progress in neurobiology

Peer Review Overview

Manuscript Title: "mTOR-driven neural circuit changes initiate an epileptogenic cascade"

Received	06-Jul-2020
1st Decision	17-Aug-2020
Revision Submitted	19-Oct-2020
Accepted	05-Dec-2020

Decision Letter

Dear Dr. Danzer,

Thank you for submitting your manuscript to Progress in Neurobiology.

We have completed our evaluation of your manuscript. The reviewers recommend reconsideration of your manuscript following major revision. We invite you to resubmit your manuscript after addressing the comments below. Please resubmit your revised manuscript by Oct 16, 2020.

When revising your manuscript, please consider all issues mentioned in the reviewers' comments carefully: please outline every change made in response to their comments and provide suitable rebuttals for any comments not addressed. Please note that your revised submission may need to be re-reviewed.

To submit your revised manuscript, please log in as an author at https://www.editorialmanager.com/proneu/, and navigate to the "Submissions Needing Revision" folder.

Progress in Neurobiology values your contribution and we look forward to receiving your revised manuscript.

Kind regards,

Jeannie Chin
Associate Editor

Sabine Kastner
Editor-in-Chief
Progress in Neurobiology

Editor and Reviewer comments:

Reviewer #1: LaSarge et al.
"mTOR-driven neural circuit changes initiate an epileptogenic cascade"

This work describes the effects of knocking out the Pten gene in increasing numbers of adult generated dentate granule neurons. Measurements of seizures, synaptic transmission, and field potential responses are then regressed against %KO to determine if there is a graded worsening of the phenotype. In most cases the answer is interpreted as "yes," with a few exceptions. Interneuron number is also assessed in animals with high %KO and reductions are found in PV and NPY/SST expressing neurons. The results are then interpreted as providing evidence for both the onset of epilepsy appearing abruptly after an insult and progressively.
This is an interesting model to study given the ability to vary the %KO and the relevance of mTOR signaling to epilepsy. The experiments are technically impressive and appear well done with a few exceptions. The immediate interpretations of the experiments are also mostly sound (e.g. increased % PTENko leads to increased line length). The overall framing of the paper and the larger interpretations of the authors, however, are confusing. I disagree that the data supports conclusions about whether epilepsy onset appears "abruptly after an insult or progressively, in incremental steps." This seems like a retrofitted hypothesis. In my opinion the study isn't designed or analyzed to address this question. The

paper needs to be rewritten to better reflect the data, which are inherently interesting without trying to spin them into this translational framework.

Instead of straightforwardly saying that they are studying how Pten loss or mTOR activation causes seizures, the authors use PTENko in newborn granule neurons as a "tool to introduce abnormal granule cells into the dentate," reasoning that "abnormal granule cells are a hallmark pathology of temporal lobe epilepsy," in an attempt to model an "injury-induced" epilepsy that develops "over months to years." The conflation of trying to understand how PTENko causes epilepsy and extrapolating these results to protracted epileptogenesis in an injury-induced epilepsy contributes to the imprecise nature of the paper's conclusions. The assumption that these two things are equivalent is a strong one.

The results of the correlation analyses should be interpreted cautiously, especially when trying to compare across different experiments (like saying some changes are abrupt while others are graded), and using different types of correlation (Pearson, Spearman, linear).The question may be better suited to asking what type of equation best describes the data (linear. Loglinear, etc.)

Figure 1: "Analyses of the combined data sets reveals the presence of distinct KO cell load thresholds for different phenotypes." There is no analysis done here that supports this claim. Even if an analysis was designed to test this, there aren't enough data points here to make the conclusion that there are "distinct thresholds" for the electrographic phenotypes. The only conclusion the data supports is the qualitative one that low %KO is sufficient to cause hippocampal pathology while high %KO is needed to cause generalized seizures.

Figure 2: The relationship between %KO and sEPSC frequency is not strong, and may be driven primarily by the one large outlier (800, 35) and the group at %15 KO. Between these values the fit is pretty flat.
It is unclear whether the recorded cells are control cells or KO cells. From the supplementary table it looks like some animals had a fluorescent reporter and others did not. If unknown, then %KO may be correlated with sEPSC frequency simply because there is a higher chance of getting a KO cell.
Example traces should be included.
What about sEPSC amplitude? I assume this data is available.
The sIPSC data should be removed from the paper. With the internal and external solutions used by the authors and the holding potential (-30 — -40 mV), the driving force for Cl will be only about 25 mV, which is too low to detect most IPSCs. I suspect many of these detected events are noise, which is another reason example traces should be shown.

