activation of group 1 metabotropic glutamate receptors (mGluR-LTD), which is thought to underlie the weakening of synaptic connections, was substantially attenuated in P23-24 Atg7$^{CamKCre+}$CKO mice immediately following the expression of Cre recombinase and Atg7 gene depletion. Our findings confirmed an increase in functional excitatory synapses and a blunting of hippocampal synaptic plasticity in Atg7$^{CamKCre+}$CKO mice that specifically lack autophagy in pyramidal neurons, suggesting that normal autophagy is required for the development of hippocampal synaptic plasticity. Membrane properties of CA1 neurons, such as resting membrane potentials, membrane resistance, and membrane capacitance, were similar between control and Atg7$^{CamKCre+}$CKO mice. The NMDA current was significantly enhanced in the Atg7$^{CamKCre+}$CKO mice, resulting in an increase in NMDA:AMPA ratio. We also recorded the miniature EPSCs and found that the amplitude was similar between control and Atg7$^{CamKCre+}$CKO mice, suggesting that the average synaptic density of AMPA receptors, and thus the synaptic weight of CA1 synapses, remained intact in the Atg7$^{CamKCre+}$CKO mice. The frequency of mEPSC was however significantly higher in the Atg7$^{CamKCre+}$CKO mice than in controls, indicating that the number of synaptic contacts or release probability may be substantially increased. Using DiOlistic labeling, we confirmed a remarkable increase in dendritic spine density in the Atg7$^{CamKCre+}$CKO CA1 neurons that was due to a blockade in postnatal dendritic spine pruning. We have also examined the pre- and post- synaptic mechanisms for impaired hippocampal synaptic plasticity, e.g. CA3-CA1 LTP. As an initial step in our analysis, we examined basal synaptic function by plotting the stimulus voltages (V) against the slope of field excitatory postsynaptic potentials (fEPSPs) to generate input-output relations before each experiment. We observed a trend towards enhanced basal synaptic response in the Atg7$^{CamKCre+}$CKO mice, which was statistically significant at stimulus intensities of 40-60V compared to that of controls. Post-tetanic potentiation (PTP) and pare pulsed facilitation (PPF), both are synaptic parameter indicating presynaptic function, were not different between control and Atg7 KO mice, suggesting that presynaptic neurotransmitter release is not compromised in Atg7 knockouts. We then examined the expression of Cre recombinanse in different hippocampal areas. The CamKII-Cre mice were crossed to the HA-tagged Ribotag mice. By co-immunostaing for HA and neuronal marker NeuN, we found that CamkII-Cre does not express at CA3 pyramidal neurons, suggesting that Atg7 alleles were not floxed out from the presynaptic neurons in the CA3-CA1 circuit. We also examined synaptic fatigue (SF), a form of short-term plasticity that occurs during high frequency stimulation and consists of a decrease in synaptic strength. SF could be caused by a depletion of the release ready pool of presynaptic vesicles or desensitization of postsynaptic glutamate receptors. We found that SF was more pronounced in Atg7 KO mice. Given that presynaptic transmission remained intact in the Atg7CKO mice, the enhanced SF may suggest a decrease in synaptic

localization of AMPA receptors or enhanced desensitization of AMPA receptor at postsynaptic membrane. Together, our tests of basal synaptic function and short-term forms of synaptic plasticity demonstrate that neuronal autophagy deficiency perturbs the ability of CA1 neurons to respond to controlled presynaptic stimulation. Given the increase in excitatory synaptic density, enhanced basal synaptic transmission and impaired LTP induction, we hypothesize that neuronal autophagy deficiency may occlude further attempts to elicit LTP.

Task 3 (Aim 3). Identify molecular mechanisms of impaired autophagy for synaptic dysfunction in *Tsc1/2* deficient mice

Plan: Task 3a To explore neuronal substrates of autophagy downstream of mTOR in *Tsc1/2* deficient mice **Task 3b** To explore synaptic substrates of autophagy downstream of mTOR in *Tsc1/2* deficient mice

Hypothesis: We hypothesize that impaired autophagy contributes to disrupted hippocampal CA3-CA1 synaptic plasticity by regulating levels of neuronal proteins required for the development of CA1 neurons and synapses, and so regulating the molecular composition of synapses. We will employ a candidate strategy to explore the autophagic mechanisms for synaptic and cognitive deficits in *Tsc1/2* deficient mice.

