BRIEF REPORT

REVISED Neuronal subset-specific *Pten*-deficient mice do not exhibit deficits in sensorimotor gating processes [version 2; peer review: 2 approved with reservations]

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Abstract

Background: Deficits in sensorimotor gating have been reported in individuals with autism spectrum disorder (ASD), as well as in ASD murine models. However, this behavior has not been examined in the neuronal subset-specific (NS)-*Pten* knockout (KO) model of ASD. NS-*Pten* KO mice exhibit hyperactivity of the PI3K/AKT/mTOR signaling pathway which is implicated in the onset of autistic deficits. This study investigates the potential relationship between PI3K/AKT/mTOR signaling and deficits in sensorimotor gating.

Methods: To assess sensorimotor gating in NS-*Pten* KO mice we utilized a three-day paradigm. On day 1 (habituation) the mice were administered 80 repetitions of a 120-dB startle stimulus. On day 2, prepulse inhibition was measured with 90 trials of the startle stimulus that was paired with a smaller (2, 7, or 12 dB) prepulse stimulus. Day 3 was assessed one week later, consisting of randomized startle trials and trials with no stimulus and was used to determine the startle threshold.

Results: No significant difference between NS-*Pten* KO or wildtype (WT) mice was found for habituation ($p > 0.05$). No significant differences were found between groups when assessing the percentage of prepulse inhibition at 2, 7, and 12 dB ($p > 0.05$). There was also no difference in startle threshold between groups ($p > 0.05$).

Conclusion: Our study found that the NS-*Pten* KO model does not display significant deficits in sensorimotor gating processes. The present findings help to elucidate the relationship between PI3K/AKT/mTOR hyperactivation and sensory reactivity.

Keywords

autism, pten, macrocephaly, ASD, sensorimotor

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Any reports and responses or comments on the article can be found at the end of the article.

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Author roles: **Binder MS:** Data Curation, Formal Analysis, Investigation, Writing – Original Draft Preparation, Writing – Review & Editing; **Nolan SO:** Data Curation, Writing – Original Draft Preparation, Writing – Review & Editing; **Lugo JN:** Conceptualization, Formal Analysis, Funding Acquisition, Project Administration, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing

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REVISED Amendments from Version 1

The updated version of the manuscript is in response to the comments from reviewers 1 and 2. We provided more justification for the inclusion of males in the study and elaborated on the details of the sensorimotor gating assessment used in the experiments. We added the weights of the wildtype and knockout mice over the three testing days and included the raw data. The additional data resulted in the addition of an additional graph in Figure 1. Figure 1d is new. We included the statistical results for the weights in the results section of the paper. In the discussion section, we elaborated on the differences between the results found in our study and in others.

Any further responses from the reviewers can be found at the end of the article

Introduction

Sensorimotor gating is the ability of a sensory stimulus to suppress a motor response¹. It can be measured by assessing prepulse inhibition (PPI), wherein a weak auditory stimulus inhibits a startle response that is induced by the following presentation of a loud sound². Deficits in PPI have been widely reported in various neurological conditions, including autism spectrum disorder (ASD)^{3–5}. Similar to humans, impairments in PPI have been reported in ASD models such as *Fmr1* and *Cntnap2*-knockout (KO) mice; however, the underlying mechanism is unknown^{6,7}. *Pten* mutant mice are another model of autism and can be used to investigate the connection between a cell signaling pathway commonly implicated in ASD, the PI3K/AKT/mTOR pathway, and specific autistic-like deficits⁸. Specifically, neuronal subset specific (NS) *Pten* KO mice have previously been shown to exhibit deficits in repetitive behavior, sociability, and communication, however, prepulse inhibition has not been assessed in this model^{9,10}. In the present study, we used NS-*Pten* KO mice that exhibit hyperactivation of the PI3K/AKT/mTOR pathway in the cortex, hippocampus, and cerebellum, and assess PPI in order to further elucidate the potential relationship between PI3K/AKT/mTOR signaling and deficits in sensorimotor gating⁹.

