The Journal of Physiology

# Inhibitory interneurons show early dysfunction in a SOD1 mouse model of amyotrophic lateral sclerosis

Clarissa Fantin Cavarsan, Preston R Steele, Landon T GENRY, Emily J Reedich, Lynn M McCane, Kayleigh J LaPre, Alyssa C Puritz, Marin Manuel, Natallia Katenka, and Katharina Ann Quinlan
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*The following individual(s) involved in review of this submission have agreed to reveal their identity: Roisin Mc Mackin (Referee #2)*

## Review Timeline:


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| Submission Date: | 14-Mar-2022 |
| Editorial Decision: | 05-Apr-2022 |
| Resubmission Received: | 02-Dec-2022 |
| Editorial Decision: | 06-Dec-2022 |
| Revision Received: | 08-Dec-2022 |
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*Senior Editor: David Wyllie*

*Reviewing Editor: Gareth Morris*


## Transaction Report:

(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 Dr Quinlan,

Re: JP-RP-2022-283097 "Inhibitory interneurons show early dysfunction in a SOD1 mouse model of Amyotrophic Lateral Sclerosis" by Clarissa Fantin Cavarsan, Preston R Steele, Lynn M McCane, Kayleigh J LaPre, Alyssa C Puritz, Natallia Katenka, and Katharina Ann Quinlan

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-2022-283097..." 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,

David Wyllie
Senior Editor
The Journal of Physiology

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EDITOR COMMENTS

Reviewing Editor:

This manuscript has been closely reviewed by two expert referees, with one of the referees raising substantial concerns about the way in which the data were analysed statistically. Therefore, the current version of the manuscript is not suitable for consideration in the Journal of Physiology.

However, both referees expressed strong enthusiasm for the work and state that the data could be highly influential and fill an important gap in the research field. I would therefore recommend that the authors be given the chance to consider the feedback from Referee 1, and to re-submit a new manuscript with substantial re-analysis of their datasets.

Senior Editor:

You manuscript has received some positive comments but serious concerns have been raised about the analysis and the robustness of the conclusions that have been reached. I am will to consider a new submission provided the concerns regard the appropriate analysis have been thoroughly addressed.

Please comply with our Statistics Policy (see 'ADDITIONAL FORMATTING REQUIREMENTS' below).

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REFEREE COMMENTS

Referee #1:

Please see attached file.

Referee #2:

I am very impressed by this study. The findings are very valuable to the field of understanding pathophysiology of ALS and work performed is commendable. The figures and descriptions are clear throughout and the findings are well considered in the discussion. I would be very interested to see further work from this group investigating these measures in SOD1 mice of different ages/times relative to symptom onset and progression, as well as the same work on cortical inhibitory interneurons, and simultaneous investigation of these action potential properties in the upper/lower motor neurons nearby in the same animals.

I have a two minor comments:

Abstract: "Few studies in amyotrophic lateral sclerosis (ALS) focus on the inhibitory interneurons

synapsing onto motoneurons (MNs)" - I don't consider this to be true, there are a lot of studies now focussing on inhibitory interneurons and their role in ALS, rather there are few studies in ALS directly measuring the effects of ALS on these cells, which is the real novelty/value of this study.

Methods: "Neurons that did not repetitively fire or did not maintain a resting membrane potential below -35 mV were excluded from electrophysiological analysis." - Did the authors notice/measure any difference in the rate of exclusion due to this RMT abnormality between the animal cohorts?

Clarify the origin of the animals (location of breeding) and standard of living (food, water, cages, temperature etc.) as requested by the Journal.

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ADDITIONAL FORMATTING REQUIREMENTS:

-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.

-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.

-Please ensure that any tables are in Word format and are, wherever possible, embedded in the article file itself.

-Please ensure that the Article File you upload is a Word file.

-A Statistical Summary Document, summarising the statistics presented in the manuscript, is required upon revision. It must be on the Journal's template, which can be downloaded from the link in the Statistical Summary Document section here: https://jp.msubmit.net/cgi-bin/main.plex?form_type=display_requirements#statistics

-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

**Confidential Review** **14-Mar-2022**

The paper by Cavarsan et al. contains a large and important dataset comparing the properties of glycinergic interneurons in wild type mice and those carrying an ALS inducing mutation in the SOD gene.

The animal model is the most commonly used and its properties have been extensively described, but this paper fills an important gap, providing data on morphology and electrophysiological properties of inhibitory interneurons, a class of interneurons that other recent studies (Allodi et al, for one) indicated as potentially affected by the SOD mutation.

