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TITLE: Exploring the Interaction between TSC2, PTEN, and the NMDA Receptor in Animal Models of Tuberous Sclerosis

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14. ABSTRACT
During the first year of this award, we completed much of the work proposed under Specific Aim 1: to examine the subunit composition of the NMDA receptor in Pten and Tsc2 knockout mice. First, we examined the subunit composition of the NMDA receptor in conditional NS-Pten knockout mice at postnatal day 21. The analysis revealed that NMDA receptor subunits are expressed at similar levels in NS-Pten mutant compared to wild type mice. However, the expected Pten loss and mTOR activation was partial. Second, we generated conditional NS-Tsc2 knockout mice and conducted an initial characterization of the phenotype. Heterozygous NS-Tsc2 mice appeared normal, but most homozygous mutant mice died prematurely. We thus analyzed NS-Tsc2 mutant mice at postnatal day 10, and found that the NMDA receptor appears normal. However, there was no Tsc2 loss or mTOR activation. Therefore, we further investigated the activity of the NS promoter used for both lines, and discovered that it does not induce significant gene deletion at developing ages. Together, the results indicate that the NS promoter is not suitable for our proposed studies. Therefore, we plan to switch to another Cre driver line, the NEX-Cre, which we previously shown to cause strong Pten gene deletion in conditional knock out mice.

15. SUBJECT TERMS
Pten, NMDAR, TSC

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INTRODUCTION

Tuberous Sclerosis Complex (TSC) is a genetic disease resulting from the loss of either the Tsc1 or the Tsc2 genes. These genes produce proteins that inhibit the growth-promoting factor mTOR. This mechanism may account for tumor susceptibility, however, it is not clear whether it underlies cognitive dysfunction in TSC patients. Loss of Tsc2 also results in the increased expression of Pten, another tumor-suppressor gene that works upstream of mTOR. Since Pten affects the expression of the NMDA receptor, a neurotransmitter receptor that is very important for learning and cognition, it is possible that changes in this receptor may be relevant to cognitive dysfunction. In this study, we are examining whether the loss of Tsc2, like that of Pten, in addition to activate the mTOR signaling pathway, also causes changes in the expression of NMDA receptor subunits in animal models.

BODY

During the first year of this award, we completed much of the work proposed under Specific Aim 1: to examine the subunit composition of the NMDA receptor in Pten and Tsc2 knockout mice. The proposed work was based on the use of conditional knock out mice in which deletion of the Pten or Tsc2 gene occurs in a neuronal subset (NS) due to the expression of a ‘driver’ GFAP-Cre transgene (Kwon et al., 2001). The goal of this work was to examine and compare the effects of the loss of Pten versus that of Tsc2 in the same neuronal population. The NS-Pten mutant mouse colony was already established in my laboratory, but the NS-Tsc2 line was generated during this past year. The results of our experiments are described in detail below according to the proposed tasks.

Task 1. Establish the NMDA receptor profile of NS-Pten mutant mice (months 1-12):

1a. Breeding of Cre+, Pten heterozygous mice to generate wild type, heterozygous and homozygous mutant mice (months 1-3).

We intercrossed NS-Cre+/+;Pten wt/fl mice and generated homozygous (Cre+/+;Pten fl/fl), heterozygous (Cre+/+;Pten wt/fl) and wild type (Cre+/+;Pten wt/wt) mice at postnatal day (P) 21. We chose this age to avoid the confounding effect of spontaneous seizures, which typically begin around 1 month of age as shown in our previous studies (Ljungberg et al., 2009).

1b. Dissection of the hippocampus, protein extraction and Western blot analysis of NMDA receptor subunits (months 3-12).

