5-HT7 receptors expressed in the mouse parafacial region are not required for respiratory chemosensitivity

Yingtang Shi, Cleyton R Sobrinho, Jaseph Soto-Perez, Brenda M Milla, Daniel S Stornetta, Ruth Stornetta, Ana C Takakura, Daniel K Mulkey, Thiago S Moreira, and Douglas A. Bayliss

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Corresponding author(s): Douglas Bayliss (dab3y@virginia.edu)

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

Review Timeline:

| Event                  | Date       |
|------------------------|------------|
| Submission Date        | 27-Aug-2021|
| Editorial Decision     | 29-Sep-2021|
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Transaction Report:

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Dear Dr Bayliss,

Re: JP-RP-2021-282279 “5-HT7 receptors expressed in the mouse parafacial region are not required for respiratory chemosensitivity” by Yingtang Shi, Cleyton R Sobrinho, Brenda M Milla, Josep Soto-Perez, Daniel S Stornetta, Ruth Stornetta, Ana C Takakura, Daniel K Mulkey, Thiago S Moreira, and Douglas A. Bayliss

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.

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

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

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I hope you will find the comments helpful and have no difficulty returning revisions within 4 weeks.

If you need to check to make sure that your Methods section conforms to the principles of UK regulations, you may wish to refer to Grundy (2015):
Grundy (2015) J. Physiol. 2015 Jun 15;593(12):2547-9 https://doi.org/10.1113/JP270818

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REVISION CHECKLIST:

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- All table and figure legends with summary data must include the statistical test used in the table/figure and sample size
- Figures with summary data bars must include individual data points, or box whisker plots when n> 30.
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Yours sincerely,

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

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â€“ Your paper contains Supporting Information of a type that we no longer publish. Any information essential to an understanding of the paper must be included as part of the main manuscript and figures. The only Supporting Information that we publish are video and audio, 3D structures, program codes and large data files. Your revised paper will be returned to you if it does not adhere to our Supporting Information Guidelines.
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

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.

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EDITOR COMMENTS

Reviewing Editor:

Methods Details:
Referee 1 indicated some methodological aspects that require further clarification.

Ethics Concerns:
The manuscript misses a description of how anesthesia was monitored during the surgical procedures in adult mice.

Comments to the Author:
The study used imaging and functional procedures to investigate the expression of 5-HT7 receptors (5-HT7R) in neurons of the parafacial region and their contribution to cellular and respiratory responses to high CO2. The authors reported that 5-HT7R mRNA is found mainly in RTN neurons that may not exhibit intrinsic chemosensitive mechanisms. It was also demonstrated that the antagonism of 5-HT7R in the RTN did not affect the CO2-induced firing response of putative chemosensitive neurons in vitro and the ventilatory response to hypercapnia in freely behaving mice. The authors conclude that 5-HT7Rs in the RTN neither mediate the chemosensitivity of neurons nor contribute to the ventilatory response to hypercapnia. This conclusion contrasts a previous study that, using a different approach, reported different results.

The manuscript is straightforward, the results are well described, and the discussion is appropriate. As highlighted by the
referees, the information provided contributes to understanding the functioning and modulation of chemosensitive cells in the parafacial region. However, there is a gap between the electrophysiological and imaging data that requires authors' attention. If the expression of Htr7 was low or undetectable in most Nmb-expressing RTN neurons located ventral to the facial nucleus, it is not clear why in CO2-responsive neurons the 5-HT7R antagonist reduced the response to 5-HT. This observation parallels the referees' comments on the phenotypic characterization of the recorded neurons, the evaluation of CO2 responses in 5-HT7R-expressing neurons, and cellular targets of 5-HT7R agonists in the RTN to evoke an increase in breathing in vivo (despite the developmental-related reduction in the 5-HT7R expression). The referees also provided other important comments that, if addressed adequately, will enhance the manuscript. Other minor comments:

- provide the ethical approval protocol number of all institutions.
- indicate the animals' sex.
- describe the perfusion and histological procedures to check the microinjection sites in adult mice.
- please make sure that all data are represented as mean (plus minus) SD (Fig 5 indicates SEM, and panels D-F of Fig 6 miss this information) and that all statistical tests and results are described in the manuscript.

