

Chronic reduction of store operated Ca^{2+} entry is viable therapeutically but is associated with cardiovascular complications

Fang Yu, Raphael Courjaret, Asha Elmi, Ethel Alcantara Adap, Nelson N Orie, Fawzi Zghyer, Satanay Hubrack, Sajad Hayat, Nidal A Saad, Stefan Worgall, Manikkam Suthanthiran, Vidya Mohamed Ali, and Khaled Machaca
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The following individual(s) involved in review of this submission have agreed to reveal their identity: Ken D O'Halloran (Referee #3)

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Professor Machaca,

Re: JP-RP-2022-283711 "Chronic reduction of store operated Ca^{2+} entry is viable therapeutically but is associated with cardiovascular complications" by Fang Yu, Raphael Courjaret, Asha Elmi, Ethel Alcantara Adap, Nelson N Orie, Fawzi Zghyer, Satanay Hubrack, Sajad Hayat, Nidal A Saad, Stefan Worgall, Manikkam Suthanthiran, Vidya Mohamed Ali, and Khaled A Machaca

Thank you for submitting your manuscript to The Journal of Physiology. It has been assessed by a Reviewing Editor and by 2 Referees and the reports are copied below.

Please let your co-authors know of the following editorial decision as quickly as possible.

As you will see, in its current form, the manuscript is not acceptable for publication in The Journal of Physiology. In comments to me, the Reviewing Editor expressed interest in the potential of this study, but much work still needs to be done (and this may include new experiments) in order to satisfactorily address the concerns raised in the reports.

In view of this interest, I would like to offer you the opportunity to carry out all of the changes requested in full, and to resubmit a new manuscript using the "Submit Special Case Resubmission for JP-RP-2022-283711..." on your homepage.

We cannot, of course, guarantee ultimate acceptance at this stage as the revisions required are substantial. However, we encourage you to consider the requested changes and resubmit your work to us if you are able to complete or address all changes.

A new manuscript would be renumbered and redated, but the original referees would be consulted wherever possible. An additional referee's opinion could be sought, if the Reviewing Editor felt it necessary. A full response to each of the reports should be uploaded with a new version.

I hope that the points raised in the reports will be helpful to you.

Yours sincerely,

Bjorn Knollmann
Senior Editor
The Journal of Physiology

EDITOR COMMENTS

Reviewing Editor:

Comments for Authors to ensure the paper complies with the Statistics Policy:

Please present data in the form of mean \pm standard deviation as this is a requirement of the journal. Tables include data presented this way, but most graphs are currently presented as mean \pm SEM.

Comments to the Author:

Congratulations on a compelling article. The referees were enthusiastic with this manuscript and I agree that it is very high impact. We all agree that your characterization of the novel SOCE hypomorph mouse and discoveries of important complications would be of interests to a wide variety of researchers given the importance of SOCE in autoimmune and inflammatory diseases. There are a few concerns that the referees and I would like you to address (please see their comments). In addition, there is missing information regarding animal ethics and the journal requires all statistical data to be presented as standard deviations. Please attend to these comments and consider resubmitting your manuscript.

Senior Editor:

Comments for Authors to ensure the paper complies with the Statistics Policy:

Please read and follow the instructions in our statistics policy (i.e., use SD not SEM, show all data if $n < 31$, etc).

Please make sure the statistical summary document is filled out correctly on resubmission

Comments to the Author:

The MS is of potential interest, but major deficiencies will need to be corrected for a responsive resubmission. In particular, the experiments for figure 1 will need to be redone with a correct protocol for measuring SOCE. Ionomycin is an ionophore and should not be used by itself to measure SOCE. Please adhere to the statistics policy. No supplements are allowed, all methods have to be included in the main MS.

REFeree COMMENTS

Referee #1:

In this study, authors thoroughly evaluated a mouse genetic model of partial inhibition of store-operated Ca^{2+} entry (SOCE), called SOCE hypomorph. Different from global STIM1/Orai1 (SOCE component proteins) knockouts which are lethal postnatally, SOCE hypomorph mice reported in this study are viable and reproduce normally, therefore, this model is valuable for research. Authors validated the success of this model by a variety of biochemical, pharmacological, and functional experiments. Importantly, authors carefully and comprehensively examined potential side effects arising from the genetic manipulation (SOCE hypomorph) and demonstrated that the SOCE hypomorph mice are not susceptible to diabetes development, but are associated with cardiovascular complications, such as tachycardia, hypertension. Overall, the study is straightforward and well-designed. Conclusion is supported by the data presented. There are several concerns listed below.