Figure 3: I'm not familiar with the use of the fEPSP threshold as a measurement of "release properties of afferents from the EC." Please provide a justification for this interpretation. I think the fEPSP threshold would reflect a mixture of release properties and postsynaptic responsiveness of the granule neurons. The slope of the fEPSP is traditionally plotted against stimulation strength as a correlate of postsynaptic responsiveness. It is unclear why this wasn't done. I expect that it would be increased in the PTENko slices.

Figure 6: This figure doesn't add much and if anything undermines the conclusion that optogenetic inhibition is normalizing the field potential response. The reader has to look really closely to see any differences in the patterns at all and the differences in treatment are not apparent. A scale bar should be included as well.

Figure 7: I am confused by the fact that there appear to be far more PV neurons that coexpress SST than PV alone. These two populations are thought to be primarily non-overlapping in the cortex. I guess its possible that the hilus is very different, but this also raises the possibility of non-specific labeling or cross-reactivity. The authors should present additional evidence that they haven't misclassified these populations or included false positive staining.
Why weren't low %KO animals stained for interneuron markers? If the authors' speculation that the PtenKO neurons are directly exciting and killing hilar interneurons is correct then even low %KO animals should show some interneuron loss.

Reviewer #2:

The manuscript PRONEU-D-20-00279 entitled "mTOR-driven neural circuit changes initiate an epileptogenic cascade" describes a new model of epileptogenesis in tamoxifen-inducible Gli1-CreERT2, Ptenflox/flox mouse. With these mice, they generated a graded epileptogenic insult by single, or multiple, injections of tamoxifen at two postnatal ages to create mice with variations in the numbers of hippocampal granule cells deficient for Pten. This work is of considerable interest and builds upon a large body of evidence pointing to excessive mTOR signaling in both genetic and acquired epilepsies. Pten

deletion causes epilepsy in animal models. The novelty of this study however is that the timing and dose of tamoxifen was used to conditionally delete Pten over a range of 0% to 40% of the granule cells in the dentate gyrus. This approach provides an experimental paradigm to ask how an increased "load" of Pten knockout (KO) granule cells relates to hyperexcitable networks, granule cell hyperexcitability, and epileptogenesis. Varying the timing or dose of tamoxifen resulted in a range of deletion rates in granule cells in different mice. The number of Pten KO cells was determined by immunostaining brain sections with rabbit anti-Pten and Neurotrace Nissl, then after confocal imaging of the dentate gyrus to detect KO cells/total Neurotrace cells in the granule cell layer they obtained z-stacks and quantified the KO cells/total with the optical fractionator method and stereology. They also noted distinctive morphological changes in Pten-deficient granule cells, including abnormally large somas, increased dendritic spines, and hilar-projecting basal dendrites.

A threshold effect of Pten KO was found when comparisons were made between hippocampal and cortical seizures using EEGs and electrophysiological depth recordings. For seizures to be detected in the cortical EEG recordings, the KO load needed to be 15% or greater, whereas hippocampal depth recordings detected seizures with KO cell loads of 4-5%. These data suggest that greater KO loads were associated with spread of seizures outside of the hippocampus. Moreover, KO load was correlated with spontaneous excitatory postsynaptic currents (eEPSCs) in dentate granule cells when the KO load increased by >15%, as shown by patch-clamp electrophysiological recordings. It was increased by as much as 83% in high KO slices. These correlations suggested a gradual increase in network activity as the KO load increases. The effect was also associated with an overall decrease in spontaneous inhibitory postsynaptic currents (sIPSCs) in KO cells relative to controls. They also conducted field potential recordings from acute hippocampal slices while stimulating the lateral perforant path to target projections from the entorhinal cortex to distal dendrites of hippocampal granule cells. The excitatory postsynaptic field potential (fEPSP) produced by lateral perforant path stimulation was followed by a population spike. By expressing Archaerhodopsin in the KO neurons, they optogenetically silenced Pten KO cells, while measuring the threshold for fEPSPs. KO in granule cells did not alter the release properties of Pten-expressing afferents from entorhinal cortex in these experiments, but the population spike threshold was significantly reduced in slices from high KO animals. Interestingly, the population spike threshold dropped abruptly, even with only small numbers of KO cells. Silencing the KO cells optogenetically increased the population spike thresholds, but failed to restore the normal response in some high KO mice. Also, the population spikes increase in number, size and duration with increasing KO cell load. Thus, this model, Pten KO cells receive increased excitatory drive and decreased inhibition. Interestingly, Pten KO also induced secondary changes in the circuit, including a significant loss of NPY/SST and PV+ cells.