Accomplishments: In **Years 1 and 2** of this project, we had compared genome-wide translation efficiencies between rapamycin treated and untreated control and Tsc2+/- mice and ranked all genes based on their translation efficiency fold-change, as proposed in task 3a. Unexpectedly, we failed to find altered translation of neuronal enriched genes in the Tsc2+/-; RiboTag mice (n=4). These data confirmed that the Tsc2 heterozygous mutation does not interfere with the translational profile, and that the disrupted protein and synaptic protein homeostasis in the Tsc2+/- mice may be largely due to suppressed protein degradation, instead of exaggerated translation. When both WT and Tsc2+/- mice were treated with rapamyin, we found that only genes containing the TOP-motif are significantly enriched among the genes that are translationally downregulated by rapamycin treatment.

In **Year 3**, we had run both RNA-seq and ribosome profiling analysis in $Tsc1^{mGFAPCre}$CKO mice at the age of 5-6 weeks (not shown). We did not find significant common changes in the abundance of RNA transcripts in both knockout mouse lines. The RNA-Seq gene expression profile however show significant changes in astrocyte and synaptic proteins in 3.5month old $Tsc1^{mGFAPCre}$CKO mice with severe seizures, which we considered as a secondary response to recurrent seizures. Similar to our preliminary data in Tsc2+/- mice, we found significant changes in the translation of TOP-motif genes in rapamycin treated mice, indicative of the consequence of mTOR suppression by rapamycin.

These data were unexpected. As such, we proceed to our original research aim 3b on the proteome of total hippocampal lysates and excitatory synapses, with the hope that we can isolate the specific effects of neuronal autophagy on synapse/neuronal development. We had performed the complicate mouse breeding for excitatory synapse specific proteomics. We had crossed $Atg7^{flox/flox}$ mice to $PSD95^{flox/flox}$ mice. The resulting $Atg7^{flox/+}$:$PSD95^{flox/+}$ mice were self-crossed to obtain $Atg7^{flox/flox}$:$PSD95^{flox/flox}$ double homozygous breeders. We then crossed the $Atg7^{flox/flox}$:$PSD95^{flox/flox}$ males to $PSD95^{flox/flox}$;CamkCre+ females to obtain female $Atg7^{flox/+}$:$PSD95^{flox/flox}$;Camkcre+ breeders. By further crossing the male $Atg7^{flox/flox}$:$PSD95^{flox/flox}$ mice to the female $Atg7^{flox/+}$:$PSD95^{flox/flox}$;Camkcre+ mice, we were able to obtain $Atg7^{CamkCre+}$CKO;$PSD95^{flox/flox}$ and $PSD95^{flox/flox}$:CamKCre control mice at both ages of postnatal day 23-24 (P23) and 34-35 (P35). During the no cost extension period, we will continue to collect brain tissues from each group at different ages for synapse proteomics, synaptic affinity purification and western blot analysis.

