Activation of histamine type 2 receptors enhances intrinsic excitability of medium spiny neurons in the nucleus accumbens

Giuseppe Aceto, Luca Nardella, Simona Nanni, Valeria Pecci, Alessia Bertozzi, Claudia Colussi, Marcello D’Ascenzo, and Claudio Grassi

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Corresponding author(s): Marcello D’Ascenzo (marcello.dascenzo@unicatt.it)

The following individual(s) involved in review of this submission have agreed to reveal their identity: Helmut L. Haas (Referee #1); Jing-Ning Zhu (Referee #2)

Review Timeline:

| Event                              | Date       |
|-----------------------------------|------------|
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| Editorial Decision                | 23-Dec-2021|
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| Accepted                          | 21-Mar-2022|

Senior Editor: David Wyllie

Reviewing Editor: Tommas Ellender

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 D'Ascenzo,

Re: JP-RP-2021-282608 “Activation of histamine type 2 receptors enhances intrinsic excitability of medium spiny neurons in the nucleus accumbens” by Giuseppe Aceto, Luca Nardella, Simona Nanni, Valeria Pecci, Alessia Bertozzi, Claudia Colussi, Marcello D'Ascenzo, and Claudio Grassi

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-2021-282608..." 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:

The paper has now been assessed by two Reviewers and editor.

They agree the experiments are well executed and findings are interesting but note further experiments that would increase the impact of this paper (e.g. protein expression of histamine receptors in Nac and effect of H1R activation on Nac neurons). Although H2R activation has been studied in isolation here the physiological effects of histamine in Nac likely reflect activity at H1R and H2R simultaneously. Is the effect of histamine acting at H2R ultimately the dominant one in Nac? Is the effect of histamine acting at H1R also to increase excitability?

The Lahiri & Bevan 2020 paper is cited. Here they demonstrated that recording in whole-cell patch-clamp mode vs. perforated patch-clamp was key for what effect of dopamine was observed on striatal neuron excitability. Experiments reported in the current manuscript are all made in whole-cell patch-clamp mode. One interpretation is that histamine signalling is less sensitive to washout of intracellular factors during patching - this needs to be discussed.

In addition several key publications that also demonstrate excitatory effects of histamine on neurons should be included and discussed such as Atzori et al 2000 and Ellender et al 2014 and those as outlined by Reviewer #2. There might also be a missing reference to Possier et al 2020 as described in methods? Reference to H2 receptor expression in Nac should likely not be Takagi et al. 1986 (p.14) as they don't discuss H2R.
Check manuscript for spelling, addition to points made by Reviewer #2, e.g. exanimated (p8), scopes (p.16), ...to a somatic current pulse a series of 1 (p.19).

Additional changes are needed to comply with our policies:

Statistics: please present your data with SD rather than SEM; please give exact p-values.

Ethics: there is currently no mention of anaesthesia before euthanasia and the ethics approval number is not included. Please provide these details in a revised version.

Senior Editor:

Your manuscript has been assessed by two Referees and a Reviewing Editor. Referee 2 and the Reviewing Editor have each made comments on the current version of the manuscript that indicate to me that you will need to carry out further experiments to address the concerns raised. As such, your manuscript is not acceptable in its current form but with the changes it may be suitable for publication in The Journal of Physiology. Please also ensure that any revised manuscript complies fully with our Statistics Reporting policy. As I have noted above, please ensure you document n values appropriately and report SD and not SEM. I cannot see what "n" refers to - is it animal, slice or cell? Repeated measures from multiple cells in a slice, or from multiple slices from an animals is not a true biological replicate. In many cases the parameters from cells from the same animals should be pooled and treated as N=1. I suspect that if you do this you may find some of your experiments under-powered. If you decide to re-submit a revised manuscript please ensure it is formatted in the style of The Journal of Physiology - only initial submissions can be 'format free'.