Overall, this is an excellent study that presents novel findings. This work will be of high interest to many neuroscientists. The new observation is that conditional ablation of Pten in granule cells in the adult hippocampus using different times and doses, allows a tightly-controlled experimental insult to the granule cells and leads to circuit-wide changes in excitability, inhibition, and interneuron loss. The authors have nicely demonstrated that this genetic manipulation leads to an accumulation of abnormal granule cells, which instigates the development of hyperexcitability. The title and abstract accurately reflect the content. The highlights are adequate. The statistical analyses are appropriate and the authors have included very good controls. Overall, the figures are clear and of high quality. I have only minor suggestions for improvement.

1. The rationale and background for using Gli1-CreERT2 +/-, Ptenflox/flox, DREADDflox/wt mice is not described in the text, apart from the methods and Supplemental Table 1, Supplemental Table 2, and Supplemental Table 3. Were these mice used as controls? If so, the authors should show the data (possibly with a separate symbol) and/or revise the findings in the text. If not, the description in the methods and the tables should be removed.
2. A sentence or two describing the specificity of the Pten deletion just in granule cells is needed. Were other brain regions examined? How widespread is Pten expression in other brain regions?
3. A sentence or two about the rationale for using the Gli1-CreERT2 driver line would help to clarify why this transgenic line is used to drive Pten deletion in granule cells and whether it potentially can result in complete deficiency in all granule cells.
4. The authors should why 40% was the maximum KO achieved. Does this approach delete Pten in postnatal developing granule cells (if so, when?) and/or mature granule cells or both? Are the Pten deficient granule cells dispersed throughout the granule cell layer or are they more restricted?
5. The data presented in Figure 3D, E, H, are difficult to see because of the extensive overlap of the symbols. Can the symbols be larger in panel D? Similarly, the symbols are very small and overlapping, and difficult to see in panels E and H.

Author Response Letter

October 19, 2020

Dear Drs. Chin and Kastner

We would be pleased if you would consider our revised manuscript entitled "**mTOR-driven neural circuit changes initiate an epileptogenic cascade"** for publication in Progress in Neurobiology.

We have responded point-by-point to each reviewer critique, and include new data and analyses in the revision. Notable changes include new analyses of physiology data, and inclusion of a new cohort of mice to confirm cell loss and correlate loss with Pten KO cell load. The text has also been extensively revised to provide better focus and clarity for the reader, as requested by reviewer #1.

We greatly appreciate your time and attention. Please contact me if any additional information is required.

Sincerely,

Steve Danzer, PhD

Professor of Anesthesia

Director, Center for Pediatric Neuroscience

C. Nelson Melampy Chair in Anesthesia Basic Science Research

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Email: Steve.Danzer@cchmc.org

Response to Review
Manuscript Number: PRONEU-D-20-00279
mTOR-driven neural circuit changes initiate an epileptogenic cascade

We thank the reviewers for their time and helpful feedback. Point-by-point responses to each reviewer critique are presented in blue below, and changes throughout the text are highlighted.

Reviewer #1:

The overall framing of the paper and the larger interpretations of the authors, however, are confusing. I disagree that the data supports conclusions about whether epilepsy onset appears "abruptly after an insult or progressively, in incremental steps." This seems like a retrofitted hypothesis. In my opinion the study isn't designed or analyzed to address this question. The paper needs to be rewritten to better reflect the data, which are inherently interesting without trying to spin them into this translational framework. Instead of straightforwardly saying that they are studying how Pten loss or mTOR activation causes seizures, the authors use PTENko in newborn granule neurons as a "tool to introduce abnormal granule cells into the dentate," reasoning that "abnormal granule cells are a hallmark pathology of temporal lobe epilepsy," in an attempt to model an "injury-induced" epilepsy that develops "over months to years." The conflation of trying to understand how PTENko causes epilepsy and extrapolating these results to protracted epileptogenesis in an injury-induced epilepsy contributes to the imprecise nature of the paper's conclusions. The assumption that these two things are equivalent is [not??] a strong one.
AR: The reviewer raises a fair point. To make the study goals and results clearer, we have extensively revised the abstract, introduction and discussion. The revised text now focuses on the implications of our study for mTORopathies, rather than the broader issue of temporal lobe epileptogenesis.