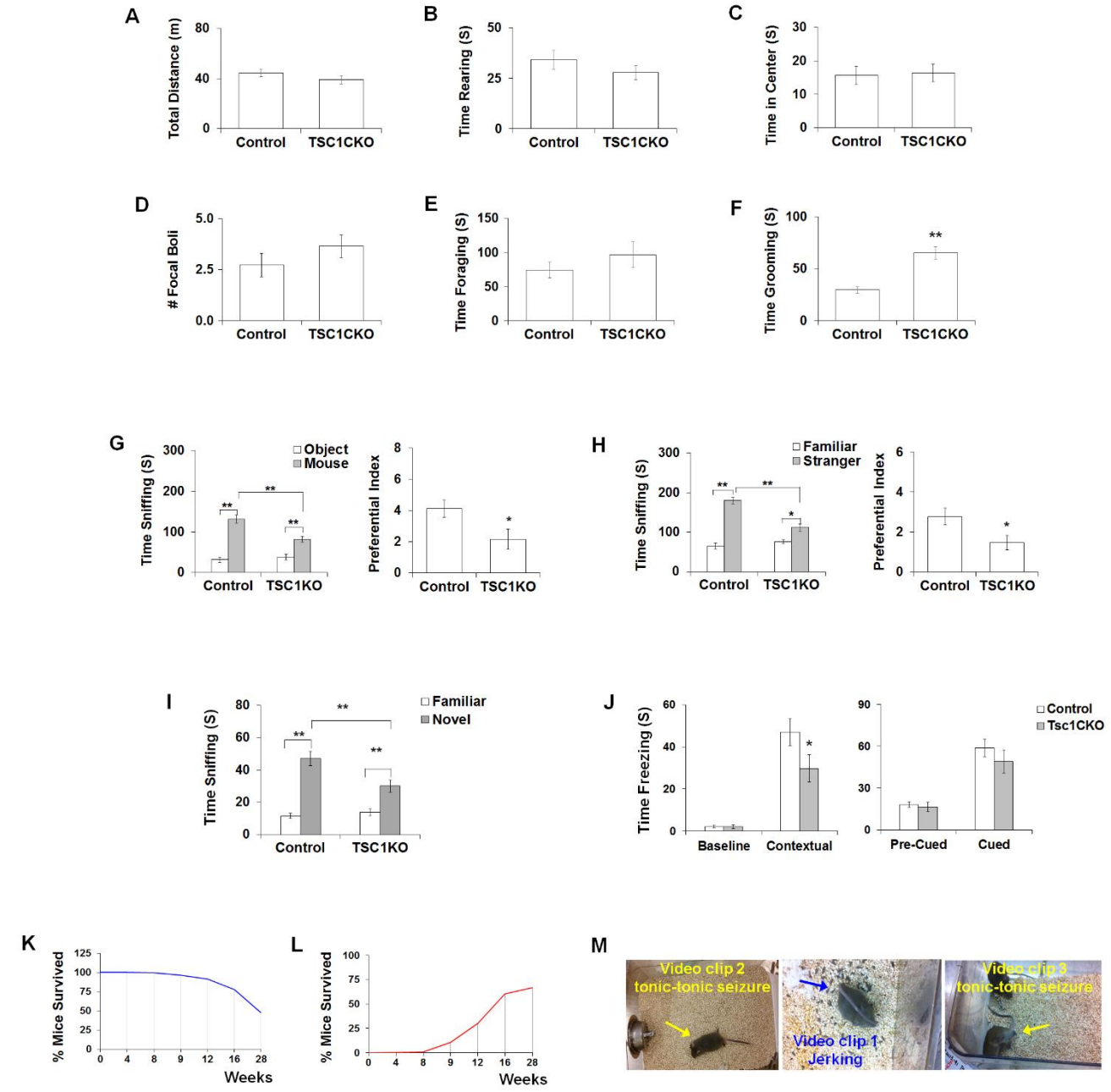


Figure 1. Neurological symptoms in Tsc1^{mGFAPCre}CKO mice.
(**A-F**) Open field behaviors; (**G, H**) Three chamber social interaction test. G, preference for social target; **H**, preference for social novelty; (**I**) Novel object recognition test; (**J**) Fear conditioning test. Left, contextual feared memory; right, cued memory; (**K**) Survival rate in Tsc1^{mGFAPCre}CKO mice; (**L**) Seizure onset in Tsc1^{mGFAPCre}CKO mice; (**M**) Seizure behaviors in Tsc1^{mGFAPCre}CKO mice. (**N**) Rapamycin (P19-P42) rescues contextual feared memory in Tsc1^{mGFAPCre}CKO (Tsc1CKO) but not in Atg7^{mGFAPCre}CKO (Atg7KO) mice. Compared to control, *, $P<0.05$; **, $p<0.01$.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

We plan to complete all proposed tasks, and to prepare and submit two scientific manuscripts during the next reporting period. We will continue to address the molecular mechanisms for altered synaptic and cognitive changes in autophagy deficient mice by analyzing the protein composition of excitatory synapses in Atg7CKO mouse hippocampus.

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report

5. CHANGES/PROBLEMS: The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

We were unable to complete the proposed task 3b in the third year of funding period due to an unexpected move of laboratory space (2019.02-2019.06) and that we could not find a qualified postdoc with required technical skillsets in the lab during the third year. We have requested a No-cost extension in order to complete the tasks proposed in our specific aim 3b, and to re-run crucial experiments that have to be validated for publication, and to prepare, submit, and publish scientific manuscripts.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

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: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

Publications, conference papers, and presentations
Report only the major publication(s) resulting from the work under this award.

Journal publications.

N/A

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

Other publications, conference papers, and presentations.

Nothing to report

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?
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). If information is unchanged from a previous submission, provide the name only and indicate "no change."

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: *Guomei Tang*
Project Role: *Principle investigator*
Researcher Identifier (e.g. ORCID ID): 0000-0001-9479-5331
Nearest person month worked: *2.4 months*
Contribution to Project: Dr. Tang supervised the project, performed work in mouse breeding, behavioral analysis, molecular biology/RNA analysis and histology.
Funding Support: Dr. Tang's funding portfolio currently includes: The Simons Foundation Autism Research Initiative (SFARI) Pilot award (SFARI 40220); DOD award W81XWH-16-1-0263 and R01NS104390.

Name: Zhonghai Yan
Project Role: Postdoc
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: *4* months
Contribution to Project: Dr. Yan assisted with mouse breeding and behaviors.
Funding Support: Dr. Yan's funding portfolio currently includes DOD W81XWH-16-1-0263 and PDF Research Center Grant PDF RCE-1701.