We euthanized 3 sets of homozygous (KO), heterozygous (Het) and wild type (Wt) NS-Pten mice, dissected the hippocampus and prepared whole tissue protein homogenate (Hom) as well as crude synaptosomal fractions (P2) as previously described (Ventru et al., 2011). We then proceeded to analyze the levels of NMDAR subunits NR2A, NR2B and NR1 in homogenate and synaptosomal fractions by Western blotting (Fig. 1). All subunit levels were normalized to actin, and the data were statistically analyzed using the one-way ANOVA test. Contrary to our expectation, the results indicate that there are no significant differences between genotypes.
To ensure that Pten deletion and activation of the PI3K/mTOR signaling pathway had occurred at least in homozygous mutants, we performed Western blot analysis of Pten, PI3K and mTOR targets such as phosphoAkt (for mTORC2) and phosphoS6 (for mTORC1) (Fig. 2). The blots were reprobed for total Akt, total S6 and actin to normalize for protein content in each lane. Unlike previous studies utilizing adult mice (Kwon et al., 2003), the present results indicate that the levels of Pten at P21 were only modestly reduced in homozygous mutants. The levels of phosphoThr308-Akt (pAkt(T)) were significantly increased in KO mice, indicating a significant increase in PI3K signaling. However, the levels of phosphoSer473-Akt (pAkt(S)), a target of mTORC2, and phosphoSer240/244-S6 (pS6), a downstream target of mTORC1 were only marginally or not affected at all. These data suggest a partial expression of NS-Cre in the hippocampus of NS-Pten mice at P21.

Fig. 1. NMDA receptor subunit expression in NS-Pten mice

Fig. 2. Pten expression and PI3K/mTOR signaling in NS-Pten mice

Task 2. Regulatory review and approval of animal protocol

We added NS-Tsc2 mice to our protocol and received approval by IACUC. A copy of the approval letter is attached to the Appendix.

Task 3. Establish the NMDA receptor profile of NS-Tsc2 mice (months 6-12):
3a. Breeding of homozygous floxed mice with hGFAP-Cre positive mice to generate an NS-Tsc2 colony (months 4-8).

We obtained floxed Tsc2 mice described in (Way et al., 2009), and crossed them to NS-Cre+/+ mice. The initial matings of Tsc2 fl/fl males with NS-Cre +/- females generated F1 progenies consisting of double heterozygous NS-Cre+/-;Tsc2 wt/fl mice. Some of these mice were backcrossed to NS-Cre +/- mice to generate the preferred breeders (NS-Cre+/-;Tsc2 wt/fl) for colony maintenance.

3b. Breeding of Cre+, Tsc2 heterozygous mice to generate wild type, heterozygous and homozygous mutant mice (months 6-9).

While waiting for the generation of optimal NS-Cre+/-;Tsc2 wt/fl, we proceeded to backcross F1 double heterozygous NS-Cre+/-;Tsc2 wt/fl mice with the original Tsc2 fl/fl males. This mating scheme generated, as expected, approximately 25% homozygous mutants (NS-Cre+/-;Tsc2 fl/fl), 25% heterozygous mutants (NS-Cre+/-;Tsc2 wt/fl), and 50% wild type controls (NS-Cre+/-;Tsc2 fl/fl or NS-Cre-/-;Tsc2 wt/fl) (Table 1).

<table>
<thead>
<tr>
<th>Mouse Type</th>
<th>Genotype</th>
<th>n of mice born</th>
<th>% of total</th>
<th>% expected</th>
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<tbody>
<tr>
<td>Homozygous mutant</td>
<td>Cre +/-;Tsc2 fl/fl</td>
<td>16</td>
<td>24</td>
<td>25</td>
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<tr>
<td>Heterozygous mutant</td>
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<tr>
<td>Control</td>
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<tr>
<td></td>
<td>Cre +/-;Tsc2 fl/wt</td>
<td>18</td>
<td>18</td>
<td>25</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>40</td>
<td>51</td>
<td>50</td>
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Table 1. NS-Tsc2 mice born in 9 litters (67 mice total) from F1 backcross matings

However, many of the homozygous mutants die prematurely between postnatal day 8 and 15. When a cohort of 16 homozygous mutants was observed, only 1/3 survived until P19 (Fig. 3). No premature death was observed in heterozygous mutants or controls.

3c. Dissection of the hippocampus, protein extraction and Western blot analysis of NMDA receptor subunits (months 9-12).