The results of the correlation analyses should be interpreted cautiously, especially when trying to compare across different experiments (like saying some changes are abrupt while others are graded), and using different types of correlation (Pearson, Spearman, linear).The question may be better suited to asking what type of equation best describes the data (linear. Loglinear, etc.)
AR: We have revised the text to present the correlation analyses in a more balanced fashion. We did also examine the suggested statistical approaches with the help of a statistician, but the results did not further inform the interpretation, and so are not included in the revision.

Figure 1: "Analyses of the combined data sets reveals the presence of distinct KO cell load thresholds for different phenotypes." There is no analysis done here that supports this claim. Even if an analysis was designed to test this, there aren't enough data points here to make the conclusion that there are "distinct thresholds" for the electrographic phenotypes. The only conclusion the data supports is the qualitative one that low %KO is sufficient to cause hippocampal pathology while high %KO is needed to cause generalized seizures.
AR: We agree and have revised the text accordingly in line with the reviewer's suggestions.

Figure 2: The relationship between %KO and sEPSC frequency is not strong, and may be driven primarily by the one large outlier (800, 35) and the group at %15 KO. Between these values the fit is pretty flat.
AR: We reanalyzed the data with the outlier removed and confirmed the reviewer's impression that the outlier drove the significant effect. We were not able identify any technical reasons to justify removing this outlier (other than it being an outlier), so in the interest of transparency we now present statistical findings with and without the outlier, and include additional caveats (Lines 417 and 424).

It is unclear whether the recorded cells are control cells or KO cells. From the supplementary table it looks like some animals had a fluorescent reporter and others did not. If unknown, then %KO may be correlated with sEPSC frequency simply because there is a higher chance of getting a KO cell.
AR: All cells were confirmed to be KO cells, in accord with immunohistochemically-validated (PTEN-ih) morphological criteria (LaSarge et al., 2019). This is now clarified in the methodology (Line 192).

Example traces should be included.
AR: Example sEPSC traces have been added to figure 2.

What about sEPSC amplitude? I assume this data is available.
AR: The revised manuscript now includes sEPSC amplitude data, which is described in the methods (line 196) and presented in the results (Line 427) and in figure 2. No difference in sEPSC amplitude was observed between control and KO cells, although a modest positive correlation between sEPSC amplitude and KO cell load was found. This significant effect was also driven by a couple outliers, so we also interpret it cautiously.

The sIPSC data should be removed from the paper. With the internal and external solutions used by the authors and the holding potential (-30 — -40 mV), the driving force for Cl will be only about 25 mV, which is too low to detect most IPSCs. I suspect many of these detected events are noise, which is another reason example traces should be shown.
AR: sIPSC data has been removed from the paper as requested.

Figure 3: I'm not familiar with the use of the fEPSP threshold as a measurement of "release properties of afferents from the EC." Please provide a justification for this interpretation. I think the fEPSP threshold would reflect a mixture of release properties and postsynaptic responsiveness of the granule neurons.
AR: We agree that the statement was unclear and have removed it from the revised text.

The slope of the fEPSP is traditionally plotted against stimulation strength as a correlate of postsynaptic responsiveness. It is unclear why this wasn't done. I expect that it would be increased in the PTENko slices.
AR: We have previously established that fEPSP slope is increased in high KOs, and this prior work is referenced. In addition, we include new supplemental data (Line 463; supplemental figure 1) showing fEPSP slopes and amplitudes at each stimulus intensity, and the impact of KO cell silencing on these measures.

Figure 6: This figure doesn't add much and if anything undermines the conclusion that optogenetic inhibition is normalizing the field potential response. The reader has to look really closely to see any differences in the patterns at all and the differences in treatment are not apparent. A scale bar should be included as well.
AR: In accord with the reviewer's comments, figure 6 has been eliminated.