Methods

Subjects

Male and female mice on a FVB based mixed background were obtained from Baylor College of Medicine and have been bred for more than 10 generations at Baylor University. Heterozygous NS-*Pten* males ($n=6$) and females ($n=12$) were used to breed NS-*Pten* wildtype (WT) and KO pups (RRID: MGI:3714016). The housing for the breeders consisted of two females housed with one male. Genotype was determined from toe clippings taken on postnatal day (PD) 10 (performed by Mouse Genotype, Escondido, CA, USA). On PD 21, animals were weaned and housed with mixed genotype littermates in groups of $n=3–5$ in cages (Allentown Caging PC7115HT, Allentown, PA, USA) filled with sani-chip bedding (7090 Teklad, Envigo, Somerset, NJ, USA) kept in a room on a 12-hr light/dark diurnal cycle held at 22°C. Mice had *ad libitum* access to food and water. All animals were tested at 9–10 weeks of age between

the hours of 10:00 and 11:30 a.m. Only males were assessed in this study to be in accordance with the literature, as previous behavioral phenotyping studies in the NS-*Pten* adult model were done in males^{9,10}. Furthermore, a similar study investigating sensorimotor gating in neuron-specific enolase (Nse)-*Pten* KO mice also only assessed males¹¹. Therefore, in order to make an accurate comparison and to provide similar context with past studies we did not assess females. A total of 29 male mice were assessed, 17 NS-*Pten* KO and 12 WT mice. The target sample size was determined by, and is in accordance with, the PPI literature^{11–13}. The final sample sizes were as follows: day 1: $n=12$ WT, $n=17$ KO, day 2: $n=12$ WT, $n=13$ KO, day 3: $n=9$ WT, $n=9$ KO. All test procedures were carried out in compliance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by Baylor University's Institutional Animal Care and Use Committee. Once the experiment concluded, mice were placed into a CO₂ chamber and euthanized.

Sensorimotor gating assessment

Sensorimotor gating was assessed via the SR-LAB system, which consists of a 15 × 14 × 18 inch isolation cabinet, a plexiglass cylinder (3.2-cm diameter) mounted on a sensor platform, and a speaker that generated white noise, as well as the stimuli (San Diego Instruments, San Diego, CA, USA). The paradigm consisted of three separate testing days: habituation, prepulse inhibition, and startle response, and was conducted as previously described⁶.

For habituation, the animal was acclimated to the room for 30 minutes then was placed inside the cylinder for a 5-minute habituation period, which was followed by 80 startle stimuli delivered at a fixed interval of 15 seconds. The startle stimulus was a 40 ms, 120 dB noise burst, with a rise/fall time of less than 1 ms. Prepulse inhibition testing occurred 24 hours after day 1 and consisted of a 5-minute habituation phase that was followed by 20 presentations of a 40 ms, 120 dB noise burst. In the prepulse phase, mice were presented with 90 trials consisting of three prepulse intensities that were 2, 7, and 12 dB over the 68 dB background noise. The onset of the prepulse occurred 100 ms before the onset of the startle pulse. Each prepulse was 20 ms in duration and were spaced an average of 15 seconds apart (7–23 s). One week after the prepulse session, the startle threshold was assessed. Following the 5-minute habituation period, the mice were presented with 99 trials of 11 trial types. These included a no stimulus trial and 10 startle stimuli trials ranging from 75–120 dB at 5 dB intervals. The startle stimuli were 40 ms noise bursts. The 11 trial types were pseudorandomized, with each trial type being presented once in a block of the 11 trials. To eliminate potential confounds during testing, background sound levels were maintained at 68 dB and the experimenter was not present.

Statistical analysis

GraphPad Prism 7 software (La Jolla, CA) or SPSS 21.0 (IBM, USA) were used to analyze the data. Repeated-measure ANOVAs were run for habituation, prepulse inhibition, and startle threshold. The within subject factors for habituation were the trials (1-10, 11-20, 21-30, 41-50, 51-60, 61-70, and

71-80) with genotype as the between subjects factor (wildtype and knockout). For prepulse inhibition, the within-subjects factors were the prepulse intensities (2, 7, and 12 dB), with genotype as the between subjects factor. For the startle threshold, the between-subjects factors were the stimulus intensities (no stimulus, startle at 75, 80, 85, 90, 95, 100, 105, 110, 115, and 120 dB), with the between subject factor of genotype. To provide a general indicator of the overall health of each animal throughout the study, the subject's weights were assessed with a repeated measures ANOVA that had a within subjects factor of day (weight of the subject for testing days 1, 2, and 3) and a between subjects factor of genotype. No post-hoc tests were performed. A total of $n=4$ KO mice were excluded from the day 2 analysis and $n=11$ mice (3 WT and 8 KO) were excluded from the day 3 analysis due to protocol malfunction or death as a result of the

severity of the knockout. A value of $p < 0.05$ was considered significant for each statistical test.