Senior Editor:

Comments for Authors to ensure the paper complies with the Statistics Policy:
Define n with animal numbers as well as number of samples. Use SD throughout. All tables and figures must be incorporated into the manuscript.

Comments to the Author:
Please indicate how the level of anesthesia was assessed and maintained, and how animals were terminated.

Supplementary tables and figures must be incorporated into the manuscript. Material such as figures, tables, text (e.g. expanded/detailed methods or results), equations, and other material must be incorporated into the manuscript itself as part of the text or as standard figures or tables and not supplied as Supporting Information.

Please indicate the number of animals as well as the number of preparations when describing 'n' sample size.

Please ensure all summary data variance is described as Standard Deviation.

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REFEREE COMMENTS

Referee #1:

The manuscript by Shi and colleagues explores the expression and function of 5HT7 receptors in the anatomically defined (PHOX2B+/NMB+) chemosensitive RTN neurons in mice. This manuscript follows up and challenges previously published work (Wu et al, 2007) that suggests that chemosensitivity of RTN neurons is in part mediated by 5HT and 5HT7 receptors expressed on RTN neurons.

The authors use previously collected transcriptomics data from isolated RTN neurons, multiplex single cell RT-PCR and RNA scope to demonstrate that the 5HT7 receptors are only weakly expressed in Phox2b cells that co-express high level of NMB and low levels of either GPR4 and Kcnk5. Cells expressing Phox2b/NMB and GPR4 and/or Kcnk5 (typical of chemosensitive RTN neurons) had no or low levels of Htr7.

Furthermore, the authors demonstrate that the expression of Htr7 is developmentally regulated. By using the same 5HTR7 receptor antagonists (as in Wu et al) they demonstrate that in slices containing RTN neurons, chemosensitivity is not affected by blockade of 5HT7 receptor and in vivo, the chemoreflex response is also not affected by local application of the same antagonist.

The manuscript is well written, the data is well presented. Although the expression of the protein/receptor may be quantitatively different from its mRNA, it is reasonable to assume that very low level of 5HT7 receptors are present in the parafacial region.

I have some comments on the manuscript, as follows:

Introduction
Page 6, "it was recently suggested instead that the chemosensitivity of RTN neurons is largely conferred indirectly -", I would substitute "largely" with "in part" since there is extensive and compelling literature that supports direct chemosensitive properties of RTN neurons.

Methods

page 8. Single-cell molecular biology of RTN neurons. It would be interesting to provide details on how single cells were harvested for sc RT-PCR.

"We rapidly isolated the brainstem from anesthetized mice (as above), extracted RNA..." Was the entire brainstem used for this analysis or was the ventral portion of the brainstem selected for this analysis? Please clarify and provide further details.

Results.

Page 11. Single cell mRNA quantification reveals limited Htr7 expression in RTN neurons. There is no mention on how transcriptomics data was collected or analyzed. It would be useful to report details in the methods section, even though it is a secondary analysis of previous work published by the authors.

The authors report a multiplex analysis for Htr7, Kcnk5, Gpr4 and NMB. Did the authors assess also the relative expression of PHOX2B? Since previous work reports the link between 5HT7 receptor and PHOX2B, these results could contribute to assess this relationship in the current study.

Page 13, "At caudal levels in the neonate....". If this is referred to P12 data, I would consider the term "juvenile" or simply P12 rather than neonate.