Comments to ensure the paper complies with the Statistics Policy:
Please ensure you document n values appropriately and report SD and not SEM. I cannot see what "n" refers to - is it animal, slice or cell? Repeated measures from multiple cells in a slice, or from multiple slices from an animals is not a true biological replicate. In many cases the parameters from cells from the same animals should be pooled and treated as N=1. I suspect that if you do this you may find some of your experiments under-powered.

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REFEREE COMMENTS

Referee #1:

Dear authors

You provided detailed new information on histamine actions on an important group of neurons.
Professional in vitro electrophysiology, new information in an interesting field, logically presented.

High quality recordings demonstrating clear results. Conclusions: identification of the involved channels.

See attached file for further comments.

Referee #2:

The study revealed a powerful enhancement of histamine H2R activation on NAc MSNs firing through PKA-dependent modulation of A-type potassium currents carried by Kv4.2 channels, by using whole-cell patch-clamp recordings combined with single-cell qPCR. Although previous studies have reported the expression of H2Rs in NAc MSNs (Chronister et al., J Neurobiol, 1982; Honrubia et al., Synapse, 2000; Zarrindast et al., Iran J Psychiatry, 2010), as well as the enhancement effect of H2R activation on neuronal excitability by suppressing the potassium currents (Haas and Panula, Nat Rev Neurosci, 2003), the role of H2Rs on the neuronal excitability of NAc MSNs has not yet been well known. This study reported the direct postsynaptic electrophysiological effect of H2Rs on NAc MSNs, but some details need to be further clarified.
Major points:

1. NAc includes both the core and the shell. Have the authors tested the effect of H2R activation on both of these two regions or one of the specific regions? Please show it clearly and provide the cell number recorded in the core and shell, respectively.

2. The authors observed that the levels of mRNA for H2R were significantly higher compared to those attributable to H1R on single NAc MSNs. However, the protein expression of these two receptor subtypes has not been provided. Moreover, whether the H1R activation has a similar effect on the neuronal excitability of NAc MSNs need to be investigated.

3. The author observed the effect of dimaprit on the evoked action potentials, but what is its effect on the resting membrane potential? And whether its enhancement effect can be washed out or blocked by the selective antagonist for H2R?

4. It has been reported widely that H2R activation enhances neuronal firing through the modulation of potassium currents (Haas and Panula, Nat Rev Neurosci, 2003). In myenteric neurons from small intestine, the suppression of A-type potassium currents by H2R activation has also been studied (Starodub and Wood, J Pharmacol Exp Ther, 2000). The authors identified Kv4.2 channels targeted by H2R downstream signaling in detail. However, the co-expression of H2Rs and Kv4.2 channels in the same NAc MSNs need to be determined, the mechanism underlying the internalization of Kv4.2 channels and the physiological or pathological significance of Kv4.2 channels in the NAc MSNs also need to be further discussed.

Minor points:

1. Page 13 line 14, "Fig. 6" should be changed into "Fig.7".
2. Fig. 7G and H, "H2R" should be changed to "Dimaprit".
3. The grammar and syntax throughout the manuscript need to be refined. For instance:
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   - Page 8 line 19, a comma needs to be added after "(Kim et al., 2005)".
   - Page 9 line 1, full name of "mAHP" should be given.
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   - Page 9 line 17, a comma needs to be added before "thus".
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   - Page 19 line 22, "SD" should be changed into "SDs".
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ADDITIONAL FORMATTING REQUIREMENTS:
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.

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. 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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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 \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

-A Data Availability Statement is required for all papers reporting original data. This must be in the Additional Information section of the manuscript itself. It must have the paragraph heading "Data Availability Statement". All data supporting the results in the paper must be either: in the paper itself; uploaded as Supporting Information for Online Publication; or archived in an appropriate public repository. The statement needs to describe the availability or the absence of shared data. Authors must include in their Statement: a link to the repository they have used, or a statement that it is available as Supporting Information; reference the data in the appropriate sections(s) of their manuscript; and cite the data they have shared in the References section. Whenever possible the scripts and other artefacts used to generate the analyses presented in the paper should also be publicly archived. If sharing data compromises ethical standards or legal requirements then authors are not expected to share it, but must note this in their Statement. For more information, see our Statistics Policy.

