

Acute morphine blocks spinal respiratory motor plasticity via long-latency mechanisms that require toll-like receptor 4 signaling

Arash Tadjalli, Yasin B Seven, Abhisheak Sharma, Christopher R McCurdy, Donald C Bolser, Erica S Levitt, and Gordon S Mitchell

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The following individual(s) involved in review of this submission have agreed to reveal their identity: Glenn Matthew Toney (Referee #1)

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Submission Date:	14-Mar-2021
Editorial Decision:	16-Apr-2021
Revision Received:	24-May-2021
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Senior Editor: Harold Schultz

Reviewing Editor: Daniel Zoccal

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 Tadjalli,

Re: JP-RP-2021-281362X "Acute morphine blocks spinal respiratory motor plasticity via long-latency mechanisms that require toll-like receptor 4 signaling" by Arash Tadjalli, Yasin B Seven, Abhisheak Sharma, Christopher R McCurdy, Donald C Bolser, Erica S Levitt, and Gordon S Mitchell

Thank you for resubmitting your revised Research Paper to The Journal of Physiology. It has been assessed by the original Reviewing Editor and Referees and has been well received. Some final revisions have been requested.

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.

I hope you will find the comments helpful and have no difficulty returning your revisions within one week.

Your revised manuscript should be submitted online using the links in Author Tasks Link Not Available.

Any image files uploaded with the previous version are retained on the system. Please ensure you replace or remove all files that have been revised.

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- Article file, including any tables and figure legends, must be in an editable format (eg Word)
- Upload each figure as a separate high quality file
- Upload a full Response to Referees, including a response to any Senior and Reviewing Editor Comments;
- Upload a copy of the manuscript with the changes highlighted.

You may also upload:

- A potential 'Cover Art' file for consideration as the Issue's cover image;
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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,

Harold D Schultz
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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

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

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-Statistics Summary Document completed appropriately upon revision

EDITOR COMMENTS

Reviewing Editor:

If the Statistical Summary Document has errors please describe what is incorrect?:

The SSD is incomplete (it does not cover all results) and contains incorrect information (it may apply to the original submission).

Comments to the Author:

I thank the authors for addressing all comments. This revised version contains an extensive review of the previous data and includes new experiments covering the initial submission's main caveats. I agree with the referees that this study presents new observations that advance our understanding of the adverse effects of opioid use on breathing control. Referee #2 suggests some minor changes in the manuscript. Moreover, I recommend describing the units in the Y-axis of the graphs in Fig. 6B and C. Additionally, please revise the Statistical Summary Document (SSD) carefully and provide the correct information of all the data presented in the manuscript.

Senior Editor:

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

In tables and figure legends, please state and define sample sizes and state the statistical test(s) used for comparisons.

Figures and tables must state the actual P values in the figure or legend. Asterisks should not be used to denote significance within figures.

Figure 9N should include data points.

If the statistical summary document has errors please describe what is incorrect. (Required):
It does not appear that all statistical comparisons are included in the stats document.

Comments to the Author:

Please correct requirements for statistical reporting.

REFEREE COMMENTS

Referee #1:

This resubmitted manuscript by Tadjalli et al. reports that low dose treatment of rats with the opioid morphine ~4.5 hours prior to being exposed to AIH show blunted pLTF. They further report little if any evidence of ongoing opioid effects on resting respiratory activity or the response to acute hypoxia at the time of AIH exposure. They show that the TLR4 antagonist +naloxone effectively prevents the long-latency pLTF depressing effect of low-dose morphine, consistent with morphine activation of an innate immune response rather than activation of opioid receptor signaling. Consistent with this, the authors show that morphine increased phospho-p38 MAPK activity in microglia, but neurons, in the cervical spinal cord, ostensibly near phrenic motor neurons and that this effect is prevented by the TLR4 blocker +naloxone. This is an interesting study that provides strong evidence that morphine actions to suppress respiratory plasticity very likely hinge on TLR4 signaling. Although mechanistic investigation did not extend to possible intermediary actions of cytokines, the authors have responded thoughtfully to my previous comments and have extensively revised the presentation of data, restrained certain interpretations and expanded the discussion as requested. I have no further comments or concerns.

Referee #2:

The authors provide an updated version of their manuscript with new experiments and new figures. They have addressed most of the issues raised. For instance, they showed in Figure 6 that blocking +/-naloxone do not block the acute action of morphine while naloxone at a concentration 10x lower did. This is encouraging data that supports their original hypothesis. I only have minor comments and typos to highlight.

Line 106. A comma is missing.

Line 135. Missing comma.

Line 155. Which is an opioid-inactive isomer.

Line 814. Use same abbreviation than other figures. Fig. 10.

Line 864. I would remove the last sentence and replace with a general statement about the results and their clinical implications.

Figure 3. Legends and number in figures are difficult to read. If there was a way to improve figure so it can be easily read, it would be great.

Figure 6. Make sure it is obvious that panel B and C are different experiments and how they differ.

END OF COMMENTS

Dear Reviewing Editor and Referees: Tadjalli et al., JP-RP-2021-281362X

Thank you for your thoughtful review and the positive feedback. We are delighted that our revisions have been well received and that the reviews have been satisfied with addressing their original comments. We now have taken into consideration any additional comments/concerns provided by the reviewers and the Editor. Below is the summary of additional changes that have been made following our original revision #1 submission. We genuinely believe our study became stronger and the quality of the paper was enhanced because of the critical feedback provided by the reviewers. This was one of the best critical reviews received in recent memory. We look forward to submitting additional future studies to the Journal of Physiology.

