1st Decision letter

Reference: CRNEUR-D-22-00051
Title: Murine Model of Triosephosphate Isomerase Deficiency with Anemia and Severe Neuromuscular Dysfunction
Journal: Current Research in Neurobiology

Dear Dr. Palladino,

Thank you for submitting your manuscript to Current Research in Neurobiology. I am sorry for the delay in returning these reviewer's comments. A second reviewer had agreed to provide a review but did not do so despite numerous requests. Fortunately, a third reviewer was able to provide a timely review and this has allowed me to complete my evaluation of your manuscript.

The reviewers recommend reconsideration of your manuscript following major revision. I invite you to resubmit your manuscript after addressing the comments below. Please resubmit your revised manuscript by Oct 24, 2022.

When revising your manuscript, please consider all issues mentioned in the reviewers' comments carefully: please outline in a cover letter 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.

Current Research in Neurobiology values your contribution and I look forward to receiving your revised manuscript.

CRNEUR aims to be a unique, community-led journal, as highlighted in the Editorial Introduction. As part of this vision, we will be regularly seeking input from the scientific community and encourage you and your co-authors to take the survey.

Kind regards,

Anna S Mitchell, Ph.D.
Editor in Chief
Current Research in Neurobiology
Comments from Editors and Reviewers:

The two expert reviewers and I were in agreement that this manuscript describes an interesting new mouse model of TPI deficiency. It could be a valuable model for further research into disease mechanisms and potential therapeutic approaches of this rare genetic disorder. However, the data presented for model characterization concerning the mutant phenotype are rather weak and do not allow drawing the reported conclusions. As both reviewers indicate, the manuscript would need to be supplemented by additional data, or results and discussion have to include more clearly the limitations of the study. In addition, there are no raw data provided yet for this manuscript.

Reviewer #1:

The authors describe a new KI/KO mouse model of TPI deficiency recapitulating in contrast to earlier models most of the characteristics of the human disease, including neurological and hematological symptoms as well as short lifespan. Therefore this model appears quite interesting and could contribute to the further understanding of pathogenesis of TPI Df and might be used for testing therapeutic approaches.

The manuscript is well and clearly written and the data presented appear reliable, but in several aspects preliminary. Data provided are sufficient to demonstrate, that the model is clinically stronger affected than previously reported models, but limited concerning the conclusions that can be drawn about the nature of the pathological effects.

The n-number of animals per group (4 per sex and genotype, 8 per genotype, when pooled) is quite low for mouse phenotyping experiments. There is no information, whether only this number of animals was investigated or data of animals were excluded. However, still the differences can be seen, since they are large for most of the parameters presented.

Raw data seem to be not accessible but should be provided, since scales in the figures do not allow exact conclusion on measured values.

Observations described for behavioural/neurological phenotype analysis are not sufficient to conclude on neuro-muscular impairement. Most of described phenotypes (haunched body position, inactivity, jerk-like movements) are frequently observed in moribund mice independent of the underlying cause. It seems obvious, that mutant animals are severely impaired. However, failure to thrive and hydrocephalus development is sometimes also observed in C57BL/6J wild-type animals. Therefore the number of animals investigated for each group should be increased, the observation of hydrocephalus development confirmed by imaging or pathology and number of animals affected should be reported for each group, mutants and controls. However, hydrocephalus is no typical finding in TPI deficiency in humans, but neurodegeneration, which might be detectable by brain or nerve histology.

In the method description of hematological analyses, relevant information is missing. For example the device used for hematological analyses and sample processing steps performed by IDEXX are not reported. However, without this information it is not possible, whether the data presented are comparable to previously published data. Please provide more details in this respect.

To me the MCV values appear high compared to other published values ranging between 44 and 55 fl dependent on age of the mice investigated and device that was used.

Especially concerning hematological phenotyping and interpretations of hematological data and inclusion of KI/KI phenotypic data, there are some weaknesses and room for improvement.