-Please include an Abstract Figure. The Abstract Figure is a piece of artwork designed to give readers an immediate understanding of the research and should summarise the main conclusions. If possible, the image should be easily 'readable' from left to right or top to bottom. It should show the physiological relevance of the manuscript so readers can assess the importance and content of its findings. Abstract Figures should not merely recapitulate other figures in the manuscript. Please try to keep the diagram as simple as possible and without superfluous information that may distract from the main conclusion(s). Abstract Figures must be provided by authors no later than the revised manuscript stage and should be uploaded as a separate file during online submission labelled as File Type 'Abstract Figure'. Please ensure that you include the figure legend in the main article file. All Abstract Figures should be created using BioRender. Authors should use The Journal's premium BioRender account to export high-resolution images. Details on how to use and access the premium account are included as part of this email.

Confidential Review 16-Nov-2021
 Activation of histamine type 2 receptors by Aceto et al. provides a thorough neuro-, electrophysiological and pharmacological analysis of histamine H2R mediated excitation in an important group of neurons in the nucl. accumbens. The histaminergic neurons are located in the tuberomamillary (not mammillary, derived from mamilla, not mamma) nucleus and a particularly interesting target of these neurons is studied. The mechanisms of excitation of the medium spiny neurons through H2R activation are studied, state of the art, also by local administration of histamine or H2R agonist, which caused excitation. Results of the professionally analyzed molecular mechanisms are not surprising. A PKA inhibitor blocked these actions. A sophisticated investigation identified the involvement of specific A type potassium channels (Kv4.2), through determination of histamine H1- and H2- receptor mRNA levels in combination with single cell electrophysiology. It is clearly shown that the long lasting excitatory actions are mediated mainly by the downregulation Kv4.2 channels in the medium spiny neurons.
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RESPONSE TO REFEREES

We are very grateful to the Senior Editor, Reviewing Editor and Referees for their valuable comments and suggestions that were very useful to improve the quality of our manuscript. New experiments were performed to address the referees’ concerns, and all their comments were taken into consideration in the manuscript revision.

point by point response to the Referees’ comments

Replies to the Comments of Referee #1

Referee: Dear author, you provided detailed new information on histamine actions on an important group of neurons. Professional in vitro electrophysiology, new information in an interesting field, logically presented. High quality recordings demonstrating clear results. Conclusions: identification of the involved channels.

See attached file for further comments.

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Authors: We greatly appreciate the positive comments of the Referee on our work.
Replies to the Comments of Referee #2

Referee: The study revealed a powerful enhancement of histamine H2R activation on NAc MSNs firing through PKA-dependent modulation of A-type potassium currents carried by Kv4.2 channels, by using whole-cell patch-clamp recordings combined with single-cell qPCR. Although previous studies have reported the expression of H2Rs in NAc MSNs (Chronister et al., J Neurobiol, 1982; Honrubia et al., Synapse, 2000; Zarrindast et al., Iran J Psychiatry, 2010), as well as the enhancement effect of H2R activation on neuronal excitability by suppressing the potassium currents (Haas and Panula, Nat Rev Neurosci, 2003), the role of H2Rs on the neuronal excitability of NAc MSNs has not yet been well known. This study reported the direct postsynaptic electrophysiological effect of H2Rs on NAc MSNs, but some details need to be further clarified.

Authors:
We thank the Referee for the overall positive comments on our study. The referee’s comments and suggestions, listed below, have been very useful to improve the quality of our study.

Major points:

Referee:
1. NAc includes both the core and the shell. Have the authors tested the effect of H2R activation on both of these two regions or one of the specific regions? Please show it clearly and provide the cell number recorded in the core and shell, respectively.

Authors:
We have followed the Referee’s suggestion and included, in the revised version of the manuscript, quantification of dimaprit’s effect on evoked firing based on MSN localization in NAc core and shell. We also increased the number of observations. As shown in the new Fig. 2 and highlighted below, similar dimaprit-induced increases in evoked firing were found in the two NAc subterritories.