Results

When assessing the sensorimotor gating paradigm, there were no main effects of genotype for habituation ($F[1,27] = 0.17$, $p > 0.05$), prepulse inhibition ($F[1,23] = 2.65$, $p > 0.05$) or startle threshold ($F[1,16] = 2.33$, $p > 0.05$). There was also no interaction between genotype and trial for habituation ($F[7,189] = 0.91$, $p > 0.05$), genotype and prepulse intensity for prepulse inhibition ($F[2,46] = 0.71$, $p > 0.05$), or genotype and stimulus intensity for the startle threshold ($F[10,160] = 1.94$, $p > 0.05$) (Figure 1a–c). When assessing the weight of each subject throughout the study, no main effect for the within subjects factor of day was found ($F[2,30] = .11$, $p > .05$), nor was there a day by genotype interaction ($F[2,30] = .17$,

Figure 1. Habituation, prepulse inhibition, and startle threshold in NS-*Pten* KO mice. (a) We found that there was no significant difference in habituation between KO and WT mice ($p > 0.05$). (b) We found no difference in the percentage of prepulse inhibition between groups following prepulses that were 2, 7, and 12 dB over the 68 dB background noise ($p > 0.05$). (c) We observed no difference in startle threshold between NS-*Pten* KO and WT mice ($p > 0.05$). (d) We observed no differences in weight between NS-*Pten* KO and WT mice ($p > 0.05$). Data are presented as the mean $\pm$ standard error of the mean (SEM).

$p > .05$). There was also no between subjects effect of genotype ($F[1,15] = 1.16$, $p > .05$) (Figure 1d). Raw results for each procedure on each day for every animal are available as *Underlying data*¹³.

Discussion

The NS-*Pten* KO mice did not exhibit significantly different sensorimotor gating from WT mice. A previous study by Kwon *et al.* (2006) assessed neuron-specific enolase (Nse)-*Pten* KO mice in a variation of the PPI protocol and reported a decrease in percent inhibition at 4 dB but no differences at 8 or 16 dB¹¹. Our study assessed percent inhibition at 2, 7, and 12 dB, per established protocol, and found no differences at these intensities⁶. This discrepancy in sensorimotor gating between similar models may be due to the timing of the induction of cre expression. Cre expression is induced following neuronal differentiation in Nse-*Pten* KO mice, however, in NS-*Pten* KO mice, the model we assessed, cre expression is induced prior to neuronal differentiation. Therefore, our study indicates that a premitotic expression of cre does not result in sensorimotor deficits. Additionally, in accordance with our study, no differences in prepulse inhibition have been reported in the BTBR and *Shank1* mouse models of autism^{14,15}. Altogether, this indicates that alterations in sensory reactivity may be a less sensitive measure of an autistic-like phenotype and may also only be present in particular ASD models.

Overall, the current study found that hyperactivity of the PI3K/AKT/mTOR pathway does not result in sensorimotor gating deficits in NS-*Pten* KO mice, suggesting that the pathway may not directly affect prepulse inhibition. This conclusion is supported by a prior study that assessed PPI in a transgenic mouse model of tuberous sclerosis complex, another model of ASD and mTOR hyperactivation, which similarly reported no deficits in prepulse inhibition between WT and KO mice¹⁶. Taken together, these studies indicate that despite mTOR's

contribution to an autistic-like phenotype, it does not significantly contribute to the onset of sensorimotor gating deficits in several different ASD models. Ultimately, our study is in support of the literature and helps to further elucidate the relationship between hyperactivation of the PI3K/AKT/mTOR pathway and deficits in sensory reactivity.

Data availability

Underlying data

Figshare: Neuronal subset-specific *Pten*-deficient mice do not exhibit deficits in sensorimotor gating processes. <https://doi.org/10.6084/m9.figshare.9885401.v2>¹³.

This project contains the following underlying data:

- PPI Day1 *Pten* Raw Data 9-6.xlsx (raw data from all experiments performed for all animals; day 1).
- PPI Day 2 *Pten* Raw Data 9-6.xlsx (raw data from all experiments performed for all animals; day 2).
- PPI day 3 *Pten* Raw Data 9-6.xlsx (raw data from all experiments performed for all animals; day 3).
- *Pten* data weights *Pten* ppi weights.xlsx (raw data for weights across testing dates).

Data are available under the terms of the [Creative Commons Zero “No rights reserved” data waiver](#) (CC0 1.0 Public domain dedication).

Acknowledgements

We would like to thank Samantha Hodges and Paige Womble for their critical review of the paper. The authors do not have any conflicts of interest to declare.

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Version 2

Reviewer Report 12 February 2020

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Benjamin K. Yee

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While the interpretation that neuron-specific deletion of (NS)-Pten did not substantially alter the acoustic reflex startle (ASR) and PPI of ASR largely agrees with the impression of the data presented, the reporting of the data is not sufficiently stringent. Some responses to the previous comments are inadequate.

Please rephrase the sentence: "When assessing the sensorimotor gating paradigm, there were no main effects of genotype for habituation ($F[1,27] = 0.17, p > 0.05$), prepulse inhibition ($F[1,23] = 2.65, p > 0.05$) or startle threshold ($F[1,16] = 2.33, p > 0.05$)."