1. Some information on mice was missed, such as age and sex of mice used in the study.
2. In Figs. 1 E&F, and 3 B, SOCE was evaluated by Ca^{2+} imaging using Ca^{2+} re-admission protocol. Ionomycin was used in Fig. 1 and thapsigargin was used in Fig. 3. Thapsigargin is a classical activator of SOCE, but ionomycin is a Ca^{2+} ionophore, which make Ca^{2+} enter cell not through any specific Ca^{2+} channels. Therefore, the data presented in Fig. 1 E&F may not fully represent SOCE. In addition, the concentration of ionomycin should be specified.
3. In Fig. 3D, it is very hard to see the difference between each panel.
4. In Fig. 3 I&J, the summary data presented in Fig. 3J compared the difference between ConKI and KI groups, however, the representative images in I only showed the response in ConKI MEF cells. The images from KI MEF cells should also be included.

Referee #2:

The current manuscript examines the various phenotypes of STIM1 mutant (mutations in phosphorylation sites and duplication of the C-terminal end) knock-in mice showing SOCE hypomorph. The authors found that T cells from this novel genetic mouse model had defects in SOCE and cytokine production with normal T cell development and homeostasis. These results are consistent with the other groups' findings and prove that this model can provide valuable information. However, the authors found that these mice developed cardiovascular complications, including hypertension and tachycardia caused by increased sympathetic autonomic nervous system activity. Overall, the manuscript has novelty in examining the phenotypes of hypomorph mice since global Stim1 knockout mice are not viable at this age. This manuscript can also impact the therapeutic use of SOCE inhibition in the long term by providing potential complications. The techniques and interpretation of the results are straightforward, and I do not have major issues. To strengthen their conclusions, I have the following recommendations:

1. The description of KI mice in Figure 1 is confusing because expression of higher molecular weight STIM1 mutant was not expected. The mutations replacing S/T to A should be indicated in Figure 1B. Does the duplicated C-terminal end contain these substitutions? Is it correct whether small bars in this figure indicate substitutions?
2. In Figure 2D, I suggest putting the actual numbers of these cells in addition to the percent.
3. In Figure 2E, instead of ConKI T cells, WT T cells were used. Do the authors have any data that compare WT and ConKI T cells?

4. The subheading "The SOCE hypomorph is not susceptible to diabetes" on page 7 is misleading.

5. In the discussion section, I suggest more description of the potential mechanism of how this mutation has a hypomorph phenotype in SOCE. This description will help future researchers who want to analyze this hypomorph model.

ADDITIONAL FORMATTING REQUIREMENTS FOR RESUBMISSION:

-Include a [Key Points](#) list in the article itself, before the Abstract.

-Author photo and profile. First (or joint first) authors are asked to provide a short biography (no more than 100 words for one author or 150 words in total for joint first authors) and a portrait photograph. These should be uploaded and clearly labelled with the revised version of the manuscript. See [Information for Authors](#) for further details.

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-You must start the Methods section with a paragraph headed [Ethical Approval](#). A detailed explanation of journal policy and regulations on animal experimentation is given in [Principles and standards for reporting animal experiments in The Journal of Physiology and Experimental Physiology](#) by David Grundy J Physiol, 593: 2547-2549. doi:10.1113/JP270818.). A checklist outlining these requirements and detailing the information that must be provided in the paper can be found at: <https://physoc.onlinelibrary.wiley.com/hub/animal-experiments>. Authors should confirm in their Methods section that their experiments were carried out according to the guidelines laid down by their institution's animal welfare committee, and conform to the principles and regulations as described in the Editorial by Grundy (2015). The Methods section must contain details of the anaesthetic regime: anaesthetic used, dose and route of administration and method of killing the experimental animals.

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-You must upload original, uncropped western blot/gel images (including controls) if they are not included in the manuscript. This is to confirm that no inappropriate, unethical or misleading image manipulation has occurred <https://physoc.onlinelibrary.wiley.com/hub/journal-policies#imagmanip> These should be uploaded as 'Supporting information for review process only'. Please label/highlight the original gels so that we can clearly see which sections/lanes have been used in the manuscript figures.

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-Papers must comply with the Statistics Policy https://jp.msubmit.net/cgi-bin/main.plex?form_type=display_requirements#statistics

In summary:

-If $n \leq 30$, all data points must be plotted in the figure in a way that reveals their range and distribution. A bar graph with data points overlaid, a box and whisker plot or a violin plot (preferably with data points included) are acceptable formats.