These results have been reported in the main text (page 5, lines 9-13) and in figure 2 of the revised manuscript.
Referee:
2. The authors observed that the levels of mRNA for H2R were significantly higher compared to those attributable to H1R on single NAc MSNs. However, the protein expression of these two receptor subtypes has not been provided. Moreover, whether the H1R activation has a similar effect on the neuronal excitability of NAc MSNs need to be investigated.

Authors: To test whether H1R activation also affected evoked firing we performed the following experiments:

- First, we locally applied the H1R agonist 2PyEA (100 μM) that failed to significantly affect firing proprieties of MSNs (number of APs: pre = 8.23 ± 2.34; post = 9.21 ± 3.00; n = 13; P = 0.066; paired Student’s t test).

- We then hypothesized that pharmacological blockade of H2R would counteract the histamine-induced increase in evoked firing. In accordance to our hypothesis, evoked firing was significantly increased by histamine (Fig. 4 C and D; number of Aps: pre = 6.25 ± 2.18; post = 10.22 ± 2.75; n = 10 from four mice; P = 0.0002; paired Student’s t test), an effect that was prevented by prior application of the H2R antagonist ranitidine (Fig. 4 E and F; number of APs: pre = 8.21 ± 1.39; post = 8.94 ± 2.29; n = 14; P = 0.275; paired Student’s t test).

Collectively, these findings suggest that histamine elicits long-lasting positive modulation of MSN excitability by acting on H2 receptors only. These new findings have been included in the “result section” (Page 6, lines 1-12) and in figure 4 of the revised manuscript. Moreover, we integrated the “discussion section” accordingly (Page 14, lines 1-7).

Figure 4 has also been reported below to facilitate the reading.
Figure 4. Pharmacological activation of H1R failed to affect the frequency of evoked firing in MSNs. (A) Representative traces showing current-evoked firing of a MSN before and after local perfusion of H1R agonist (2-PyEA; 100 µM). (B) Normalized mean ± S.D. for 13 experiments in which 2-PyEA was applied (P = 0.066; paired Student’s t test). (C) Representative traces and summary plot (D) illustrating that histamine application increased evoked firing (n = 10; P = 0.0002; paired Student’s t test). (E) Representative traces showing that histamine-dependent modulation of evoked firing was not observed when slices were pre-incubated (5-20 min) with the H2R antagonist ranitidine (100 µM). (F) Bar graph depicting quantification of evoked firing in the experimental conditions reported in panel E (n = 14; P = 0.275; paired Student’s t test).

According to the Referee’s request, we performed Western Blot analysis for H2R and H1R protein expression in NAc lysates. As shown in the figure reported below, WB data were in agreement with mRNA results, suggesting higher expression of H2R than H1R also at the protein level. However, we would like to point out that the reliability of quantitative comparisons of WB data obtained with two different antibodies, that may have different affinity and specificity for the targets, may be limited. For these reasons, we would prefer not to include WB analyses in the revised manuscript. However, we are ready to incorporate these new data if the Referee considers them strictly required.

Expression of H1R and H2R proteins in NAc tissues. (A) Representative Western blots of NAc tissues showing levels H1R and H2R. (B) Densitometry analysis for the blots probed with anti H1R and H2R and normalized to actin is shown.
Referee:
3. The author observed the effect of dimaprit on the evoked action potentials, but what is its effect on the resting membrane potential? And whether its enhancement effect can be washed out or blocked by the selective antagonist for H2R?

Authors: Previous literature reports have shown that dimaprit excites striatal MSNs (Ellender et al., 2011; Zhuang et al., 2018). To evaluate whether a similar effect could be observed also in NAc MSNs we performed a new set of experiments, in voltage-clamp configuration, and found that slice superfusion with dimaprit elicited slow, tonic, inward currents (−61.6 ± 27.3 pA; n = 10) in these cells. Furthermore, in current-clamp recordings, dimaprit caused mild depolarization of MSNs (from -84.8 ± 3.5 mV to -74.5 ± 4.3 mV, n = 8; P < 0.001). These findings, summarized in the figure reported below, are fully in agreement with those previously reported (Ellender et al., 2011; Zhuang et al., 2018).