This sentence is awkward because of the word "for", I think. It simply intends to indicate that the main effect of genotype is not significant in the ANOVAs of the data obtained on the habituation test on day 1, the prepulse inhibition test on day 2, and the startle reactivity profile on day 3. My comment before focuses on the fact that the main effect of genotype cannot inform on the habituation of startle response.

Legends of Figure 1: Description of (a) and (c) have been mixed up. Either correct the legends or the placement/labels of the two plots.

The reasons behind the drop of animals from Day 1 to Day 3 should be provided in the text, although it is available from the links to the raw data files.

Mouse 2355 was reportedly dead on Day 2 but provided body weight on Day 3 (see data file - pten ppi weights.xlsx in the underlying data).

It appears that the total number of animals included in the weights analysis across days was 17. It was specified that Day 3 test comprised $9 + 9 = 18$ mice. One mouse's data was missing. I suspect the missing mouse was the one reported dead on Day 2 but not dead on Day 3.

The data distribution of the data set for "threshold analysis" was highly skewed. It may be appropriate to log transform the data set, which incidentally strengthen the impression that the

two genotypes were closed matched in their reactivity profile (as a function of stimulus intensity).

The response to the critique concerning the lack of any attempt to index (indeed, or to define) “threshold” is inadequate. The group by intensity interaction in the ANOVA evaluate the extent to which the two reactivity curves (as function of stimulus intensity) are parallel. The main effect of genotype evaluated the average “height” of the reactivity curve between groups. While it may be true that the genotype and genotype by stimulus intensity did not reach statistical significance. Neither of them directly addresses “threshold”. One may admit that if the two lines are indeed identical and thus no difference in threshold would be possible (no matter how one may define it), then neither the main effect of genotype nor the interaction would achieve statistical significance. The reverse argument does not necessarily stand.

The authors may consult this article by Hince *et al.*¹ for curve fitting methods to obtain proxies for a startle threshold value (e.g., stimulus intensity that yield 50% of the maximum reaction generated by the most intense stimulus) for individual animals.

My previous comment was not clear because I had examined the data set in question trial-by-trial, rather than just block-by-block in the reported ANOVA of data on Day 1. Below is the plot that clearly shows that the startle reaction obtained in trial 1 was notably weaker than the rest. This anomaly warrants an explanation. First, this is highly unusual. Typically we expect the highest response in the first few trials. Second, Day 1 was designed to examine habituation, yet it produced a strong sensitization effect from trial 1 to trial 2. Some checking on the SR-LAB programme or derivation of raw data may be warranted, even though it is not likely that a systemic error in, and if limited to, a single trial could substantially change the statistical outcomes.

Plot

The final concluding sentence warrants revision. it reads “...our study is in support of the literature and helps to further elucidate the relationship between hyperactivation of the PI3K/AKT/mTOR pathway and deficits in sensory reactivity”.

However, the present null results, if anything, are not supportive of a functional link between hyperactivation of the PI3K/AKT/mTOR pathway (as a result of the genetic knockout) and any deficits in sensory reactivity as exemplified by the acoustic startle reflex and its modulation in the form of PPI.

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Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Prepulse inhibition, Behavioural phenotyping of mutant mice, animal models of schizophrenia

I confirm that I have read this submission and believe that I have an appropriate level of

expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 30 Mar 2020

Joaquin Lugo, Baylor University, Waco, USA

Dear Reviewer,

Please see our rebuttal for your comments. We appreciate your input and believe the paper has been improved as a result of your input. Thank you.

1. Thank you for your comment, the sentence has been modified to state: "When assessing the sensorimotor gating paradigm, the main effect of genotype was not significant in the ANOVAs of the data obtained on the habituation test on day 1 ($F[1,27] = 0.17, p > 0.05$), the prepulse inhibition test on day 2 ($F[1,23] = 2.65, p > 0.05$) or the startle response test on day 3 ($F[1,16] = 2.10, p > 0.05$)" to clarify our results.
2. The figure legend has been changed to reflect that the habituation data is depicted in figure 1a and the startle response data is depicted in figure 1c.
3. Thank you for your suggestion the following sentence has been added to the subjects section of the methods. "A subset of $n = 4$ KO mice were excluded from the day 2 analysis and $n = 11$ mice (3 WT and 8 KO) were excluded from the day 3 analysis due to either a protocol malfunction or the death of the KO animal caused by the occurrence of spontaneous seizures."
4. Per your insight, mouse 2355's weight for day 2 has been added to the excel weight spreadsheet, as the animal survived throughout testing.
5. The excel page has been updated to include the missing weight data to reflect the sample size used in analysis.
6. The data was transformed using a log transformation per your insight, and analysis for the startle analysis were rerun. No differences were detected, the results and methods section have been updated to reflect this transformation.
7. Thank you for clarifying your previous comment. You raise a terrific point; we should not have referred to graph as startle threshold as it depicts the average startle response at each decibel level which does not in and of itself constitute a threshold. Therefore, the graph title has been changed to: "Average Startle Response." All other references to this data as startle threshold have been replaced with "Startle Response". Importantly, the analysis we ran for startle amplitude is in line with other papers in the field such as Frankland et al., (2004), Brody & Geyer (2004) etc. which was done to maximize our paper's points of comparison.