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

The PI has a new R01 grant that began September 2018:

R01NS104390 (Tang, PI) 9/30/2018 – 7/31/2023 3.6 CM
National Institute of Health
Title: Cellular and Molecular basis for cognitive impairment associated with Glucocerebrosidase (GBA1) mutation
Role: Principle Investigator

The PI has a previously active grant closed in Sept 2018:

W81XWH-15-1-0112 (Goldman, PI) 09/01/2015 – 09/14/2018 0.6 CM
Department of Defense (DOD)
Title: Molecular mechanisms underlying the epileptogenesis and seizure progression in Tuberous Sclerosis Complex deficient mouse models
Role: Co-investigator

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

Nothing to Report

9. APPENDICES:

Task 1 (Aim1). To determine the role of impaired mTOR-autophagy in cognitive dysfunction in *Tsc1 or Tsc2 (Tsc1/2)* deficient mice

Hypothesis: Autophagy deficiency downstream of overactive mTOR is implicated in cognitive impairment in *Tsc1/2* deficient mouse models.

Research Plan: We planned to assess learning and memory cognitive functions using Morris water maze and fear conditioning tests in control and autophagy gene Atg7 conditional knockout (CKO) Tsc2 mutant (Tsc2+/-) and mGFAPCre mediated Tsc1 knockout ($Tsc1^{mGFAPCre}$CKO) mice. Mice were tested at the age of 1 and 3 months to determine the progression of cognitive impairment. Behaviors were compared among control, *Tsc2+/-* , and rapamycin treated *Tsc2+/- mice and Tsc2+/-:*$Atg7^{CamKCre+}$CKO double mutant (Tsc2-Atg7 DKO) mice, with the goal to isolate the role of mTOR-regulated autophagy in cognitive dysfunction in *Tsc2+/-* mice, and among $Tsc1^{flox/flox}$ control, $Tsc1^{mGFAPCre}$CKO, and rapamycin treated $Tsc1^{mGFAPCre}CKO$ and Tsc1-Atg7 double knockout (Tsc1-Atg7 DKO) mice to identify the role of mTOR-autophagy in cognitive deficits in *Tsc1* deficient mice.

Accomplishments: In years 1&2, we assessed hippocampus dependent associative (Fear conditioning test) and spatial (Morris water maze test) learning and memory in Tsc2 mutant mice, and found that while Tsc2+/- and $Atg7^{CamKCre+}$CKO mice both exhibited hippocampus-related cognitive impairment at 3 months of age, they do not exhibit cognitive deficits at the age of 1 month, the developmental time period when excitatory dendritic spine synapses are exuberant due to the insufficient synapse pruning (Tang et al., Neuron, 2014;83(5):1131-43. doi:10.1016/j.neuron.2014.07.040). We therefore consider that the increase in excitatory synapses due to autophagy deficiency contributes to cognitive impairment that occurs in a later life in TSC. To disentangle autophagy from other downstream effectors of mTOR, we assessed the effects of rapamycin on hippocampus dependent learning and memory. In vehicle treated mice, compared to $Atg7^{flox/flox}$ controls, all mutant mice (Tsc2+/-, $Atg7^{CamKCre+}$CKO and Tsc2-Atg7 DKO) showed deficits in learning and memory. Rapamycin treatment significantly improved learning in the Tsc2+/- mice but not in the Atg7CKO or Tsc2-Atg7 DKO mice. Our data strongly support that autophagy deficiency underlies impaired hippocampus dependent cognitive function in the Tsc2+/- mice.

Neurocognitive function was assessed in a second neuroglia Tsc1 deficient ($Tsc1^{mGFAPCre}CKO$) mouse line. In years **1 &2**, we have focused on the breeding of the $Tsc1^{flox/flox}$ mice to the $Atg7^{flox/flox}$ mice to obtain $TSC1^{flox/flox}$:$Atg7^{flox/flox}$ double homozygous mice, then to the mouse GFAP promoter driven Cre (mGFAPCre) expressing mouse line to obtain $TSC1^{flox/+}$:$Atg7^{flox/+}$:mGFAPCre+ triple heterozygous mice. Male $TSC1^{flox/flox}$:$Atg7^{flox/flox}$ mice were crossed to female triple heterozygous mice to obtain Tsc1-Atg7 double knockout mice. Due to the involvement of multiple genetic loci, we failed to obtain sufficient double knockout mice for all planned experiments, and most of those double knockout mice were not viable by the age when all experiments were proposed.