The paper as such is interesting and important, experiments well executed and the data well worth publishing.

However, before I can properly judge the details of the results, I would like to see an entirely revised version that takes into account the following important points about statistics.

Before I enter into the details of the statistics, please note that Standard Deviations should be reported in all the tables and not standard error. SD gives information about the biological variability of the sample (across cells in this case) while the SE (otherwise, and more properly, known as SD of the mean) tells us what would be the standard deviation of the n estimated means if the entire experiment was repeated n times on n different samples. Therefore, SD is a descriptive parameter, while SE is an inference parameter, that depends on the 'true' mean (that of course is unknown). In the context of reporting variability across cells, the SE has no meaning.

The authors show differences (or lack of) basing their conclusion on 'threshold' p-values. While this is commonly accepted (and often acceptable), there are increasing concerns about the applicability and overall meaning of p-values. I will discuss the two most important points that I think apply specifically to this paper: large sample size and pseudo-replication.

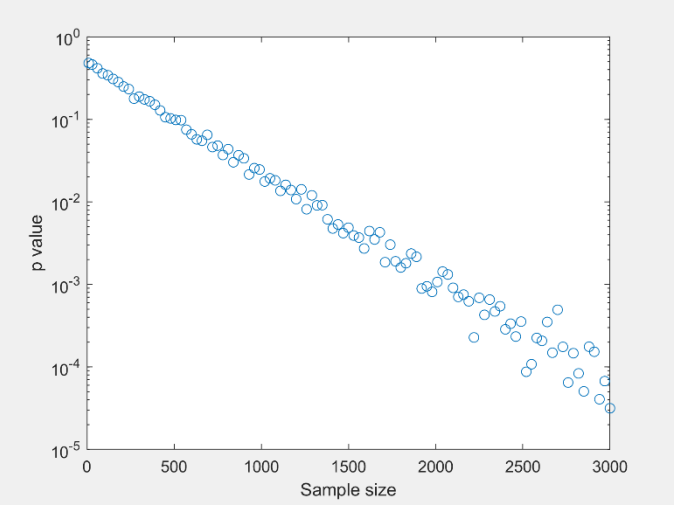
**Large sample size**

I will use the data in Table 1 as an example, in particular the surface area, but the same reasoning applies for the volume.

The authors report a difference between the means of approximately 30 $\mu m^2$ between wt and SOD. The difference, unsurprisingly, is highly significant. But going back to biology, is a 30 $\mu m^2$ difference in area biologically relevant? It would correspond to ~0.7 µm difference in radius, or 2 pixels, using the reported resolution of the optical system.

Now it is well known, but not often acknowledged, that the p-value in any statistical test, by construction, tends to 0 for increasing sample size. A nice explanation of the phenomenon is described in this paper (https://www.jstor.org/stable/24700283 ), and a further description can be found here (https://doi.org/10.1038/s41598-021-00199-5), even though the solution proposed by the authors is debatable.

Using the reported n values I took the freedom of estimating the SD for the surface area measurements. The mean surface area is 459±207 $\mu m^2$ for wt and 430±195 $\mu m^2$ for SOD. Assuming a




normal distribution (I know the data distribution is not normal in this case, but the reasoning below applies equally), one can see that taking random samples of different sizes, the p-value slowly (and exponentially) decreases towards zero (see figure below, p was calculated using a standard t-test, but the same applies for Kruskal-Wallis). This known phenomenon can lead to the paradox of finding significant differences between measurements, when the actual difference between means is below the resolution of the experiment (not the case here, but very close).

As a first approach, I would ask the authors to report also the effect size. The reasoning behind this (and other suggestions for interpreting data) can be found in this paper (http://dx.doi.org/10.1098/rsbl.2019.0174) and a simple guide to the interpretation of effect size can be found here (http://dx.doi.org/10.4300/JGME-D-12-00156.1). Any measure of effect size could be used (Cohen, Hedges, or else). Another approach would be to use estimation statistics, as recommended in a recent eNeuro editorial (see https://www.eneuro.org/content/8/2/ENEURO.0091-21.2021 and references therein to the original papers), even though this approach may equally suffer from the large sample size.

For the authors' data on surface area, it turns out that the effect size (Cohen's d in this case) is 0.14 (and it is 0.17 for the volume). Such a small effect size should lead to the conclusion that the two parameters are not different between wt and SOD and by relying on p values the authors are making a Type II error (false positive). Further proof of this is that in the set of patched neurons (that could be viewed as a sub-sample of the full dataset) the size of the recorded cells does not change between phenotypes.