-If $n > 30$, then the entire raw dataset must be made available either as supporting information, or hosted on a not-for-profit repository e.g. FigShare, with access details provided in the manuscript.

-'n' clearly defined (e.g. x cells from y slices in z animals) in the Methods. Authors should be mindful of pseudoreplication.

-All relevant 'n' values must be clearly stated in the main text, figures and tables, and the Statistical Summary Document (required upon revision)

-The most appropriate summary statistic (e.g. mean or median and standard deviation) must be used. Standard Error of the Mean (SEM) alone is not permitted.

-Exact p values must be stated. Authors must not use 'greater than' or 'less than'. Exact p values must be stated to three significant figures even when 'no statistical significance' is claimed.

-Statistics Summary Document completed appropriately upon revision

-Include a [Key Points](#) list in the article itself, before the Abstract.

-Author photo and profile. First (or joint first) authors are asked to provide a short biography (no more than 100 words for one author or 150 words in total for joint first authors) and a portrait photograph. These should be uploaded and clearly labelled with the revised version of the manuscript. See [Information for Authors](#) for further details.

-The contact information provided for the person responsible for 'Research Governance' at your institution is an author on this paper. Please provide an alternative contact who is not an author on this paper or confirm that the author whose email was provided has sole responsibility for research governance. This is the person who is responsible for regulations, principles and standards of good practice in research carried out at the institution, for instance the ethical treatment of animals, the keeping of proper experimental records or the reporting of results.

-You must start the Methods section with a paragraph headed [Ethical Approval](#). A detailed explanation of journal policy and regulations on animal experimentation is given in [Principles and standards for reporting animal experiments in The Journal of Physiology and Experimental Physiology](#) by David Grundy J Physiol, 593: 2547-2549. doi:10.1113/JP270818.). A checklist outlining these requirements and detailing the information that must be provided in the paper can be found at: <https://physoc.onlinelibrary.wiley.com/hub/animal-experiments>. Authors should confirm in their Methods section that their experiments were carried out according to the guidelines laid down by their institution's animal welfare committee, and conform to the principles and regulations as described in the Editorial by Grundy (2015). The Methods section must contain details of the anaesthetic regime: anaesthetic used, dose and route of administration and method of killing the experimental animals.

-Please upload separate high-quality [figure files](#) via the submission form.

-You must upload original, uncropped western blot/gel images (including controls) if they are not included in the manuscript. This is to confirm that no inappropriate, unethical or misleading image manipulation has occurred <https://physoc.onlinelibrary.wiley.com/hub/journal-policies#imagmanip> These should be uploaded as 'Supporting information for review process only'. Please label/highlight the original gels so that we can clearly see which sections/lanes have been used in the manuscript figures.

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-Papers must comply with the Statistics Policy https://jp.msubmit.net/cgi-bin/main.plex?form_type=display_requirements#statistics

In summary:

-If n {less than or equal to} 30, all data points must be plotted in the figure in a way that reveals their range and distribution. A bar graph with data points overlaid, a box and whisker plot or a violin plot (preferably with data points included) are acceptable formats.

-If $n > 30$, then the entire raw dataset must be made available either as supporting information, or hosted on a not-for-profit repository e.g. FigShare, with access details provided in the manuscript.

-' n ' clearly defined (e.g. x cells from y slices in z animals) in the Methods. Authors should be mindful of pseudoreplication.

-All relevant ' n ' values must be clearly stated in the main text, figures and tables, and the Statistical Summary Document (required upon revision)

-The most appropriate summary statistic (e.g. mean or median and standard deviation) must be used. Standard Error of the Mean (SEM) alone is not permitted.

-Exact p values must be stated. Authors must not use 'greater than' or 'less than'. Exact p values must be stated to three significant figures even when 'no statistical significance' is claimed.

-Statistics Summary Document completed appropriately upon revision

4 Sep 2022

Response to Referees and Editors comments

We thank the Referees and Editors for their constructive critics, which have improved the manuscript. Below is a detailed response to the individual comments by both the Editors and Reviewers. The comments are listed in italics between quotes followed by our response. We have in addition provided a track changes document for the test to allow the Referees and Editors to easily follow the changes made. We however excluded the Figure legends and Methods sections from the track changes as the changes were very extensive making it difficult to follow.

Response to issues raised by the Editors

“Please present data in the form of mean +/- standard deviation as this is a requirement of the journal.

“...there is missing information regarding animal ethics and the journal requires all statistical data to be presented as standard deviations.”