Importantly, these H2R-related effects on the resting conductances are not related to the increased evoked firing we observed in our study. Indeed, in our experiments membrane potential was kept, during interpulse intervals, at the resting value (~ -80 mV) by injecting hyperpolarizing/depolarizing currents.

Since the novelty of our study is centered on the role of H2R in the modulation of evoked firing and because of the already large number of figures in our manuscript (9 figures; supplementary figures are not allowed by the journal policy), we would prefer to only describe these results in the text (see page 5, lines 22-31 of the revised manuscript). However, we are ready to increase the number of figures if the Referee will consider it necessary.

H2R activation induces tonic currents in voltage-clamp configuration and mild depolarization in current-clamp configuration.

(A) Representative trace recorded in the voltage-clamp configuration showing the inward current elicited by 10 μM dimaprit (B) Bar graph showing the mean amplitudes of currents evoked by dimaprit. C. Summary plot illustrating that dimaprit effect on membrane potential.

With regard to the second question of the comment (i.e., whether the enhancement of evoked firing can be washed out or blocked by the selective antagonist for H2R), we performed new experiments in which we bath applied the H2R antagonist ranitidine (100 μM) prior to dimaprit application. As shown in the figure reported below, in these experimental conditions dimaprit had no effect on evoked firing (number of APs: pre = 7.28 ± 2.34; post = 7.85 ± 2.01; n = 11 from 4 mice; P = 0.102; paired Student’s t test).
These data have been included in the main text of the manuscript (page 5, lines 6-9) and shown in figure 2 G.

**Referee:**

4. It has been reported widely that H2R activation enhances neuronal firing through the modulation of potassium currents (Haas and Panula, Nat Rev Neurosci, 2003). In myenteric neurons from small intestine, the suppression of A-type potassium currents by H2R activation has also been studied (Starodub and Wood, J Pharmacol Exp Ther, 2000). The authors identified Kv4.2 channels targeted by H2R downstream signaling in detail. However, the co-expression of H2Rs and Kv4.2 channels in the same NAc MSNs need to be determined, the mechanism underlying the internalization of Kv4.2 channels and the physiological or pathological significance of Kv4.2 channels in the NAc MSNs also need to be further discussed.

**Authors:** We thank the Referee for this comment that gave us the opportunity to better reconcile our findings with the current literature. In the revised manuscript we included the above citations. Accordingly, we extended the discussion on: i) mechanism underlying the internalization of Kv4.2 (Page 13, lines 6-19) and ii) pathological significance of Kv4.2 channels in NAc MSNs (Page 12; lines 11-29).

We also performed a new set of single-cell qRT-ddPCR experiments to test whether H2R and Kv4.2 are co-expressed in MSNs. As expected, we found that the vast majority of screened MSNs (8 out of 9) expressed both H2R and Kv4.2 transcripts. Noteworthy, this finding is in agreement with those reported in our recent study (Aceto et al., PNAS 2020) in which Kv4.2 was found in almost all MSNs tested (17/18, 94.4%).

Results of these experiments are reported below and in figure 9 F of the revised manuscript.
**H2R and Kv4.2 mRNA levels in single NAC MSNs.** Box plot showing H2R and Kv4.2 mRNA levels by ddPCR in single NAc MSNs \(n = 9\) from three mice. Noteworthy, the vast majority of screened MSNs \(8\) out of \(9\) expressed both H2R and Kv4.2 transcript.