In **Year 3**, we have examined neurocognitive behaviors in both $Tsc1^{mGFAPCre}CKO$ and $Atg7^{mGFAPCre}CKO$ mice, treated with or without rapamycin. We observed TSC-related neurological symptoms, including autism, cognitive impairment and epilepsy, in 5-6-week old adolescent male $TSC1^{mGFAPCre}$CKO mice. These KO mice however did not present motor defects or anxiety-like behaviors in open field (**Fig. 1A-E**). They instead spent more time self-grooming, suggesting autistic-like repetitive behaviors (**Fig. 1 F**). Social deficits were assessed using the traditional three-chamber social test as we described before in Tang et al., Neuron, 2014. While $TSC1^{mGFAPCre}CKO$ mice showed a preference for interacting with a social target compared with nonsocial target (**Fig. 1G**, left), the preference index (the ratio of time sniffing mouse versus nonsocial target) was decreased (**Fig. 1G**, right). In the social novelty test, the $TSC1^{mGFAPCre}CKO$ mice spent more time sniffing the familiar social targets (**Fig. 1H**, left). The preference index (the ratio of time sniffing a stranger mouse versus a familiar mouse; **Fig. 1H**, right) decreased, suggesting a reduced preference for social novelty. Cognitive function in the $TSC1^{mGFAPCre}CKO$ mice was assessed using the novel object recognition test. Compared to control littermates, the $TSC1^{mGFAPCre}CKO$ mice spent significant less time exploring the novel object, with no difference in time spent exploring the familiar object (**Fig 1 I**), indicative of cognitive impairment. We also assessed feared learning and memory in the $TSC1^{mGFAPCre}CKO$ mice, and found significantly impaired contextual but not cued fear memory (**Fig. 1 J**). $TSC1^{mGFAPCre}$CKO mice grew normally for the first 6-8 weeks postnatally (**Fig. 1 K**), but began to exhibit noticeable spontaneous behavioral seizures between postnatal 2-2.5months (**Fig. 1 L**), which were especially evident after mechanical and/or emotional stress due to cage changes, transfer into new surroundings and handling. By 3.5months of age, ~60.2% mice show detectable behavioral seizures, and ~33% of these seized mice died unexpectedly (**Fig. 1M)**. ~50 percent of $TSC1^{mGFAPCre}$CKO mice may survive until 7 months, among which ~66.7% demonstrated seizures and 50% died. Seizure behaviors ranged from frequent jerking of head and body, chewing, and infrequently progression to a tonic-clonic seizure (extension of fore- and hindlimbs, falling on one side, rearing and bouncing in the cage) (**Fig.1M**). The episodes last for 0.5-2mins and were

followed by complete physical inactivity. These mice were not tested for Morris Water Maze learning and memory due to their susceptibility to seizures upon stresses in the water maze. Atg7^{mGFAPCre}CKO autophagy deficient mice showed similar contextual fear memory deficits at 5-6 weeks of age (**Fig. 1N)**, but did not develop seizures. Rapamycin treatment can rescue cognitive dysfunction in the Tsc1^{mGFAPCre}CKO mice but not in the Atg7^{mGFAPCre}CKOmice. A limited number of Tsc1/Atg7 double knockout mice were obtained and evaluated for cognitive function, with (n=3) or without (n=2) rapamycin treatment, but the data are not interpretable due to the small sample size.

Taken together, our data strongly support that autophagy deficiency underlies impaired hippocampus dependent cognitive function in both Tsc2+/- and Tsc1^{mGFAPCre}CKO mouse models of TSC.

Task 2 (Aim2). To identify synaptic mechanisms of impaired mTOR-autophagy for cognitive dysfunction in *Tsc1/2* deficient mice

Hypothesis: Impaired neuronal autophagy interferes with hippocampal long-term potentiation (LTP) and long-term depression (LTD) (Task 2a), and that impaired neuronal autophagy disrupts hippocampal CA3-CA1 synapse maturation (Task 2b).

Research Plan: To determine the effect of impaired autophagy on hippocampal synaptic plasticity and to Characterize developmental synapse maturation in autophagy deficient mice