“...the experiments for figure 1 will need to be redone with a correct protocol for measuring SOCE. Ionomycin is an ionophore and should not be used by itself to measure SOCE. Please adhere to the statistics policy. No supplements are allowed, all methods have to be included in the main MS.”

All the Figures have been revised to comply with the statistics policy of J. Physiol. Data are now presented as means with standard deviation and the actual p values listed in the figure legends. In addition, we completed the statistics table that now lists the means, standard deviations, and p values whether significant or not for the presented data.

Furthermore, the figures have been extensively revised to incorporate the essential supplemental data in the original submission into the figures. All data are now part of the main Figures in the revised manuscript. This required us to add a new Figure to accommodate the additional datasets.

We added the missing animal ethics information.

We performed the experiments in Figure 1 using thapsigargin to measure SOCE as requested and obtained similar data as with ionomycin.

Referee 1

“1. Some information on mice was missed, such as age and sex of mice used in the study.

2. In Figs. 1 E&F, and 3 B, SOCE was evaluated by Ca^{2+} imaging using Ca^{2+} re-admission protocol. Ionomycin was used in Fig. 1 and thapsigargin was used in Fig. 3. Thapsigargin is a classical activator of SOCE, but ionomycin is a Ca^{2+} ionophore, which make Ca^{2+} enter cell not through any specific Ca^{2+} channels. Therefore, the data presented in Fig. 1 E&F may not fully represent SOCE. In addition, the concentration of ionomycin should be specified.

3. *In Fig. 3D, it is very hard to see the difference between each panel.*
4. *In Fig. 3 I&J, the summary data presented in Fig. 3J compared the difference between ConKI and KI groups, however, the representative images in I only showed the response in ConKI MEF cells. The images from KI MEF cells should also be included."*

1. We added the age and sex of the mice used in the study in the Methods section.
2. We performed the experiments in Figure 1 on T cells using thapsigargin to evaluate SOCE as requested, and the Figure has been updated accordingly.
3. We revised Fig. 3D by enhancing the contrast and intensity for all panels to make the STIM1 puncta more visible.
4. We added representative image for NFAT translocation for the KI as requested.

Referee 2

"1. The description of KI mice in Figure 1 is confusing because expression of higher molecular weight STIM1 mutant was not expected. The mutations replacing S/T to A should be indicated in Figure 1B. Does the duplicated C-terminal end contain these substitutions? Is it correct whether small bars in this figure indicate substitutions?"

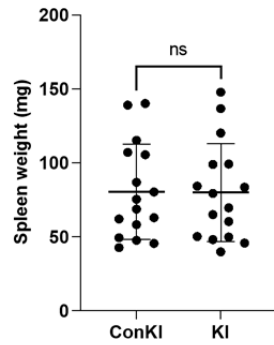
2. In Figure 2D, I suggest putting the actual numbers of these cells in addition to the percent.

3. In Figure 2E, instead of ConKI T cells, WT T cells were used. Do the authors have any data that compare WT and ConKI T cells?"

4. The subheading "The SOCE hypomorph is not susceptible to diabetes" on page 7 is misleading.

5. In the discussion section, I suggest more description of the potential mechanism of how this mutation has a hypomorph phenotype in SOCE. This description will help future researchers who want to analyze this hypomorph model."

1. The Referee is correct that the S/T to A mutations are indicated in Figure 1B by the small bars in the long chimeric STIM1 protein. This is also stated in the Figure legend.
2. We present splenocyte cell types as percent rather than absolute cell number because we did not observe any enlargement of the spleen in KI mice as compared to ConKI (Fig. 2B). The spleens in both ConKI and KI mice are of similar shape and size. We further carefully compared spleen weight between age and gender matched ConKI and KI mice and observe no difference in spleen weight (see attached Figure). Based on these observations we measured the cell type frequency and not total cell number from whole spleens in flow cytometry experiments from the same number of isolated cells (typically 10,000) for these experiments. As spleen size and weight are similar between the two genotypes, these frequency measurements are representative of the true splenocyte populations.



3. For Fig. 2E the panels were labelled for the STIM1 protein and not the strain used. The control strain for these experiments was the ConKI and the experimental was the KI as for all the rest of the experiments. So, WT in this case refers to the ConKI strain. We thank the Referee for noticing this as it can be confusing. We have renamed the panels as ConKI or KI to avoid confusion.

4. We agree with the Referee and have revised to heading to: “SOCE hypomorph susceptibility to develop diabetes.”

5. We agree with the Referee and have added a section in the discussion outlining how the chimeric STIM1 leads to the SOCE hypomorph phenotype.