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**Referee:**

Minor points:

1. Page 13 line 14, "Fig. 6" should be changed into "Fig.7".
2. Fig. 7G and H, "H2R" should be changed to "Dimaprit".

**Authors:** The main text and figures have been changed accordingly.

**Referee:**

3. The grammar and syntax throughout the manuscript need to be refined. For instance:
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**Authors:** We revised grammar and syntax throughout the manuscript.
Dear Dr D'Ascenzo,

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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 I am pleased to tell you that it is considered to be acceptable for publication following satisfactory revision.

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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,
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-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.

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EDITOR COMMENTS

Reviewing Editor:

Many thanks for the revisions. Both Reviewers agree that these have greatly improved the manuscript.

We believe that two figures reported in the Reviewer's rebuttal would be of benefit to the community if included in the main manuscript (specifically the Western Blot of H1R and H2R and dimaprit experiment). Would it be possible to include these and update the relevant Methods & Results sections and resubmit a new version?

This version will be assessed by the Reviewing editor and Senior editor only.

Senior Editor:

Your revised submission has been seen by the original referees and reviewing editor. They raise a few comments that you should consider, especially regarding an example trace - which would be a beneficial addition. Please also note the spelling - it should be “tuberomamillary nucleus”. I do, however, accept that the inclusion of the western blot data could raise concerns regarding the affinity and selectivity of the antibodies that makes comparison difficult. However, a major issue that I raised does not appear to have be addressed - if it has please clearly state. In my previous comments I noted “Repeated measures from multiple cells in a slice, or from multiple slices from an animals is not a true biological replicate. In many cases the parameters from cells from the same animals should be pooled and treated as N=1. I suspect that if you do this you may find some of your experiments under-powered.” While you have indicated the number of cells, slices and animals you do not appear to have performed the analysis of pooling data from the same animal, using the mean of these to give a single replicate and then compare between animals. This needs to be done otherwise you are artificially increasing your n value by sampling from a number of cells from the same animal - unless you can give a justification of this. I also requested that the formatting of your manuscript follows our standard formatting with the Methods section coming after the Introduction and before Results. Once I receive a further revised manuscript and depending on the outcome of the re-analysis of pooled datasets from the same animal, I will move to a final decision.

REFEREE COMMENTS

Referee #1:

You have much improved your manuscript treating a very interesting field and important question in brain research with relevance in brain research including clinical neuroscience. I have to admit that I missed some of the shortcomings pointed out by the other referee. I also suggested, without effect, to correct tuberomamillary (from mamilla, not mamma). I would have liked to see a longer lasting (continuous) record before, during and after just for one experiment at membrane potential. This is just a matter of documentation.
Referee #2:

This is a nice paper. I appreciate the efforts that the authors have made in response to my questions and concerns. The revision clarifies almost all the points I raised.

END OF COMMENTS
RESPONSE TO REFEREES

We are pleased and grateful to the Senior Editor, Reviewing Editor and Referees for their valuable and positive comments on our revised manuscript and for Senior Editor’s decision to consider our work “acceptable for publication following satisfactory revision”.

point by point response to the Referees’ and Editor’s comments

Replies to the Comments of Referee #1

Referee: You have much improved your manuscript treating a very interesting field and important question in brain research with relevance in brain research including clinical neuroscience. I have to admit that I missed some of the shortcomings pointed out by the other referee. I also suggested, without effect, to correct tuberomamillary (from mamilla, not mamma). I would have liked to see a longer lasting (continuous) record before, during and after just for one experiment at membrane potential. This is just a matter of documentation.

Authors: We have followed the Referee’s suggestion and included, in the revised manuscript, a new figure (Fig. 4) showing that H2R activation causes, in current-clamp recordings, long-lasting depolarization in MSNs. The new figure also includes results from experiments performed in voltage-clamp configuration showing inward currents induced by H2R activation.

We corrected the editing mistake about “tuberomamillary”.

Replies to the Comments of Referee #2

Referee: This is a nice paper. I appreciate the efforts that the authors have made in response to my questions and concerns. The revision clarifies almost all the points I raised.

Authors: We are very pleased for having accomplished the Referee’s requests.
Replies to the Reviewing Editor

Reviewing Editor:

Many thanks for the revisions. Both Reviewers agree that these have greatly improved the manuscript.