Frankland, P., Wang, Y., Rosner, B. *et al.* Sensorimotor gating abnormalities in young males with fragile X syndrome and *Fmr1*-knockout mice. *Mol Psychiatry* **9**, 417–425 (2004).

<https://doi.org/10.1038/sj.mp.4001432>

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factors on startle and prepulse inhibition in mice. *neurotox res* **6**, 79–90 (2004).
<https://doi.org/10.1007/BF03033300>

8. Please see preceding response.

9. To ensure clarity, is the graph in question Day 1 Habituation (Figure 1a) or is it the Day 3 Startle Response (Figure 1c)? The Day 1 Habituation graph does not appear to conform to the pattern you are describing, as the first trial (1-10) exhibits the highest startle amplitude of approximately 1500 for WT and KO mice whereas the next trial (11-20) are below 1500, making the first trial the one with the highest startle amplitude for Day 1 Habituation. Moreover, the pattern of habituation for the remainder of the trial conforms to an expected trend (Schmid et al., 2011). Furthermore, all SR-LAB protocols and data were rechecked, re-exported, and re-analyzed and the exact same figure was generated. Therefore, since the protocol is functioning as expected and the rest of the data points display the anticipated pattern, and the pattern we observed has been previously reported, it indicates that there was no systemic error present.

Meanwhile, the Day 3 Startle Response has a slightly higher no stimulus startle amplitude than the 75 dB stimulus, which may be what you are referring to. Importantly, Frankland et al., (2004) also reported a higher startle amplitude for the no stimulus trial and a lower startle amplitude for the second trial, similar to our findings, reinforcing our observations. Additionally, it is significant to note that the difference between trials 1 and 2 is slight and that the rest of the trials conformed to the expected curve (Gould et al., 2004). Once again, all SR-LAB protocols and data were rechecked, re-exported, and re-analyzed and the exact same figure was generated. Therefore, since the protocol is functioning as expected and the rest of the data points display the anticipated pattern, and the pattern we observed has been previously reported, it again indicates that there was no systemic error present. Additionally, when the 75 dB stimulus intensity trial is removed from analysis no significant effects were again found, therefore, the lack of significance reported for Day 3 Startle response was not dependent upon that one trial. Ultimately, we elected to keep the 75 dB trial in the results section of the paper and in the figure in order to be transparent with our data and to judicially report all findings, as the observed data points in question were not due to protocol malfunction or other confounds and did not bias statistical analysis.

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10. Thank you for your suggestion, the final sentence has been changed to be more precise and states: “Ultimately, our study contributes to the literature and suggests that the relationship between hyperactivation of the PI3K/AKT/mTOR pathway and deficits in sensory reactivity is modest.”

Competing Interests: No competing interests were disclosed.

Version 1

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Maarten van den Buuse

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This Brief Report shows that a specific Pten-deficient mouse model shows no change in prepulse inhibition compared to wildtype controls. This is discussed in the context of literature about changes in PPI in humans with autism and in autism animal models. Albeit negative, this report could then be of value as an additional component of this literature. Unfortunately there are some problems with the study and the way it is presented.

Abstract, background:

- "has been rarely examined" - does this mean it has been examined once before? What was the result?

Introduction:

- It would be helpful if the neuronal subset-specific Pten KO mouse was described in more detail. What do we know about behavioural changes in this mouse model. Importantly, were there deficits in social behaviour? If this has not been published yet, it would be good to add some of those additional behavioural tests here.

Methods, subjects:

- Why were only males included in the study?

Methods, sensorimotor gating:

- As far as I know, the SR-LAB system has only one speaker and there is no "high-frequency" speaker (what is that anyway) to produce the stimuli.

Methods, sensorimotor gating:

- It would be more clear if the prepulses were described as level over background, i.e. PP2, PP7 and PP12.

Methods, sensorimotor gating:

- What was the interval between the onset of the prepulse and the onset of the startle pulse (SOA)? What was the interval between various trials in the PPI protocol (ITI)? Is it, like the figure legend suggests, always 15 seconds? This would be unusual because the ITI is variable in most of the PPI literature.