Accomplishments: In **Year 3,** we characterized the temporal and spatial expression of mGFAPCre throughout embryonic and postnatal development. mGFAP-Cre mice were bred with the Ai9 Cre reporter mouse line (*Jax 007905*) to map in vivo Cre activity. These reporter mice express the *dtomato* reporter gene constitutively only after Cre-mediated recombination occurs. There was no detectable reporter expression in brains from *Ai9* mice without the *cre* transgene. The temporal expression of mGFAP-Cre was determined in brains from embryos at E12, E15, E17-18, E20, and from pups at postnatal day 4 (P4), 6 (P6) and day 14(P14). We found that tdTomato+ cells began to appear in the ventricular zone between cortex and hippocampus at E18, immediately after endogenous mouse GFAP immunoreactivity became detectable in radial glia at E17. Many tdTomato+ cells were radial glia, based on their shape and immunohistochemical profile, with some newborn neurons positive for neuronal marker TuJ1. The majority of cortical and hippocampal astrocytes showed significant Cre recombination starting from P6, which coincided with the temporal expression of endogenous mouse GFAP promoter in astrocytes. A subset of cortical and hippocampal neurons was also positive for tdTomato, indicative of Cre-mediated recombination. The recombinant neurons were mainly excitatory projection neurons, identified by the pyramidal shape of their somata, *their* prominent apical dendrites and the expression of *glutamatergic* markers. These data are not shown in this report but will be submitted for publication during next reporting period.

Given the mixed cell types that experience Cre mediated recombination in mGFAPCre mice, it is unlikely to determine the contribution of neuronal autophagy to synapse function and plasticity. As such, we chose to use the neuronal specific *Atg7$^{CamKII\text{-}Cre}$CKO* mice to study the mechanisms of neuronal autophagy in regulating synaptic function during development. As we presented in **Years 1-2** progress reports, we have characterized CA3-CA1 synaptic plasticity in P35 *Atg7$^{CamKII\text{-}Cre}$CKO* mice, and found that long-term potentiation (LTP), an activity dependent synaptic plasticity widely proposed as a cellular model for learning and memory, was impaired in Atg7$^{CamKCre+}$CKO mice, and that the development of long term depression (LTD) upon activation of group 1 metabotropic glutamate receptors (mGluR-LTD), which is thought to underlie the weakening of synaptic connections, was substantially attenuated in P23-24 Atg7$^{CamKCre+}$CKO mice immediately following the expression of Cre recombinase and Atg7 gene depletion. Our findings confirmed an increase in functional excitatory synapses and a blunting of hippocampal synaptic plasticity in Atg7$^{CamKCre+}$CKO mice that specifically lack autophagy in pyramidal neurons, suggesting that normal autophagy is required for the development of hippocampal synaptic plasticity. Membrane properties of CA1 neurons, such as resting membrane potentials, membrane resistance, and membrane capacitance, were similar between control and Atg7$^{CamKCre+}$CKO mice. The NMDA current was significantly enhanced in the Atg7$^{CamKCre+}$CKO mice, resulting in an increase in NMDA:AMPA ratio. We also recorded the miniature EPSCs and found that the amplitude was similar between control and Atg7$^{CamKCre+}$CKO mice, suggesting that the average synaptic density of AMPA receptors, and thus the synaptic weight of CA1 synapses, remained intact in the Atg7$^{CamKCre+}$CKO mice. The frequency of mEPSC was however significantly higher in the Atg7$^{CamKCre+}$CKO mice than in controls, indicating that the number of synaptic contacts or release probability may be substantially increased. Using DiOlistic labeling, we confirmed a remarkable increase in dendritic spine density in the Atg7$^{CamKCre+}$CKO CA1 neurons that was due to a blockade in postnatal dendritic spine pruning. We have also examined the pre- and post- synaptic mechanisms for impaired hippocampal synaptic plasticity, e.g. CA3-CA1 LTP. As an initial step in our analysis, we examined basal synaptic function by plotting the stimulus voltages (V) against the slope of field excitatory postsynaptic potentials (fEPSPs) to generate input-output relations before each experiment. We observed a trend towards

enhanced basal synaptic response in the Atg7$^{CamKCre+}$CKO mice, which was statistically significant at stimulus intensities of 40-60V compared to that of controls. Post-tetanic potentiation (PTP) and pare pulsed facilitation (PPF), both are synaptic parameter indicating presynaptic function, were not different between control and Atg7 KO mice, suggesting that presynaptic neurotransmitter release is not compromised in Atg7 knockouts. We then examined the expression of Cre recombinanse in different hippocampal areas. The CamKII-Cre mice were crossed to the HA-tagged Ribotag mice. By co-immunostaing for HA and neuronal marker NeuN, we found that CamkII-Cre does not express at CA3 pyramidal neurons, suggesting that Atg7 alleles were not floxed out from the presynaptic neurons in the CA3-CA1 circuit. We also examined synaptic fatigue (SF), a form of short-term plasticity that occurs during high frequency stimulation and consists of a decrease in synaptic strength. SF could be caused by a depletion of the release ready pool of presynaptic vesicles or desensitization of postsynaptic glutamate receptors. We found that SF was more pronounced in Atg7 KO mice. Given that presynaptic transmission remained intact in the Atg7CKO mice, the enhanced SF may suggest a decrease in synaptic localization of AMPA receptors or enhanced desensitization of AMPA receptor at postsynaptic membrane. Together, our tests of basal synaptic function and short-term forms of synaptic plasticity demonstrate that neuronal autophagy deficiency perturbs the ability of CA1 neurons to respond to controlled presynaptic stimulation. Given the increase in excitatory synaptic density, enhanced basal synaptic transmission and impaired LTP induction, we hypothesize that neuronal autophagy deficiency may occlude further attempts to elicit LTP.