The authors collected blood for hematological phenotyping by cardiac puncture and stored samples at
4°C until shipment for external analysis. To my experience, both - the method of sample collection as well as longer storage at 4°C can induce significant hemolysis. This can only be ruled out, if part of the sample is centrifuged and visually inspected. Hematological analysis of hemolyzed samples will result in decrease red blood cell counts (RBC) but not hemoglobin (HGB) values and increased MCH and MCHC values measured. Mean corpuscular volume (MCV) in contrast is less affected. Information on shipping conditions and method of further analysis including sample quality checks is missing (see above). Since both, samples of mutant animals as well as controls were subjected to the same procedure of sample collection, storage and analysis, the artificial effects on the outcome of hematological measurements could be regarded similar. However, if erythrocytes of mutant animals or old erythrocytes in contrast to young erythrocytes and reticulocytes are more sensitive to mechanic stress or cold induced hemolysis, the measured values of mutants might stronger deviate from in-vivo values, than those of controls. Differences found might then reflect a combination of genetic defect and artificial influences.

The authors present HGB, RBC and MCV values as indicators of anemia. Since besides RBC also HGB was decreased in mutant animals, and MCV was increased, I agree in the conclusion of macrocytic anemia found in mutant animals. However, this must not necessarily be a hemolytic anemia, since other types of macrocytic anemia present with comparable symptoms. Further, the authors present histological analysis of the spleen to demonstrate increased red blood cell production. The quantitative analysis includes spleen weight, cross-sectional area and proportion of spleen cross-sectional white pulp area after manual segmentation of white pulp area. However, especially, when normal organization of white pulp is disrupted it can be difficult to distinguish exactly white and red hematopoietic areas by eye in HE stained tissue. Therefore I suggest to include further analyses to confirm diagnosis of hemolytic anemia: The following parameters, analyses could be done to support the diagnosis:

Increased bilirubin in plasma and increased reticulocyte counts in blood samples are indicators, that a macroscopic anemia is due to in-vivo hemolysis and a Ter119-immunohistochemistry staining of spleen slides can demonstrate an increased proportion of erythroid lineage in spleen tissue. I suggest to include at least one or two of these parameters in the manuscript to support the conclusion of hemolytic anemia in the model. In fact the authors included a statement concerning increased reticulocyte counts in the graphical abstract, but do not report such data being investigated in the paper.

Without such supporting data, the conclusion can only be that the observed symptoms are in line with the assumption of hemolytic anemia as it can be expected based on human disease and earlier described models, but further tests are needed to confirm this diagnosis.

Another weakness is the missing presentation of data from the homozygous knock-in mice. The authors state, that the homozygous KI/KI animals develop similar but less severe symptoms as the KI/KO model associated with longer life span. In the paper they present exclusively data of the KI/KO model. However, taking the extremely short lifespan of the mice into account and the fact that the homozygous KI mice from the genetic point of view would match the human situation, the KI/KI model might be preferable for some investigations.

Further the authors compare the KO/KI model to the KI/KI model in the discussion without providing any data supporting their statements concerning the phenotype of these mice. Therefore, at least some data obtained from these mice concerning neuromuscular disease and lifespan should be included in the manuscript.
Reviewer #2:

In the present manuscript entitled "Murine Model of Triosephosphate Isomerase Deficiency with Anemia and Severe Neuromuscular Dysfunction" the authors described the CRISPR-Cas9 mediated generation and characterization of a novel murine model of Triosephosphate isomerase deficiency (TPI Df), a rare, autosomal recessive genetic disease with no current treatment. They showed via western blot, histology, and behavioral assays that the compound heterozygous Tpi1E105D/null mice, harboring the most common human disease-causing Tpi1 mutation, E105D, recapitulates the key characteristic phenotypes of the human disease such as shortened lifespan, neuromuscular dysfunction, hemolytic anemia, spleen pathology, and decreased body weight. The manuscript is elaborate with detailed methodology and well-designed experiments. However, there are some important concerns as given below which need to be addressed:

A. The main emphasis of the manuscript is to generate a mice model which replicates the disease phenotype of human patient. Is there any patient reported with compound heterozygous Tpi1E105D/null condition? Why the authors choose to study Tpi1E105D/null condition rather than Tpi1E105D/E105D?

B. The authors showed that Tpi1null/null mice are embryonically lethal. What is the viability of Tpi1E105D/E105D mice and how are they different from Tpi1E105D/null mice. Are there any molecular differences in Tpi1 levels between Tpi1E105D/null and Tpi1E105D/E105D mice?

C. The Tpi1 protein levels are significantly lower in Tpi1WT/null and Tpi1WT/E105D mice as compared to Tpi1WT/WT. Do the heterozygous mice show any behavioral or neurological changes compared to control?