Referee / Editor Comments:

Referee #1:

This resubmitted manuscript by Tadjalli et al. reports that low dose treatment of rats with the opioid morphine ~4.5 hours prior to being exposed to AIH show blunted pLTF. They further report little if any evidence of ongoing opioid effects on resting respiratory activity or the response to acute hypoxia at the time of AIH exposure. They show that the TLR4 antagonist +naloxone effectively prevents the long-latency pLTF depressing effect of low-dose morphine, consistent with morphine activation of an innate immune response rather than activation of opioid receptor signaling. Consistent with this, the authors show that morphine increased phospho-p38 MAPK activity in microglia, but neurons, in the cervical spinal cord, ostensibly near phrenic motor neurons and that this effect is prevented by the TLR4 blocker +naloxone. This is an interesting study that provides strong evidence that morphine actions to suppress respiratory plasticity very likely hinge on TLR4 signaling. Although mechanistic investigation did not extend to possible intermediary actions of cytokines, the authors have responded thoughtfully to my previous comments and have extensively revised the presentation of data, restrained certain interpretations and expanded the discussion as requested. I have no further comments or concerns.

Response: We are delighted that Referee #1 has been satisfied with the revisions and we like to thank the referee for their insightful comments.

Referee #2:

The authors provide an updated version of their manuscript with new experiments and new figures. They have addressed most of the issues raised. For instance, they showed in Figure 6 that blocking +-naloxone do not block the acute action of morphine while naloxone at a concentration 10x lower did. This is encouraging data that supports their original hypothesis. I only have minor comments and typos to highlight.

Response: We appreciated the additional feedback provided by referee #2 and are happy to know that they are also satisfied with our original revisions.

Line 106. A comma is missing. **Response:** Fixed

Line 135. Missing comma. **Response:** Fixed

Line 155. Which is an opioid-inactive isomer. **Response:** Fixed

Line 814. Use same abbreviation than other figures. Fig. 10. **Response:** Fixed

Line 864. I would remove the last sentence and replace with a general statement about the results and their clinical implications.

Response: Referee number one had originally asked “at which sites inflammatory related process are operating?”. Therefore, we were obligated to add a general statement, indicating that identification of the sites was beyond the scope of this study (line 851-853). In this new version, we did add a very general statement indicating:

Line-853-855

“Overall, our data suggests that downregulation of chemoreflexes by immune driven inflammatory processes secondary to opioid use should be taken into consideration in patients with unstable ventilatory chemoreflex control.”

Figure 3. Legends and number in figures are difficult to read. If there was a way to improve figure so it can be easily read, it would be great.

Response: We agree that the numbers may be difficult to read. Unfortunately, this is an issue with the software that generates the figure, and we have no way of changing the font sizes within the chromatograms.

Figure 6. Make sure it is obvious that panel B and C are different experiments and how they differ.

Response: In the figure legend, we provided additional sentences to highlight this important point, so that the reader can easily determine that panel B and C are different experiments from different rat groups.

General changes made following comments from the Reviewing Editor:

We made extensive changes in all the relevant figures and figure legends so that the presented data complies with the overall Journal Policy. Please see below for the specific changes:

#1. We modified figure number 5 (page 33) so that it now displays the individual data points from each animal subject. The original figure was a line graph showing average group data at various time points without the individual data points. In this new figure, we have plotted phrenic nerve amplitude at minute 60 following each intervention and are showing the individual data points from each rat. We also indicate individual data points for figure 9 Niii.

#2. We modified some of the figures to comply with statistics policy. In each appropriate figure (figures 2, 5, 6, 8, 9), we removed asterisks and symbols, and replaced them with the actual p values and showed the comparisons using appropriate lines pointing to the indicated groups. In the figure legends, we now define sample sizes and state the statistical test(s) used for comparisons.

#3. We now have corrected the statistical summary document to include all of the comparisons that were made. The incorrect information was due to the original submission and we had not included an updated statistical summary document following revision submission #1. We now have corrected the summary sheet so that it now complies with the new revisions and new graphs. Thank you for this important feedback.

Dear Dr Tadjalli,

Re: JP-RP-2021-281362XR1 "Acute morphine blocks spinal respiratory motor plasticity via long-latency mechanisms that require toll-like receptor 4 signaling" by Arash Tadjalli, Yasin B Seven, Abhisheak Sharma, Christopher R McCurdy, Donald C Bolser, Erica S Levitt, and Gordon S Mitchell

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

NEW POLICY: In order to improve the transparency of its peer review process The Journal of Physiology publishes online as supporting information the peer review history of all articles accepted for publication. Readers will have access to decision letters, including all Editors' comments and referee reports, for each version of the manuscript and any author responses to peer review comments. Referees can decide whether or not they wish to be named on the peer review history document.

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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 Reviewing Editor 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,

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EDITOR COMMENTS

Reviewing Editor:

I thank the authors for responding to the comments. To fully comply with the Journal of Physiology policy, please indicate the Research Resource Identifier (RRIDs) for the key biological resources used in the study (e.g., antibodies). Also, describe the method for terminal procedure/euthanasia used at the end of electrophysiological experiments.

REFEREE COMMENTS

Referee #1:

This is outstanding work. I have no further concerns.

Referee #2:

The authors provide an updated version of their manuscript with new experiments and new figures. They have addressed most of the issues raised. For instance, they showed in Figure 6 that blocking +-naloxone do not block the acute action of morphine while naloxone at a concentration 10x lower did. This is encouraging data that supports their original hypothesis. I only have minor comments and typos to highlight which can be addressed at proof stage.

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Line 910. I don't know what are the rule for JPHysiol but the proper spelling of mu-opioid receptors may be better: μ -opioid receptors?