Dear Professor Machaca,

Re: JP-RP-2022-283811X "Chronic reduction of store operated Ca^{2+} entry is viable therapeutically but is associated with cardiovascular complications" by Fang Yu, Raphael Courjaret, Asha Elmi, Ethel Alcantara Adap, Nelson N Orie, Fawzi Zghyer, Satanay Hubrack, Sajad Hayat, Nidal A Saad, Stefan Worgall, Manikkam Suthanthiran, Vidya Mohamed Ali, and Khaled Machaca

Thank you for submitting your manuscript to The Journal of Physiology. It has been assessed by a Reviewing Editor and by 3 expert Referees and I am pleased to tell you that it is considered to be acceptable for publication following satisfactory revision.

Please advise your co-authors of this decision as soon as possible.

The reports are copied at the end of this email. Please address all of the points and incorporate all requested revisions, or explain in your Response to Referees why a change has not been made.

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I look forward to receiving your revised submission.

If you have any queries please reply to this email and staff will be happy to assist.

Yours sincerely,

Bjorn Knollmann
Senior Editor
The Journal of Physiology

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-You must start the Methods section with a paragraph headed [Ethical Approval](#). A detailed explanation of journal policy and regulations on animal experimentation is given in [Principles and standards for reporting animal experiments in The Journal of Physiology and Experimental Physiology](#) by David Grundy J Physiol, 593: 2547-2549. doi:10.1113/JP270818.). A checklist outlining these requirements and detailing the information that must be provided in the paper can be found at: <https://physoc.onlinelibrary.wiley.com/hub/animal-experiments>. Authors should confirm in their Methods section that their experiments were carried out according to the guidelines laid down by their institution's animal welfare committee, and conform to the principles and regulations as described in the Editorial by Grundy (2015). The Methods section must contain details of the anaesthetic regime: anaesthetic used, dose and route of administration and method of killing the experimental animals.

-Your manuscript must include a complete [Additional Information section](#)

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-Please include a legend for the abstract figure in the main article file.

-Please include a full title page as part of your article (Word) file (containing title, authors, affiliations, corresponding author name and contact details, keywords, and running title).

EDITOR COMMENTS

Reviewing Editor:

Congratulations on an excellent body of work. Please address the animal ethics editor's comments so that your manuscript can be considered for publication.

Senior Editor:

Excellent work that is acceptable after the remaining animal issues are addressed.

REFEREE COMMENTS

Referee #1:

No further comments.

Referee #2:

The authors answered all the comments and revised the manuscript.

Referee #3 (ethics review):

Thank you for submitting your manuscript to The Journal of Physiology. There are some additional details required pertaining to animal welfare.

Please provide details of the source of the mice used in the studies.

At each juncture in the manuscript relating to procedures performed under anaesthesia, include a statement of the approaches taken that provided assurance that an adequate depth of anaesthesia was maintained during procedures. Please comment on post-procedural care for those studies involving recovery from anaesthesia. Ensure that the concentration of isoflurane is stated at all relevant junctures (eg it is missing for the sweat test). Include details of the carrier gas.

Please change 'sacrificed' to 'killed' or 'euthanised'.

At each relevant juncture, you must state the method of killing.

END OF COMMENTS

1st Confidential Review

05-Sep-2022

18 Sep 2022

Response to Referees and Editors comments

We thank the Referees and Editors for their constructive critics. We have made the requested changes to the ethical approval section in methods including stating the method of euthanasia and confirmation of the depth of anesthesia. The additional information section is complete.

We also included a legend for the abstract figure.

Finally we uploaded a cover suggestion that summarizes the main findings of our manuscript.

Dear Dr Machaca,

Re: JP-RP-2022-283811XR1 "Chronic reduction of store operated Ca^{2+} entry is viable therapeutically but is associated with cardiovascular complications" by Fang Yu, Raphael Courjaret, Asha Elmi, Ethel Alcantara Adap, Nelson N Orie, Fawzi Zghyer, Satanay Hubrack, Sajad Hayat, Nidal A Saad, Stefan Worgall, Manikkam Suthanthiran, Vidya Mohamed Ali, and Khaled Machaca

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Yours sincerely,

Bjorn Knollmann
Senior Editor
The Journal of Physiology

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EDITOR COMMENTS

Reviewing Editor:

Congratulations!

Senior Editor:

Excellent work, congratulations!

2nd Confidential Review

20-Sep-2022