**Pseudo-replication**

The other, and far more serious, issue is the question of what is the experimental unit in the cell size experiments. Is it the cell (as in the current version) or is it the spinal cord section, or the animal?

The authors pool all the cells from all the animals analyzed (and number of animals is not reported), but it is entirely possible that the variability across animals is larger than the variability within. In other words, that measures within animals are correlated. This is entirely possible, and made even more likely by the fact that animals might have been used at different ages. 2-3 days difference at this stage of development could lead to significant changes in morphology (but the authors do not give information on the age of animals used for the fixed tissue experiments). It is thus necessary to disentangle the potential effects due to correlation, in order to establish whether the differences are genuine or not. Below are some references that are good starting points.

A general description of the pseudo-replication issue can be found here (https://bmcneurosci.biomedcentral.com/articles/10.1186/1471-2202-11-5).

Among the proposed solutions, one is hierarchical bootstrap, described in this sobering Sam Sober's paper (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7906290/) and the other, more standard solution is the use of linear mixed models, carefully reviewed and described in

(https://www.sciencedirect.com/science/article/pii/S089662732100845X). For a lighter description of mixed models, I often use this website for students: https://ourcodingclub.github.io/tutorials/mixed-models/

It is my opinion, that given the structure of data, the use of linear mixed model is absolutely necessary in order to be able to draw conclusions.

The issues above have serious effects on the morphological measures, where the number of cells is high, but are of course less severe for the electrophysiology data. These are convincing, but still I would like to see them reported alongside with the effect size or with estimation statistics (Dr. Marin is already acknowledged, so he could be consulted again).

I will be happy to review this paper, but since I suspect that some of the conclusions will not hold after a proper analysis is performed, I would like to provide a full review of the whole paper once the issues above are addressed

**Referee #1:**

The paper by Cavarsan et al. contains a large and important dataset comparing the properties of glycinergic interneurons in wild type mice and those carrying an ALS inducing mutation in the SOD gene.

The animal model is the most commonly used and its properties have been extensively described, but this paper fills an important gap, providing data on morphology and electrophysiological properties of inhibitory interneurons, a class of interneurons that other recent studies (Allodi et al, for one) indicated as potentially affected by the SOD mutation.

The paper as such is interesting and important, experiments well executed and the data well worth publishing.

However, before I can properly judge the details of the results, I would like to see an entirely revised version that takes into account the following important points about statistics.

Before I enter into the details of the statistics, please note that Standard Deviations should be reported in all the tables and not standard error. …

*We have replaced SE values to SD throughout the manuscript.*

The authors show differences (or lack of) basing their conclusion on 'threshold' p-values. While this is commonly accepted (and often acceptable), there are increasing concerns about the applicability and overall meaning of p-values. I will discuss the two most important points that I think apply specifically to this paper: large sample size and pseudo-replication.

**Large sample size**

…

As a first approach, I would ask the authors to report also the effect size.

*We have now added effect sizes throughout the manuscript*

**Pseudo-replication**

The other, and far more serious, issue is the question of what is the experimental unit in the cell size experiments. Is it the cell (as in the current version) or is it the spinal cord section, or the animal? The authors pool all the cells from all the animals analyzed (and number of animals is not reported), but it is entirely possible that the variability across animals is larger than the variability within.

…

It is my opinion, that given the structure of data, the use of linear mixed model is absolutely necessary in order to be able to draw conclusions.

*We have now used a linear mixed model to analyze the reconstruction data (where there was a large n) so that the effect of the mouse each cell originated from can be accounted for. As the reviewer predicted, a portion of these anatomical significance did not hold, so we no longer conclude that soma size of GlyT2+ neurons is smaller in SOD1 mice throughout the ventral horn. However, consistent with our previous conclusions, there is a location - specific effect: GlyT2+ neurons in the ventral-most 100 microns were indeed altered in both morphology and electrophysiology.*

*Since plotting the effect sizes of these parameters shows a large difference in the two populations that would appear "significant," we have omitted the graphics related to effect sizes only for the variables that we analyzed using the linear mixed models, so as not to present an analysis that appears to show differences where there are none.*

The issues above have serious effects on the morphological measures, where the number of cells is high, but are of course less severe for the electrophysiology data. These are convincing, but still I would like to see them reported alongside with the effect size or with estimation statistics.