Task 3 (Aim 3). Identify molecular mechanisms of impaired autophagy for synaptic dysfunction in *Tsc1/2* deficient mice

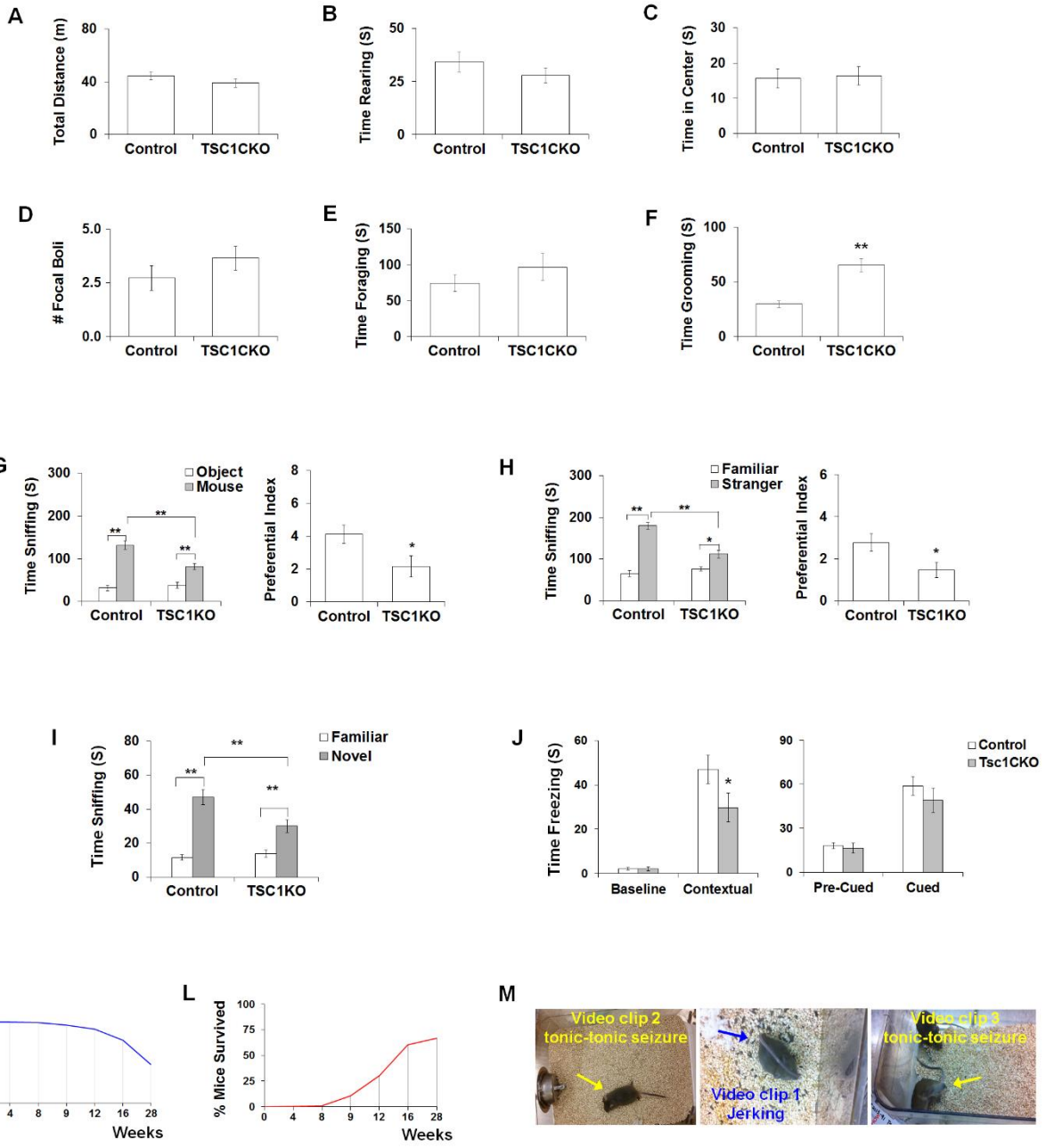
Plan: Task 3a To explore neuronal substrates of autophagy downstream of mTOR in *Tsc1/2* deficient mice **Task 3b** To explore synaptic substrates of autophagy downstream of mTOR in *Tsc1/2* deficient mice

Hypothesis: We hypothesize that impaired autophagy contributes to disrupted hippocampal CA3-CA1 synaptic plasticity by regulating levels of neuronal proteins required for the development of CA1 neurons and synapses, and so regulating the molecular composition of synapses. We will employ a candidate strategy to explore the autophagic mechanisms for synaptic and cognitive deficits in *Tsc1/2* deficient mice.