Figure 7: I am confused by the fact that there appear to be far more PV neurons that coexpress SST than PV alone. These two populations are thought to be primarily non-overlapping in the cortex. I guess its possible that the hilus is very different, but this also raises the possibility of non-specific labeling or cross-reactivity. The authors should present additional evidence that they haven't misclassified these populations or included false positive staining.
AR: We were surprised by the degree of overlap as well. We have conducted all the standard controls (systematic exclusion of primary and secondary antibodies to test for cross reactivity and bleed through). We also reviewed the literature in greater depth to look for papers specifically examining PV/SST colocalization in the hippocampus. We found surprisingly few studies that have addressed this directly, however, Jinno and Kosaka (J. Comparative Neurology, 2000; 428:377-388) do report a 15% overlap between PV and SST in the ventral hilus. This is still less than we observed. The difference could reflect greater sensitivity with newer antibodies and confocal microscopy approaches. Nonetheless, we agree that the issue deserves additional follow up and confirmation with multiple antibodies and/or genetic approaches. Since this is beyond the scope of the current study, we have revised the presentation of the data in the main text to just focus on individually labeled SST, PV and NPY neurons. In retrospect, colocalization findings are interesting, but are of

unclear significance at this point. Therefore, we removed colocalization results from the present manuscript so that we can pursue the findings in greater detail in the future.

Why weren't low %KO animals stained for interneuron markers? If the authors' speculation that the PtenKO neurons are directly exciting and killing hilar interneurons is correct then even low %KO animals should show some interneuron loss.
AR: The revised manuscript now includes a new data set examining NPY, SST and PV interneuron density in high and low KO animals. The new data replicated the prior finding, revealing significant negative correlations between all three interneuron classes and KO cell load (methods line 276; results line 629 and Figure 6). Examination of the data did not reveal strong evidence of cell loss in low KOs, however, we agree with the reviewer's earlier comment that the studies aren't designed to determine whether cell loss begins abruptly or gradually. Gradual cell loss in low KOs could be difficult to detect. Alternatively, cell loss may follow the appearance of cortical seizures. Establishing the sequence of events leading to cell loss evident in high KOs will require future studies.

Reviewer #2:

1. The rationale and background for using Gli1-CreERT2 +/-, Ptenflox/flox, DREADDflox/wt mice is not described in the text, apart from the methods and Supplemental Table 1, Supplemental Table 2, and Supplemental Table 3. Were these mice used as controls? If so, the authors should show the data (possibly with a separate symbol) and/or revise the findings in the text. If not, the description in the methods and the tables should be removed.
AR: The study included three animals carrying the DREADD receptor. These animals were used for EEG monitoring. The animals were part of another study and did not receive CNO. Other than the presence of the DREADD gene, therefore, the animals should behave identically to other controls and KOs in the study. This is now clarified in the methods (line 106 and the animals in question are now identified by unique symbols in figure 1 (#'s).

2. A sentence or two describing the specificity of the Pten deletion just in granule cells is needed. Were other brain regions examined? How widespread is Pten expression in other brain regions?
AR: The approach utilized here leads to Pten deletion from a subset of hippocampal granule cells, olfactory granule cells and small numbers of glial cells. This has been characterized extensively in our prior publication using this model (Pun et al., 2012). The specificity of PTEN deletion is now described in the text (line 348).

3. A sentence or two about the rationale for using the Gli1-CreERT2 driver line would help to clarify why this transgenic line is used to drive Pten deletion in granule cells and whether it potentially can result in complete deficiency in all granule cells.
AR: The rationale and mechanism of Gli1 targeting of granule cells is now covered in greater detail in the introduction (line 70). In principle, it should be possible to use the Gli1 driver line to target 100% of granule cells, but this would require treating pregnant dams with tamoxifen to capture granule cells generated before birth, and would lose cellular specificity, as prenatal granule cell production overlaps the production of many other neuronal types, which are also driven by Gli1-expressing progenitors.

4. The authors should why 40% was the maximum KO achieved. Does this approach delete Pten in postnatal developing granule cells (if so, when?) and/or mature granule cells or both? Are the Pten deficient granule cells dispersed throughout the granule cell layer or are they more restricted?

AR: Gli1 drives cre-mediated recombination in granule cell progenitors, but not mature granule cells. Since tamoxifen was given after the peak of granule cell neurogenesis (P7), recombination was limited to the minority of granule cells born after this point. Clarification of these issues is now provided in the introduction and results (lines 70, 360).

5. The data presented in Figure 3D, E, H, are difficult to see because of the extensive overlap of the symbols. Can the symbols be larger in panel D? Similarly, the symbols are very small and overlapping, and difficult to see in panels E and H.
AR: We thank the reviewer for the suggestion and have revised the figure accordingly.