I will be happy to review this paper, but since I suspect that some of the conclusions will not hold after a proper analysis is performed, I would like to provide a full review of the whole paper once the issues above are addressed

*The suggestions to modify our analysis has greater increased the rigor of this manuscript and we thank the reviewer for their insights.*

**Referee #2:**

I am very impressed by this study. The findings are very valuable to the field of understanding pathophysiology of ALS and work performed is commendable. The figures and descriptions are clear throughout and the findings are well considered in the discussion. I would be very interested to see further work from this group investigating these measures in SOD1 mice of different ages/times relative to symptom onset and progression, as well as the same work on cortical inhibitory interneurons, and simultaneous investigation of these action potential properties in the upper/lower motor neurons nearby in the same animals.

I have a two minor comments:

Abstract: "Few studies in amyotrophic lateral sclerosis (ALS) focus on the inhibitory interneurons synapsing onto motoneurons (MNs)" - I don't consider this to be true, there are a lot of studies now focussing on inhibitory interneurons and their role in ALS, rather there are few studies in ALS directly measuring the effects of ALS on these cells, which is the real novelty/value of this study.

*This text is now revised as suggested*

Methods: "Neurons that did not repetitively fire or did not maintain a resting membrane potential below -35 mV were excluded from electrophysiological analysis." - Did the authors notice/measure any difference in the rate of exclusion due to this RMT abnormality between the animal cohorts?

*34/37 WT interneurons and 25/29 SOD1 interneurons met the criteria for inclusion. The number of neurons that were eliminated from both groups was consistent with previous studies of patch clamped neurons, in which a small percentage of neurons are likely damaged during the process of breaking in or have bad seal. This is now stated more clearly in the methods lines 162-163.*

Clarify the origin of the animals (location of breeding) and standard of living (food, water, cages, temperature etc.) as requested by the Journal.

*This is now added to the methods section lines 120-124.*

**Other requirements from *J Physiol***

ADDITIONAL FORMATTING REQUIREMENTS:

-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.

-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.

*This is now added to the methods section.*

-Please upload separate high-quality figure files via the submission form.

-Please ensure that any tables are in Word format and are, wherever possible, embedded in the article file itself.

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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.

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-Statistics Summary Document completed appropriately upon revision

Dear Dr Quinlan,

Re: JP-RP-2022-284192X "Inhibitory interneurons show early dysfunction in a SOD1 mouse model of amyotrophic lateral sclerosis" by Clarissa Fantin Cavarsan, Preston R Steele, Landon T Genry, Emily J Reedich, Lynn M McCane, Kayleigh J LaPre, Alyssa C Puritz, Marin Manuel, Natallia Katenka, and Katharina Ann Quinlan

Thank you for submitting your manuscript to The Journal of Physiology. It has been assessed by a Reviewing Editor and by 2 expert referees and we are pleased to tell you that it is acceptable for publication following minor revision.

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

The referee reports are copied at the end of this email.

Please address all the points raised and incorporate all requested revisions or explain in your Response to Referees why a change has not been made. We hope you will find the comments helpful and that you will be able to return your revised manuscript within 4 weeks. If you require longer than this, please contact journal staff: jp@physoc.org.

Your revised manuscript should be submitted online using the link in your Author Tasks: Link Not Available. This link is accessible via your account as Corresponding Author; it is not available to your co-authors. If this presents a problem, please contact journal staff (jp@physoc.org). Image files from the previous version are retained on the system. Please ensure you replace or remove any files that are being revised.

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Check that your Methods section conforms to journal policy: https://jp.msubmit.net/cgi-bin/main.plex?form_type=display_requirements#methods.

Check that data presented conforms to the statistics policy: https://jp.msubmit.net/cgi-bin/main.plex?form_type=display_requirements#statistics.

Upload a full Response to Referees file. To create your 'Response to Referees' copy all the reports, including any comments from the Senior and Reviewing Editors, into a Microsoft Word, or similar, file and respond to each point, using font or background colour to distinguish comments and responses and upload as the required file type.

Please upload two versions of your manuscript text: one with all relevant changes highlighted and one clean version with no changes tracked. The manuscript file should include all tables and figure legends, but each figure/graph should be uploaded as separate, high-resolution files.

You may also upload:

- 'Potential Cover Art' for consideration as the issue's cover image
- Appropriate Supporting Information (Video, audio or data set: see https://jp.msubmit.net/cgi-bin/main.plex?form_type=display_requirements#supp).

We look forward to receiving your revised submission.

If you have any queries, please reply to this email and we will be pleased to advise.