D. Is the effect of Tpi1 protein on disease prognosis dosage dependent?

E. PCR validation of the mice genotype should be included as a supplemental figure for a detailed overview.

1st Author Response Letter

Response to comments from Editors and Reviewers:

We thank the reviewers for their enthusiasm for our manuscript as well as suggestions to improve it. We have revised the manuscript accordingly and believe it is significantly improved and ready for publication. We have also outlined below the changes we have made in this revision in response to each reviewer’s comments and highlighted the changes in the revised manuscript.

Comments from Reviewer 1

The authors describe a new KI/KO mouse model of TPI deficiency recapitulating in contrast to earlier models most of the characteristics of the human disease, including neurological and hematological symptoms as well as short lifespan. Therefore this model appears quite interesting and could contribute to the further understanding of pathogenesis of TPI Df and might be used for testing therapeutic approaches. The manuscript is well and clearly written and the data presented appear reliable, but in several aspects preliminary. … Observations described for behavioural/neurological phenotype analysis
are not sufficient to conclude on neuro-muscular impairment. Most of described phenotypes (haunched body position, inactivity, jerk-like movements) are frequently observed in moribund mice independent of the underlying cause.

Response: We agree that the main point is to establish that the mice model key parameters of the disease and are pleased you found the manuscript clearly written and the data reliable. We agree that the characterization of some parameters is preliminary. Unfortunately, as the animals model a severe multi-system disease fully studying every aspect of the disease will take many additional years that would unnecessarily delay this report. We agree more detailed studies of behavior and neurological impairment are important and we have begun these studies but they are beyond the scope of this first manuscript and will be the subject of future publication. We have clarified that the neuromuscular symptoms observed are of uncertain origin and that further studies are needed to distinguish which are neurogenic in nature. While end-stage animal postural defects and behavior may be due to moribund state and can be hard to distinguish from disease-specific phenotypes, the progression of symptoms suggests strong neuromuscular impairment. In Tp1\(1\text{E105D/null}\) animals, symptoms are first seen around 30 days of age. The first symptom is typically an altered gait. This then progresses to pronounced hindlimb dysfunction as evidenced by hind-limb clasp behavior and dragging of the hindlimbs. Also, around this time a strained breathing phenotype becomes readily apparent as animals struggle to get the air they need. This progression of symptoms together suggests neuromuscular impairment and is now fully described in the manuscript.

It seems obvious, that mutant animals are severely impaired. However, failure to thrive and hydrocephalus development is sometimes also observed in C57BL/6J wild-type animals. Therefore the number of animals investigated for each group should be increased, the observation of hydrocephalus development confirmed by imaging or pathology and number of animals affected should be reported for each group, mutants and controls. However, hydrocephalus is no typical finding in TPI deficiency in humans, but neurodegeneration, which might be detectable by brain or nerve histology.

Response: We agree it is premature to speculate as to whether the animals have hydrocephalus. It is possible that the altered posture of these animals simply makes it look like they could have hydrocephaly. We have removed the mention of possible hydrocephaly and have planned future studies to examine this and appreciate the reviewers suggestion to investigate this further.

The n-number of animals per group (4 per sex and genotype, 8 per genotype, when pooled) is quite low for mouse phenotyping experiments. There is no information, whether only this number of animals was investigated or data of animals were excluded. However, still the differences can be seen, since they are large for most of the parameters presented.

Response: Given the effect size the n is more than sufficient and it would be wasteful to increase the N, considering we have already shown the model is modeling the human disease well. In most cases, data from the same animals were used in all graphs – except for the spleen analysis which is mostly a different cohort of animals. In some cases, bloodwork parameters needed to be excluded due to poor sample quality upon microscopic inspection. In this case, another animal was added so that we could obtain bloodwork data and we also included other parameters for this animal such as terminal weight, but this animal did not necessarily have its TPI protein levels evaluated by western blot if the dataset was already at an N = 8. Data were rarely excluded and never for trivial reasons. A summary of which animals and data points were used in each figure can be found in the raw data for clarity. It is important
to note that at the beginning of these studies we were not getting a full bloodwork panel, so reticulocyte count is only present for samples that were collected later. We have clarified the rare events in which animal data was excluded from our studies and have ensured that the N is clearly reported for each experiment.

Raw data seem to be not accessible but should be provided, since scales in the figures do not allow exact conclusion on measured values.