Accomplishments: In **Years 1 and 2** of this project, we had compared genome-wide translation efficiencies between rapamycin treated and untreated control and Tsc2+/- mice and ranked all genes based on their translation efficiency fold-change, as proposed in task 3a. Unexpectedly, we failed to find altered translation of neuronal enriched genes in the Tsc2+/-; RiboTag mice (n=4). These data confirmed that the Tsc2 heterozygous mutation does not interfere with the translational profile, and that the disrupted protein and synaptic protein homeostasis in the Tsc2+/- mice may be largely due to suppressed protein degradation, instead of exaggerated translation. When both WT and Tsc2+/- mice were treated with rapamyin, we found that only genes containing the TOP-motif are significantly enriched among the genes that are translationally downregulated by rapamycin treatment.

In **Year 3**, we had run both RNA-seq and ribosome profiling analysis in Tsc1^{mGFAPCre}CKO mice at the age of 5-6 weeks (not shown). We did not find significant common changes in the abundance of RNA transcripts in both knockout mouse lines. The RNA-Seq gene expression profile however show significant changes in astrocyte and synaptic proteins in 3.5month old Tsc1^{mGFAPCre}CKO mice with severe seizures, which we considered as a secondary response to recurrent seizures. Similar to our preliminary data in Tsc2+/- mice, we found significant changes in the translation of TOP-motif genes in rapamycin treated mice, indicative of the consequence of mTOR suppression by rapamycin.

These data were unexpected. As such, we proceed to our original research aim 3b on the proteome of total hippocampal lysates and excitatory synapses, with the hope that we can isolate the specific effects of neuronal autophagy on synapse/neuronal development. We had performed the complicate mouse breeding for excitatory synapse specific proteomics. We had crossed Atg7$^{flox/flox}$ mice to PSD95$^{flox/flox}$ mice. The resulting Atg7$^{flox/+}$:PSD95$^{flox/+}$ mice were self-crossed to obtain Atg7$^{flox/flox}$:PSD95$^{flox/flox}$ double homozygous breeders. We then crossed the Atg7$^{flox/flox}$:PSD95$^{flox/flox}$ males to PSD95$^{flox/flox}$;CamkCre+ females to obtain female Atg7$^{flox/+}$:PSD95$^{flox/flox}$;Camkcre+ breeders. By further crossing the male Atg7$^{flox/flox}$:PSD95$^{flox/flox}$ mice to the female Atg7$^{flox/+}$:PSD95$^{flox/flox}$;Camkcre+ mice, we were able to obtain Atg7$^{CamkCre+}$CKO;PSD95$^{flox/flox}$ and PSD95$^{flox/flox}$:CamKCre control mice at both ages of postnatal day 23-24 (P23) and 34-35 (P35). During the no cost extension period, we will continue to collect brain tissues from each group at different ages for synapse proteomics, synaptic affinity purification and western blot analysis.



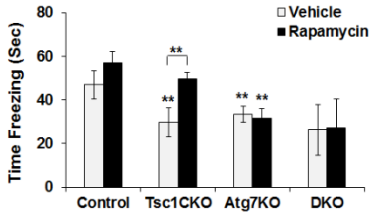


Figure 1. Neurological symptoms in Tsc1^{mGFAPCre}CKO mice. (**A-F**) Open field behaviors; (**G, H**) Three chamber social interaction test. G, preference for social target; **H**, preference for social novelty; (**I**) Novel object recognition test; (**J**) Fear conditioning test. Left, contextual feared memory; right, cued memory; (**K**) Survival rate in Tsc1^{mGFAPCre}CKO mice; (**L**) Seizure onset in Tsc1^{mGFAPCre}CKO mice; (**M**) Seizure behaviors in Tsc1^{mGFAPCre}CKO mice. (**N**) Rapamycin (P19-P42) rescues contextual feared memory in Tsc1^{mGFAPCre}CKO (Tsc1CKO) but not in Atg7^{mGFAPCre}CKO (Atg7KO) mice. Compared to control, *, $P<0.05$; **, $p<0.01$.