Yours sincerely,

David Wyllie
Senior Editor
The Journal of Physiology

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REQUIRED ITEMS:

- 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.

- The Journal of Physiology funds authors of provisionally accepted papers to use the premium BioRender site to create high resolution schematic figures. Follow this link and enter your details and the manuscript number to create and download figures. Upload these as the figure files for your revised submission. If you choose not to take up this offer we require figures to be of similar quality and resolution. If you are opting out of this service to authors, state this in the Comments section on the Detailed Information page of the submission form. The link provided should only be used for the purposes of this submission. Authors will be charged for figures created on this premium BioRender account if they are not related to this manuscript submission.

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In summary:

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- 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.

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EDITOR COMMENTS

Reviewing Editor:

Thank you to the authors for their extensive work in addressing the statistical concerns from Referee 1. As a result the manuscript is greatly improved and was well received by both referees. A few additional minor comments remain from both referees.

Senior Editor:

Your revised manuscript has been assessed by two expert referees who have raised only minor comments. I am delighted that you have implemented the linear mixed model analysis suggested by referee 1 and this undoubtedly makes your study far more robust in terms of statistical reporting. You will see they suggest you include additional data pertaining to the parameters in the LMM. I think you could include this in the Statistical Summary document - rather than the tables as it could make the table very data dense but feel free to consider either option. Aside from this, there are only minor corrections/clarifications that will be checked by the Reviewing Editor and me. Thank you for carrying out the extensive re-analysis of your data. Your manuscript is much the stronger for it.

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REFEREE COMMENTS

Referee #1:

I wish to thank the authors for submitting their extensive revision and for having taken on board my suggestions.

Now that the main issue of statistical analysis has been solved, the substance remains: these are very good and important data and they are now correctly presented, therefore I will just leave a few minor comments for the authors to consider, mainly requesting minor changes.

In general, when linear mixed models are used, it would be good to include the actual parameters in the table, rather than the summary statistics with the p value (Table 1). It wold be particularly important to report the ICC (intraclass correlation coefficient), to have an idea of the relative contribution of between animal and inter animal variations. Also in Table 1, it is not clear whether the effect size (g) for the surface and volume of unpatched neurons is calculated from the linear mixed model or from the raw data (for instance, for the raw data of surface area I get a value of g of 0.31, that is not what is in the table). Overall, I would like to see the full output of the linear mixed model and more details about it in the methods (this applies to all the subsequent tables in which LMMs are used).

Requested text changes (referring to the line numbering in the track changed version).

Line 277: it is worth explaining why LMMs are used. 'due to the large sample size' is not sufficient. Pseudoreplication and the dependency of p-values on sample size are tow independent things. LMMs addresses both issues, but both should be explained, maybe including references to the papers I mentioned in my previous report.

Line 300: Should read something like 'Analysing dendritic morphology was not possible....'

Line 300: Figure 1B does not seem to have its Cumming plot.

Line 342: 'The AHP were shorter in duration' or something along those lines. The current sentence is broken.

***

Referee #2:

The manuscript is improved with these revisions. My only query is:

In the revisions it is stated that "The total number of analyzed interneurons was over 2000, from 6 WT mice and 6 SOD1 mice" and the words "composed of 2,551 neurons from 36 WT image stacks and 1,587 interneurons from 44 SOD1 image stacks" were removed - Does the revision mean that over 2000 interneurons were analyzed from 6 WT mice and over 2000 were analyzed from 6 SOD1 mice? If so, clarify.

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END OF COMMENTS

**1st Confidential Review** **02-Dec-2022**

On behalf of all the authors, I would like to thank the editors and reviewers for the speedy review of our resubmission! The minor points remaining are all now addressed. A point by point summary of these changes is below.

KQ

In general, when linear mixed models are used, it would be good to include the actual parameters in the table, rather than the summary statistics with the p value (Table 1). It wold be particularly important to report the ICC (intraclass correlation coefficient), to have an idea of the relative contribution of between animal and inter animal variations.

Also in Table 1, it is not clear whether the effect size (g) for the surface and volume of unpatched neurons is calculated from the linear mixed model or from the raw data (for instance, for the raw data of surface area I get a value of g of 0.31, that is not what is in the table). Overall, I would like to see the full output of the linear mixed model and more details about it in the methods (this applies to all the subsequent tables in which LMMs are used).