We believe that two figures reported in the Reviewer's rebuttal would be of benefit to the community if included in the main manuscript (specifically the Western Blot of H1R and H2R and dimaprit experiment). Would it be possible to include these and update the relevant Methods & Results sections and resubmit a new version?

This version will be assessed by the Reviewing editor and Senior editor only.

Authors: As reported in the response to the referee # 1, the current version of the manuscript includes a new figure (Fig 4). Moreover, results of semiquantitative western blot analysis on H2R vs H1R protein levels in NAc tissues are shown in the new version of figure 1. Of note, for this western blot experiment, quantitative statistical comparison was not performed due to different affinity and specificity of H1R and H2R antibodies for their relative targets. Methods and Results sections have also been changed accordingly.

Replies to the Senior Editor

Senior Editor:

Your revised submission has been seen by the original referees and reviewing editor. They raise a few comments that you should consider, especially regarding an example trace - which would be a beneficial addition. Please also note the spelling - it should be "tuberomamillary nucleus". I do, however, accept that the inclusion of the western blot data could raise concerns regarding the affinity and selectivity of the antibodies that makes comparison difficult. However, a major issue that I raised does not appear to have been address - if it has please clearly state. In my previous comments I noted "Repeated measures from multiple cells in a slice, or from multiple slices from an animals is not a true biological replicate. In many cases the parameters from cells from the same animals should be pooled and treated as N=1. I suspect that if you do this you may find some of your experiments under-powered." While you have indicated the number of cells, slices and animals you do not appear to have performed the analysis of pooling data from the same animal, using the
mean of these to give a single replicate and then compare between animals. This needs to be done otherwise you are artificially increasing your n value by sampling from a number of cells from the same animal - unless you can give a justification of this. I also requested that the formatting of your manuscript follows our standard formatting with the Methods section coming after the Introduction and before Results. Once I receive a further revised manuscript and depending on the outcome of the re-analysis of pooled datasets from the same animal, I will move to a final decision.

Authors:

As highlighted above, a new figure (Fig. 4) and an integrated figure 1 are now included in the revised manuscript. We also provided to correct the editing mistake about “tuberomamillary”.

As requested by the Senior Editor data analysis and statistical comparisons were repeated by pooling data obtained from the same mice and considering the averaged values as N=1. The new analysis confirmed the statistical significance between parameters measured before and after drugs application. We also performed power analysis in all experiments in which paired-t test were used and found that the number of biological samples required for 80% power was accomplished even for N=4, since data showed a > 20% effect size with low SD. In the revised figures, the results of such analysis are reported in bar graphs along with single cell data shown on the left side of bar graphs.
Dear Dr D’Ascenzo,

Re: JP-RP-2022-282962XR1 “Activation of histamine type 2 receptors enhances intrinsic excitability of medium spiny neurons in the nucleus accumbens” by Giuseppe Aceto, Luca Nardella, Simona Nanni, Valeria Pecci, Alessia Bertozzi, Claudia Colussi, Marcello D’Ascenzo, and Claudio Grassi

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

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All queries at proof stage should be sent to TJP@wiley.com

Yours sincerely,

David Wyllie
Senior Editor
The Journal of Physiology

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authors DO NOT NEED to pay to publish and DO NOT NEED to post their accepted papers on PMC.

EDITOR COMMENTS

Reviewing Editor:

Many thanks for making these final changes to the manuscript. We believe these further strengthen the experimental findings and now also provide the readers with all necessary information (e.g. number of animals/neurons) to fully assess the manuscript.

Senior Editor:

Thank you for addressing the remaining concerns of the Referees, Reviewing Editor and the point I raised regarding cell and animal n, N. I think the revised figures are now completely transparent in their data presentation and I will use your examples when referring others to the most appropriate way to illustrate data of this nature. I greatly appreciate your efforts in addressing this point. Thank you for submitting this work to The Journal of Physiology.

2nd Confidential Review

16-Mar-2022