Response: We have compiled the raw data for all figures into an excel sheet for the reviewers and editor to review.

In the method description of hematological analyses, relevant information is missing. For example the device used for hematological analyses and sample processing steps performed by IDEXX are not reported. However, without this information it is not possible, whether the data presented are comparable to previously published data. Please provide more details in this respect. To me the MCV values appear high compared to other published values ranging between 44 and 55 fl dependent on age of the mice investigated and device that was used.

Especially concerning hematological phenotyping and interpretations of hematological data and inclusion of KI/KI phenotypic data, there are some weaknesses and room for improvement.

The authors collected blood for hematological phenotyping by cardiac puncture and stored samples at 4°C until shipment for external analysis. To my experience, both - the method of sample collection as well as longer storage at 4°C can induce significant hemolysis. This can only be ruled out, if part of the sample is centrifuged and visually inspected. Hematological analysis of hemolyzed samples will result in decrease red blood cell counts (RBC) but not hemoglobin (HGB) values and increased MCH and MCHC values measured. Mean corpuscular volume (MCV) in contrast is less affected. Information on shipping conditions and method of further analysis including sample quality checks is missing (see above).

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Therefor I suggest to include further analyses to confirm diagnosis of hemolytic anemia: The following parameters, analyses could be done to support the diagnosis: Increased bilirubin in plasma and increased reticulocyte counts in blood samples are indicators, that a macroscopic anemia is due to in-vivo hemolysis and a Ter119-immunohistochemistry staining of spleen slides can demonstrate an
increased proportion of erythroid lineage in spleen tissue. I suggest to include at least one or two of these parameters in the manuscript to support the conclusion of hemolytic anemia in the model. In fact the authors included a statement concerning increased reticulocyte counts in the graphical abstract, but do not report such data being investigated in the paper. Without such supporting data, the conclusion can only be that the observed symptoms are in line with the assumption of hemolytic anemia as it can be expected based on human disease and earlier described models, but further tests are needed to confirm this diagnosis.

Response: We have provided additional data about the testing performed by IDEXX, including their quality control procedures to ensure the samples were of good quality and that control and experimental samples were age-matched and were treated the same. Additionally, we have provided, as requested, reticulocyte data that are consistent with the conclusions we have reported. We have also addressed the limitation of visual/manual inspection of H&E spleens. Hemolysis was assessed by IDEXX BioAnalytics and it was reported in the sample comments if it was present. Only one sample in the included data points was reported to have hemolysis.

Another weakness is the missing presentation of data from the homozygous knock-in mice. The authors state, that the homozygous KI/KI animals develop similar but less severe symptoms as the KI/KO model associated with longer life span. In the paper they present exclusively data of the KI/KO model. However, taking the extremely short lifespan of the mice into account and the fact that the homozygous KI mice from the genetic point of view would match the human situation, the KI/KI model might be preferable for some investigations.

Further the authors compare the KO/KI model to the KI/KI model in the discussion without providing any data supporting their statements concerning the phenotype of these mice. Therefore, at least some data obtained from these mice concerning neuromuscular disease and lifespan should be included in the manuscript.

Response: We agree that KI/KI animals may prove useful for future studies and we have added a more complete description of the KI/KI phenotype, as well as data on the TPI protein levels observed in these animals as requested. That said, KI/KO animals model TPI Df, a severe early-onset childhood disease with childhood mortality, extremely well and are thus the focus of this report.

Comments from Reviewer 2

In the present manuscript entitled "Murine Model of Triosephosphate Isomerase Deficiency with Anemia and Severe Neuromuscular Dysfunction" the authors described the CRISPR-Cas9 mediated generation and characterization of a novel murine model of Triosephosphate isomerase deficiency (TPI Df), a rare, autosomal recessive genetic disease with no current treatment. They showed via western blot, histology, and behavioral assays that the compound heterozygous Tpi1E105D/null mice, harboring the most common human disease-causing Tpi1 mutation, E105D, recapitulates the key characteristic phenotypes of the human disease such as shortened lifespan, neuromuscular dysfunction, hemolytic anemia, spleen pathology, and decreased body weight. The manuscript is elaborate with detailed methodology and well-designed experiments. However, there are some important concerns as given below which need to be addressed:

A. The main emphasis of the manuscript is to generate a mice model which replicates the disease phenotype of human patient. Is there any patient reported with compound heterozygous Tpi1E105D
null condition? Why the authors choose to study Tpi1E105D /null condition rather than Tpi1E105D /E105D?