*In Table 1, the effect size for unpatched neurons was calculated from the raw data without considering the effect of the mouse. Because this was not reflective of the results of the LMMs, we removed the effect size / Hedge's g values for unpatched neurons throughout the manuscript. The details of the linear mixed models are now included more thoroughly in the statistics summary table, including ICC values.*

Requested text changes (referring to the line numbering in the track changed version).

Line 277: it is worth explaining why LMMs are used. 'due to the large sample size' is not sufficient. Pseudoreplication and the dependency of p-values on sample size are tow independent things. LMMs addresses both issues, but both should be explained, maybe including references to the papers I mentioned in my previous report.

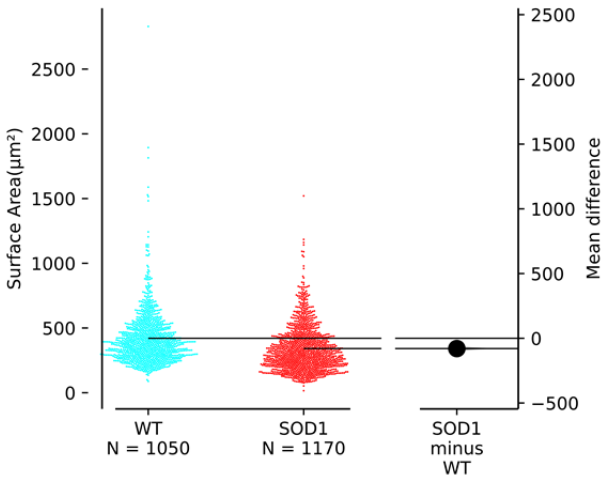
*The concepts of pseudoreplication and dependency of p values on sample size are now cited on lines 263-265. Pertinent references are included.*

Line 300: Should read something like 'Analysing dendritic morphology was not possible....'

*This is corrected.*

Line 300: Figure 1B does not seem to have its Cumming plot.

*The Cumming plots were purposefully omitted in the data reanalyzed using linear mixed models. The text mistakenly referred to a cumming plot in this figure, but that has been corrected. It was not possible to see the 95% CI in these graphs since it was so small, as shown in the example to the right, thus it may*



*have (inaccurately) appeared to show differences in these populations when there was none. Also the calculation of effect size did not take into account repeated measures (cells) from a single mouse as the linear mixed model did. Therefore, in order to be consistent, we also removed the data that we had calculated on effect sizes for unpatched neurons. This data is presented along with the best analysis method – the linear mixed models.*

Line 342: 'The AHP were shorter in duration' or something along those lines. The current sentence is broken.

*I cannot locate this error.*

***

Referee #2:

The manuscript is improved with these revisions. My only query is:

In the revisions it is stated that "The total number of analyzed interneurons was over 2000, from 6 WT mice and 6 SOD1 mice" and the words "composed of 2,551 neurons from 36 WT image stacks and 1,587 interneurons from 44 SOD1 image stacks" were removed - Does the revision mean that over 2000 interneurons were analyzed from 6 WT mice and over 2000 were analyzed from 6 SOD1 mice? If so, clarify.

*This is now clarified in line 246-247*

## 2nd Revision - Editorial Decision 09-Dec-2022

Dear Dr Quinlan,

Re: JP-RP-2022-284192XR1 "Inhibitory interneurons show early dysfunction in a SOD1 mouse model of amyotrophic lateral sclerosis" by Clarissa Fantin Cavarsan, Preston R Steele, Landon T GENRY, Emily J Reedich, Lynn M McCane, Kayleigh J LaPre, Alyssa C Puritz, Marin Manuel, Natallia Katenka, and Katharina Ann Quinlan

We are pleased to tell you that your paper has been accepted for publication in The Journal of Physiology.

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The last Word (or similar) version of the manuscript provided will be used by the Production Editor to prepare your proof. When this is ready you will receive an email containing a link to Wiley's Online Proofing System. The proof should be thoroughly checked and corrected as promptly as possible.

Authors should note that it is too late at this point to offer corrections prior to proofing. The accepted version will be published online, ahead of the copy edited and typeset version being made available. Major corrections at proof stage, such as changes to figures, will be referred to the Editors for approval before they can be incorporated. Only minor changes, such as to style and consistency, should be made at proof stage. Changes that need to be made after proof stage will usually require a formal correction notice.

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Yours sincerely,

David Wyllie
Senior Editor
The Journal of Physiology

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EDITOR COMMENTS

Many thanks for addressing these final queries and comments. Happy to accept this manuscript. Thank you for submitting to The Journal of Physiology.

## 2nd Confidential Review 08-Dec-2022