Results:

- Details of the statistical analysis are missing. In the Statistical Analysis section in the Methods, or in the Results section, it has to be explained what the between-group and within-group factors are and main effects and importantly interactions between those factors have to be detailed. For example, for PPI, was there a prepulse intensity x genotype interaction?

Discussion:

- Again, it would be more clear if the prepulses were described as level over background, i.e. PP2, PP7 and PP12. This would allow better comparison with previous studies.

Figure 1:

- Along the horizontal axis do not use labels at an angle.

Figure 1, legend:

- A lot of technical detail here should be included in the Methods section, not in a figure legend. There is also no need to constantly repeat rise-fall times. This can be mentioned once in the Methods as a feature of all stimuli.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

No

Are all the source data underlying the results available to ensure full reproducibility?

No source data required

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Prepulse inhibition, animal models of psychiatric disease.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 04 Feb 2020

Joaquin Lugo, Baylor University, Waco, USA

Reviewer 2. The original comments are first followed by a bullet point with our response.

Abstract, background:

"has been rarely examined" - does this mean it has been examined once before? What was the result?

- Thank you for your comment. The sentence has been modified to clarify that no other study has assessed prepulse inhibition in neuronal subset specific KO mice.

Introduction:

It would be helpful if the neuronal subset-specific Pten KO mouse was described in more detail. What do we know about behavioural changes in this mouse model. Importantly, were there deficits in social behaviour? If this has not been published yet, it would be good to add some of those additional behavioural tests here.

- Per your insight, a sentence has been added into the introduction that details the established behavioral phenotype of NS-*Pten* KO mice, clarifying that this model presents with deficits in repetitive behavior, sociability, and communication.

Methods, subjects:

Why were only males included in the study?

- Previous studies investigating the adult phenotype in the NS-*Pten* model only assessed males (see citations 9 and 10 in the document). Moreover, a similar study that assessed PPI using the Nse-*Pten* mouse also only investigated males (citation 11). In order to best align our study with other pertinent studies, and to provide similar points of comparison, we also only assessed male mice, acting in accordance with the literature.

Methods, sensorimotor gating:

As far as I know, the SR-LAB system has only one speaker and there is no "high-frequency" speaker (what is that anyway) to produce the stimuli.

- It has been clarified in the methods that 1 speaker was used to generate the white noise and startle stimuli.

Methods, sensorimotor gating:

It would be more clear if the prepulses were described as level over background, i.e. PP2, PP7 and PP12.

- Thank you for your suggestion, the methods have been changed to discuss the

prepulses used as being relative to the background noise.

Methods, sensorimotor gating:

What was the interval between the onset of the prepulse and the onset of the startle pulse (SOA)? What was the interval between various trials in the PPI protocol (ITI)? Is it, like the figure legend suggests, always 15 seconds? This would be unusual because the ITI is variable in most of the PPI literature.

- The methods section has been modified to state that the SOA was 100 ms and to clarify that the ITI in the PPI procedure was an average of 15 seconds with individual trials ranging from 7-23 seconds.

Results:

Details of the statistical analysis are missing. In the Statistical Analysis section in the Methods, or in the Results section, it has to be explained what the between-group and within-group factors are and main effects and importantly interactions between those factors have to be detailed. For example, for PPI, was there a prepulse intensity x genotype interaction?

- Per your insight, the statistical analysis section now clarifies what the between subjects and within subjects' factors are for each test day. Additionally, the results section has been amended to make it clearer that there were no main effects or interactions present for any test day.

Discussion:

Again, it would be more clear if the prepulses were described as level over background, i.e. PP2, PP7 and PP12. This would allow better comparison with previous studies.

- The discussion section now refers to the prepulses in terms of their increase over the background level (ppi 2,7, and 12 dB) in order to make clearer comparisons to other studies.

Figure 1:

Along the horizontal axis do not use labels at an angle.

- The labels on the x axis for all figures are now horizontal.

Figure 1, legend:

A lot of technical detail here should be included in the Methods section, not in a figure legend. There is also no need to constantly repeat rise-fall times. This can be mentioned once in the Methods as a feature of all stimuli.

- The detail concerning each testing day has been moved to the methods section. Also, the rise fall times are now only mentioned once.

Competing Interests: No competing interests were disclosed.