Because of the unexpected early lethality of homozygous NS-Tsc2 mice, we sampled a cohort of NS-Tsc2 mice at P10. First, we dissected the neocortex (CX), hippocampus (HC), and cerebellum (CB) of 2 NS-Tsc2 homozygous (KO) and 1 control mouse (C) and analyzed NMDAR
NR2 subunits (Fig. 4). The data show that there are no differences between mutants and control.

Next, we checked for Tsc2 loss and PI3K/mTOR activation by Western blotting in a larger set of P10 littermates. The data show that there is no detectable loss of Tsc2 or activation of the signaling pathway in homozygous or heterozygous mutant mice in any of the analyzed brain structures at this age (Fig. 5).

Thus, our Western blot analysis failed to identify defects in NMDA receptor subunit expression in NS-Ts2 mice as well as in NS-Pten mice. However, because the data also failed to detect the expected gene deletion and mTOR signaling activation in mutant mice (particularly in younger NS-Tsc2 mice), we hypothesized that the expression of Cre in these NS lines may be too low at the ages analyzed to cause detectable effects. Therefore, we conducted an additional experiment where we crossed the original NS-Cre driver line with a tdTomato fluorescent reporter line (B6.Cg-Gt(Rosa)26Sortm9(CAG-tdTomato)Hze/J) we obtained from the Jackson Laboratories. The F1 progeny (NS-Cre+/--; tdTomato+/−) was sacrificed at P14 and P30, and brain sections were imaged using a fluorescence microscope (Fig. 6). The image (overlaid with the DAPI nuclear stain, blue) shows that the tdTomato reporter (red) is expressed in few cells of the cerebral cortex and cerebellum, and is limited to the dentate gyrus in the hippocampus at P14. At later time points (P30), the reporter is expressed in many more cells of the cerebral cortex and cerebellum, although it remained limited to the dentate gyrus in the hippocampus.
To further investigate mTOR signaling at a cellular level, we sectioned the brain of an additional set of homozygous NS-Tsc2 mutant and a control littermate at P10, and conducted pS6 immunofluorescence. The data reveals that overall mTOR activation is not significantly increased in mutant mice at this age, except perhaps for few isolated cells.

These findings indicate that NS-Cre driven conditional mutants are not suitable for the proposed studies, and that cell population analysis, such as Western blotting, are not informative in developing mice.

Adult time points could not be collected in NS-Tsc2 mice due to early lethality of the mutants, and in NS-Pten due to spontaneous seizures. Therefore, we are forced to modify our proposed research strategy for the upcoming year of support by this grant.

In our original proposal, we had envisioned the following Tasks for the second year of support:

**Task 4.** Establish the NMDA receptor profile of NS-Pten/Tsc2 double mutant mice (months 12-24).

**Task 5.** Establish the NMDA receptor profile of NS-Tsc2 mutant mice treated with rapamycin (months 12-24).

These tasks were based on the assumption that NS-Tsc2 mice would have abnormal NMDA receptor subunit expression, and were designed to understand whether this defect was mediated by changes in Pten or mTOR activity. However, in light of the results obtained so far, we propose New Tasks for the remaining period of this award. The general goal of the new Tasks is the same as before: to identify and compare NMDA receptor abnormalities in similar Pten and Tsc2 conditional mutant mice, and to gain insights into the molecular mechanism. However, the Cre driver line will be different. Instead of using NS-Cre, we will use the NEX-Cre driver, which is expressed in virtually all excitatory neurons of the cerebral cortex and hippocampus since perinatal ages (Goebbels et al., 2005). NEX-Pten mice have already been generated in my laboratory. In a recent publication (Kazdoba et al., 2012), we already demonstrated that, even though homozygous Pten mutants die during the first postnatal week, defects in NR2A and NR2B expression are detectable by Western blotting in the perinatal forebrain. These defects are accompanied by a strong activation of PI3K/mTOR signaling in homozygous Pten mutants. Interestingly, we also noted a tendency to increased signaling and NR2A and NR2B subunit expression in heterozygous Pten mutants (Kazdoba et al., 2012). However, additional animals are needed to determine statistical significance. Building on these previous studies, we now propose the following new Tasks:

**New Task 4:** To complete the analysis of mTOR signaling and NMDA receptor subunits in heterozygous and homozygous NEX-Pten mice (months 12-15). Significant NR2 subunit defects were previously found in homozygous mutants. Next, we will determine whether NR1 defects also exists, and whether heterozygous mutants express an intermediate, but significant phenotype. We will dissect the forebrain from newborn wild type, heterozygous and homozygous NEX-Pten mice and conduct Western blot analysis. To confirm gene deletion we
will first probe the blot with Pten antibodies. To confirm activation of the PI3K/mTOR signaling, we will probe with pAkt(T), pAkt(S) and pS6 antibodies and measure the ratio of phosphorylated to total proteins. To examine the NMDA receptor subunits we will use antibodies against NR2A, NR2B and NR1. To determine whether defects are specific for the NMDA receptor, we will also examine AMPA receptor subunits GluR1 and GluR2/3. At least 5-6 sets of animals of each genotype will be used to obtain statistical significance using one-way ANOVA to directly compare all three genotypes.

New Task 5: To analyze mTOR signaling and NMDA receptor subunits in heterozygous and homozygous NEX-Tsc2 mice (months 12-18). We will generate a NEX-Tsc2 mutant line as we have done previously for NEX-Pten (Kazdoba et al., 2012). We will examine the forebrain of newborn wild type, heterozygous and homozygous NEX-Tsc2 mice by Western blotting exactly as described above for NEX-Pten mice.

If NMDA receptor defects are present in both NEX-Pten as well as NEX-Tsc2 mutant mice, we will investigate whether these defects are mediated by mTORC1:

New Task 6. To examine mTORC1 activity and NMDA receptor expression in NEX-Tsc2 and NEX-Pten mutant mice treated with rapamycin (months 18-24). Mutant mice will be treated perinatally with the mTORC1 inhibitor rapamycin or a control buffer for 2 days as described (Kazdoba et al., 2012). The forebrain will be analyzed as described above by Western blotting. The levels of mTORC1 signaling will be determined first to ensure that the rapamycin treatment is effective. Second, we will examine the expression levels of all NMDA receptor subunits. If normal levels are restored by rapamycin we will conclude that they depend on mTORC1 signaling. If they are not, we will determine whether Pten and Akt are altered in NEX-Tsc2 mice, and design further studies to investigate the mechanism. If NMDA receptor defects are present only in NEX-Pten but not in NEX-Tsc2 mutants, we will omit the rapamycin treatment of NEX-Tsc2 from this Task. Additional treatments of NEX-Pten mutants with Pten or Akt inhibitors will be performed if time allows it.

**KEY RESEARCH ACCOMPLISHMENTS**

During year 1 of this exploratory grant we have accomplished the following:

1. We have analyzed NS-Pten mice for Pten loss, PI3K/mTOR signaling and NMDA receptor expression at a late postnatal (P21) age by Western blotting. Unexpectedly, we found partial Pten loss, partial activation of the PI3K/mTOR signaling, and no abnormalities in the NMDA receptor.
2. We generated a new NS-Tsc2 line. Unexpectedly, we found that homozygous mutant mice died prematurely mostly during the second postnatal week. The cause of death is unexplained as no obvious brain abnormality is present.
3. We therefore analyzed Tsc2 loss, PI3K/mTOR signaling and NMDA receptor expression at a younger (P10) age. However, no abnormalities at all were found.
4. We suspect that our negative data are due to a technical problem with the NS-Cre driver line (insufficient Cre expression), rather than a lack of effect of Pten and Tsc2 on the NMDA receptor. Using a reporter gene approach and immunofluorescence we have confirmed that limited Cre expression is a significant problem in the NS lines.