B. The authors showed that Tpi1null/null mice are embryonically lethal. What is the viability of Tpi1E105D/E105D mice and how are they different from Tpi1E105D/null mice. Are there any molecular differences in Tpi1 levels between Tpi1E105D/null and Tpi1E105D /E105D mice?

Response: TPI Df is a spectrum with E105D homozygous patients being the most common. There are less severe patients recently reported (VanDemark et al) and more severe that were identified and diagnosed shortly after birth (Roland et al). The latter identified in an Italian patient is an extremely strong loss-of-function with E105D allele that is the most similar to the KI/KO model we are reporting. We believe it is useful to have a model of severe TPI Df as the phenotypes emerge quickly and efficacy testing in the most severe model available will ensure therapies will work across the spectrum of the disease. The KI/KI mice develop severe motor dysfunction beginning ~ day 50 that is very similar to that observed in the KI/KO mice. Consistent with the slightly higher protein levels observed (but still markedly reduced from WT) the onset of these symptoms is delayed. We have now added a supplemental figure showing representative Western blots to demonstrate the effect on protein levels of the KI/KI compared to the KI/KO and controls. As the KI/KI mice also have severe locomotor dysfunction it is neither surprising their lifespans are reduced nor that they are less severe than KI/KO. We cannot include lifespan data on the KI/KI animals as they are incomplete and will take years to complete with an n suitable for publication (but have included the ongoing lifespan and weight trend curves for reviewers to evaluate).

C. The Tpi1 protein levels are significantly lower in Tpi1WT/null and Tpi1WT/E105D mice as compared to Tpi1WT/WT. Do the heterozygous mice show any behavioral or neurological changes compared to control?

D. Is the effect of Tpi1 protein on disease prognosis dosage dependent?

Response: The TPI Df disease is fully recessive, and no symptoms are observed in heterozygous family members. Consistent with this, the heterozygous mice also lack phenotypes, despite an ~ 50% reduction in TPI protein levels. We have emphasized this point in the manuscript.

E. PCR validation of the mice genotype should be included as a supplemental figure for a detailed overview.

Response: We have added representative sequencing chromatographs of the KI/KI and KI/KO mice.
Dear Dr. Palladino,

Thank you for submitting your manuscript to Current Research in Neurobiology.

I am pleased to inform you that your manuscript has been accepted for publication. Congratulations.

My comments, and any reviewer comments, are below.

Your accepted manuscript will now be transferred to our production department. We will create a proof which you will be asked to check, and you will also be asked to complete a number of online forms required for publication. If we need additional information from you during the production process, we will contact you directly.

We appreciate and value your contribution to Current Research in Neurobiology. We regularly invite authors of recently published manuscript to participate in the peer review process. If you were not already part of the journal’s reviewer pool, you have now been added to it. We look forward to your continued participation in our journal, and we hope you will consider us again for future submissions.

CRNEUR aims to be a unique, community-led journal, as highlighted in the Editorial Introduction. As part of this vision, we will be regularly seeking input from the scientific community and encourage you and your co-authors to take the survey.

We would also like to invite you to take part in our CRNEUR Author Question & Answer (Q&A), which could get published alongside your article and help to promote it. We suspect you might have an interesting story of perseverance or team work that was required for the research study to complete, or a diversity of perspectives that you might share, as a way of inspiring others about neuroscience.

Kind regards,

Anna S Mitchell, Ph.D.
Editor in Chief
Current Research in Neurobiology

Editor and Reviewer comments:

Reviewer 1: The manuscript describes a new mouse model of TPI Df that reflects most of the human severe TPI Df disease characteristics. The authors addressed all comments of the reviewers and added sufficient supportive data and more detailed phenotype description that allow for the conclusions stated in the discussion. Further the authors now include comments regarding the limitations of the presented work.

The only change I recommend is, to include the numbers of animals investigated in the material and methods part, such as: In total xy animals were used for... At the moment it is available from the figure legend or from the supplemental raw data, while in the material and methods part there is just
information about the number of samples excluded.

Reviewer 2: The authors have addressed most of the concerns and made appropriate changes in the manuscript. The strength of the manuscript has enormously improved so I recommend the article for publication.

-------- End of Review Comments --------