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Benjamin K. Yee

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Whether sensorimotor gating, as evaluated by the prepulse inhibition (PPI) of the acoustic startle reflex paradigm, is attenuated or exaggerated in ASD is still controversial. The present study attempted to investigate this using a mutant mouse model. Specifically, neuronal deletion of *Pten* in the mouse is expected to result in P13K/AKT/mTOR hyperactivity implicated in ASD onset. The study may potentially clarify whether this genetic manipulation would be sufficient to modify PPI expression. No difference between mutants and wild type (WT) mice was reported. Indeed, the magnitude, habituation and threshold of the startle response as such were reported to be highly comparable between genotypes. The null results led the authors to conclude that the contribution of the mTOR pathway to ASD-related PPI deficits is limited. Closer examination of the methods and data reveal significant concerns that undermine confidence in the reliability and robustness of the reported findings.

1. No attempt was made to examine sex difference, while it is highly relevant to ASD.
2. Methodology details were not sufficient. Essential test parameters such as ITI and SOA in prepulse-pulse trials were not reported. Wide of response window was not reported, although it could be discerned from the raw data file.
3. Apparently, 8 (out of 17) mutant mice died from Experiment 1 to Experiment 3. This led one to suspect that the mutant mice had serious and widespread physiological defects, which could undermine any meaningful comparison. One would like to see body weights reported at least. Were the mutants significantly lighter?
4. Statistical results are poorly reported. Only “main effects” (supposedly the genotype effects) were considered. Statistics towards ascertaining the presence of startle habituation (e.g., Trials or blocks of 10 trials effects), and prepulse inhibition (the effect of prepulse intensities) etc. are not provided. To report that “no main effects were found for habituation $F(1,27)=...$ ” is inappropriate, because the comparison of habituation between genotypes could only be meaningfully evaluated by reference to the Genotype x Blocks of 10 trials interaction. Reporting the main effect of Genotype does not allow an effective assessment of the habituation profile, merely the overall magnitude of startles.
5. The plot shown in Figure 1a cannot be reproduced from the raw data provided.
6. It is also observed that the first trial of Day 1 data were all very low (in all mice). This is highly unusual and may indicate a protocol failure, or misalignment of data.
7. Examination of Day 2 data for PPI assessment also reveals another anomaly. At least 4 mice (ID: 2081, 2084, 2085, 2072) exhibited very weak startle values (well under 100) in all

“120startle” trials – substantially lower than the startle magnitude obtained on the previous startle habituation test. The change is massive and inexplicable. The problem may be more extensive and include other mice. The authors should exercise due diligence in examining their data before analysis.

8. In their discussion of Kwon et al.'s (2006) reported findings of a PPI deficit (Nse)-Pten KO mice, the authors mistook the prepulses at 4dBm 8dB and 16dB as the actual magnitude of the prepulses used by Kwon et al. In fact, these refer to prepulse of intensity at 4, 6 and 18 decibels units above background. The use of 70, 75 and 80dB prepulses here were presented against a background noise level of 68dB, and thus effectively be +2, +7 and +12 decibel units above background. Hence, it is incorrect to conclude (by comparison between the present study and Kwon et al.) that “there may only be changes in percent inhibition in Pten mutant mice when the prepulse in comparatively quiet”. If anything, the +2 (or 70 dB) condition here was even lower than the weakest prepulse used by Kwon et al.
9. The authors evaluated the startle reactivity curve as a function of increasing pulse intensity – as a means to examine the “startle threshold”. Yet no attempt was made to measure individual startle threshold for comparison between genotypes. Otherwise, it is misleading to conclude that threshold did not differ when only the group's average profile was presented.

Hence, although the available data tend to support the overall lack of an effect of the gene KO on PPI, the methods, presentation, data analysis are clearly inadequate.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Prepulse inhibition, Behavioural phenotyping of mutant mice, animal models of schizophrenia

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 04 Feb 2020

Joaquin Lugo, Baylor University, Waco, USA

Reviewer comments are first followed by a bullet point of our response.

No attempt was made to examine sex difference, while it is highly relevant to ASD.

- Thank you for your comment. Previous studies investigating the adult phenotype in the *NS-Pten* model only assessed males (see citations 9 and 10 in the document). Moreover, a similar study that assessed PPI using the *Nse-Pten* mouse also only investigated males (citation 11). In order to best align our study with other pertinent studies, and to provide similar points of comparison, we also only assessed male mice, acting in accordance with the literature.

Methodology details were not sufficient. Essential test parameters such as ITI and SOA in prepulse-pulse trials were not reported. Wide of response window was not reported, although it could be discerned from the raw data file.

- The methods section has been modified to state that the SOA was 100 ms and to clarify that the ITI in the PPI procedure was an average of 15 seconds with individual trials ranging from 7-23 seconds.

Apparently, 8 (out of 17) mutant mice died from Experiment 1 to Experiment 3. This led one to suspect that the mutant mice had serious and widespread physiological defects, which could undermine any meaningful comparison. One would like to see body weights reported at least. Were the mutants significantly lighter?