**REPORTABLE OUTCOME**

We developed a novel NS-Tsc2 conditional knock out mouse line.
CONCLUSIONS

The goal of this proposal was to investigate the relationship between Pten or Tsc2 and the NMDA receptor using NS promoter-driven conditional knock out mice. However, the studies conducted during this past year failed to establish the proposed link. We considered two alternative possibilities: 1) Loss of Pten or Tsc2, and the consequent activation of PI3K/mTOR signaling has no impact on the NMDA receptor. This conclusion would disprove our hypothesis. However, it would be contrary to the observation that Pten loss in different NEX-Pten conditional knock out mice alters NR2 subunit expression (Kazdoba et al., 2012). 2) The loss of Pten or Tsc2 is limited to too few cells in the NS promoter-driven conditional knock out mice, hindering the interpretation of the negative results. Given that there is limited or no detectable Pten and Tsc2 gene loss, and limited or no activation of PI3K/mTOR signaling in these mutants, together with cell-restricted reporter gene and pS6 expression in developing brain structures, we conclude that NS-Cre driven conditional mutants are not suitable for the proposed studies. We therefore plan to switch to the analysis of NEX-Cre driven lines, which induce massive genetic deletion in the perinatal forebrain. A NEX-Pten line has already been generated in my laboratory and homozygous mutants have been shown to have NMDA receptor defects. A NEX-Tsc2 line will be generated and analyzed during the second year of this award. The role of mTORC1 in the etiology of NMDA receptor abnormalities in Pten and possibly also in Tsc2 mutant mice will be further established. Since NMDA receptor abnormalities are relevant to the expression of cognitive defects in TSC patients, our study will provide a better understanding of this clinical aspect of the disease, and potentially suggest new targets for therapy.

REFERENCES


APPENDICES
The protocol identified below, has been reviewed and approved by the Rutgers University Institutional Animal Care and Use Committee, IACUC.

This approval is given with the following stipulations:
1. The materials submitted to the IACUC contain a complete and accurate description of all the ways in which animals are used in your research, demonstration or teaching. All procedures involving live vertebrate animals outlined in grants, which reference this protocol, must correspond to this protocol.
2. Research or instruction will be conducted according to the plans and protocols submitted.
3. Any emergent problems, proposed significant procedural changes or changes in personnel working with animals will be immediately communicated to the IACUC.
4. The principal investigator must maintain continuance of Occupational Health approval. Personnel assigned to this protocol will not commence work with animals until approved by the Occupational Health Program for animal workers. A current listing is attached.
5. Personnel will not commence work with animals until they have completed Animal Orientation Training.
6. Visitors, including the media, will not be taken into the animal facility, nor will they be allowed to take photographs or video tapes of animals without the prior approval of the IACUC.
7. A copy of the protocol and all subsequent correspondences will be maintained by the principal investigator.
8. The additional conditions noted below will be followed.

Failure to comply with these terms will result in the withdrawal of this approval.

**Principal Investigator:** D'Arcangelo, Gabriella

**Protocol Number:** 07-037

**Title of Protocol:** MOUSE BREEDING FOR BRAIN RESEARCH

**Stress Level:** D

**Species:** Mouse

**Period of Approval:** 03/25/2013 to 03/24/2014

**Listing of Approved Occupational Health Personnel:** See attached.

**Additional Condition(s):**

Note: Applicable Standard Operating Procedures and IACUC documents noted in the conditions below can be found at https://acfc.rutgers.edu

1. Each and every shipment of mice from other than approved commercial vendors must be approved in advance by LAS.
2. Follow the IACUC document: "CO2 Euthanasia"
3. Follow the IACUC document: "Custom Formulated Compounds for Use in Animals"
4. Follow the IACUC document: "Performing Rodent Survival Surgery"
5. Follow the IACUC document: "Tissue Collection for Genotyping"
6. LAS to observe electroporation and Plasmid DNA injection into brain ventricles.

Rutgers University maintains an Assurance with the Office of Laboratory Animal Welfare, the assurance number is A3262-01. Rutgers has maintained accreditation with the Association for the Assessment and Accreditation of Laboratory Animal Care-International since July 8, 1994.

At least one month prior to the end of the period of approval, PI will be sent a copy of the form Request for Continuing Review, which should be submitted online at https://acfc.rutgers.edu. If the project ends before that time, please notify Lauren Zizza in writing.

Contact Lauren Zizza at 848-932-4012 with any questions about this approval.