- Per your suggestion, the weight data for WT and KO mice across each testing timepoint were analyzed. No differences in weight were found between WT and KO mice at any test point. *NS-Pten* KO mice do present with spontaneous seizures that can result in death, however, the KO mice that did not die prematurely did not display a significantly different weight from the controls, indicating that their constitution was sufficient to reliably assess the effects of PPI. Furthermore, our timepoints of testing are in accordance with the literature, making our comparison with other studies valid. Lastly, a graph of the weight data comparing WT to control mice per each testing timepoint has been created and has been uploaded.

Statistical results are poorly reported. Only “main effects” (supposedly the genotype effects) were considered. Statistics towards ascertaining the presence of startle habituation (e.g., Trials or blocks of 10 trials effects), and prepulse inhibition (the effect of prepulse intensities) etc. are not provided. To report that “no main effects were found for habituation $F(1,27)=...$ ” is inappropriate, because the comparison of habituation between genotypes could only be meaningfully evaluated by reference to the Genotype x Blocks of 10 trials

interaction. Reporting the main effect of Genotype does not allow an effective assessment of the habituation profile, merely the overall magnitude of startles.

- Thank you for your input, the results section has been updated to better specify the statistical tests run and the corresponding results. Additionally, the overall statistical design of the study has been added to the statistical analysis section in the methods. Regarding the statistical measure used, we agree with you that a main effect by itself is not sufficient to best assess the data, that is why we also included the statistics for the interactions of each test. For habituation, no interactions were found, indicating that the stated results are an effective assessment of the habituation profile and that our statistics were not improper. In light of your comment, the results section has been reworded in order to better highlight this and to clarify any ambiguity.

The plot shown in Figure 1a cannot be reproduced from the raw data provided.

- The plot shown in figure 1 a was created by taking the data in the T-AB columns in the excel document for the habituation day then pasting them into a grouped data file in Graphpad. The x axis for the grouped file was the trials 1-10, 11-20, etc and group A was the WT whereas group B was the KO. All data analyzed and graphed came from the corresponding excel documents.

It is also observed that the first trial of Day 1 data were all very low (in all mice). This is highly unusual and may indicate a protocol failure, or misalignment of data.

- We do not understand this comment. The first trial in day 1 shows the largest startle response. Please clarify your comment.

Examination of Day 2 data for PPI assessment also reveals another anomaly. At least 4 mice (ID: 2081, 2084, 2085, 2072) exhibited very weak startle values (well under 100) in all "120startle" trials – substantially lower than the startle magnitude obtained on the previous startle habituation test. The change is massive and inexplicable. The problem may be more extensive and include other mice. The authors should exercise due diligence in examining their data before analysis.

- We ran additional analysis to examine this, specifically, the mice in question were removed from analysis and the analysis was rerun excluding them, no difference between genotype was found ($F(1,20) = .17, p = .69$). Therefore, the results and conclusions in the paper remain consistent. Additionally, the protocol run for those mice was in compliance with all of the other trials and no oddities were documented, indicating that the lower values may be an artifact of the mouse that was being run. Due to this, and to avoid undue manipulation within the groups, all of the mice were included.

In their discussion of Kwon et al.'s (2006) reported findings of a PPI deficit (Nse)-Pten KO mice, the authors mistook the prepulses at 4dBm 8dB and 16dB as the actual magnitude of the prepulses used by Kwon et al. In fact, these refer to prepulse of intensity at 4, 6 and 18 decibels units above background. The use of 70, 75 and 80dB prepulses here were presented against a background noise level of 68dB, and thus effectively be +2, +7 and +12 decibel units above background. Hence, it is incorrect to conclude (by comparison between

the present study and Kwon et al.) that “there may only be changes in percent inhibition in Pten mutant mice when the prepulse is comparatively quiet”. If anything, the +2 (or 70 dB) condition here was even lower than the weakest prepulse used by Kwon et al.

- Thank you for pointing this out. The discussion has been amended with a more specific interpretation of Kwon et al’s (2006) findings. Furthermore, additional explanations have been made to explain any differences in results.

The authors evaluated the startle reactivity curve as a function of increasing pulse intensity – as a means to examine the “startle threshold”. Yet no attempt was made to measure individual startle threshold for comparison between genotypes. Otherwise, it is misleading to conclude that threshold did not differ when only the group’s average profile was presented.

- We believe that the repeated measures ANOVA with genotype as the between-subjects factor and stimulus intensity as the within subjects factor is sufficient to adequately, and thoroughly, assess the data. Specific details further explaining our statistics have been added to the paper to help clarify any confusion.

Competing Interests: No competing interests were disclosed.

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