Page 13. At caudal levels in the neonate, strong Htr7 labeling often coincided with Phox2b+ neurons that were Nmbnegative and dorsomedially displaced from the main cluster of Nmb+ cells that comprise the RTN (Fig. 2A-C; red circles in maps, purple arrows in photomicrographs). I am a bit confused by this description. Do you mean purple/magenta circles in map and purple arrows in photomicrographs? The quality of the photomicrograph seems a bit low - not sure if this is a submission issue or there is space for improvement of resolution. From these images it is hard for me to determine if Hrt7 is actually present in the NMB positive cells.

Discussion

Page 16, "Therefore, Nmb-high cells may be a distinct subset of RTN neurons that participates in other breathing functions (e.g., sighing) (Li et al., 2016), which may be particularly sensitive to 5-HT7-mediated modulation". If this is the case, did the authors observe any change in respiratory parameters following LP-44 administration? Did sigh rate in increase?

Referee #2:

Shi and collaborators examined the potential role of the 5-HT7 receptors in CO2 chemosensitivity in the RTN. This was accomplished via an impressive array of techniques including RNASeq, scPCR, in situ hybridization via RNAscope, electrophysiology and microinjection with plethysmography. While the data on the molecular work is fairly complete, the electrophysiology data is relatively thin and the in vivo data may have additional caveats to consider.

One issue that needs addressed is the divergent ages in which the electrophysiology and in vivo breathing studies was examined. Given the authors identification of developmental changes in 5-HT7R expression, performing these two experiments at such different ages is problematic. It is understandable from a feasibility aspect why this occurred but a concern nevertheless. In addition to the age concern, the authors should comment on the recording of CO2-induced discharge at room temperature rather than closer to physiological temperature to better correlate with their in vivo studies.
While acknowledged by the authors, the inability to examine 5-HTR's role in chemosensitivity in the major expressing dorsal Nmb neurons is a major limitation. While an appropriate marker is not possible for targeted recording, it does not appear in the present study that the in vitro recordings occurred in the same region with the highest Nmb-5HT7R expression. Thus, while 5HT7R may not contribute to ventralmedial chemosensitivity, it may in these other neurons.

Vehicle controls are missing in the control data. Does repetitive application of 5-HT induce similar discharge in the absence of the SB antagonist?

The microinjection sites provided in Fig 6G (especially on right side) are larger than only the ventral RTN, and likely the any injectate will include adjacent regions. Such possibility should be addressed.

Minor-

The concentration of Ketoflex in the methods is given for a rat rather than a mouse.

END OF COMMENTS
REQUIRED ITEMS:

-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.

We have provided a separate file containing pictures and biographies for Drs. Yingtang Shi and Cleyton Sobrinho, the co-first authors.

-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.

We have attended to the checklist provided. The Methods section begins with an “Ethical Approval” section, and we indicated that our experiments were approved by the relevant institutional animal care and use committees and conform to the above-listed guidelines, principles, and regulations. The Methods sections also note details of anesthetic use and methods for humane euthanasia in terminal experiments.

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We have now moved the previous supplemental images from adult mice into the main paper. In combination with the images from neonatal mice, this now provides a depiction of the postnatal development of Htr7 expression specifically in RTN neurons, and provides a molecular neuroanatomical context for interpreting 5-HT7 receptor contributions to in vitro electrophysiology experiments (neonate) and in vivo ventilatory measures (adult). It also documents the decrease in Htr7 expression in
adult mice, a developmental pattern that is in contradistinction to the well-known increase in respiratory chemosensitivity (and is thus inconsistent with proposed 5-HT7 receptor contributions).

-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

The Statistical Summary Document is provided.

-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.

Data points are provided on all figures.

-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.

The data presented in Fig. 1A is available at the NCBI Gene Expression Omnibus site (GEO: GSE163155), as indicated in the text (p. 8) and figure legend. We now provide “Supporting Information” for Fig. 1B.

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

For the electrophysiology experiments, we have indicated the \( n \), cells and \( N \), animals in the manuscript. Note that one cell was recorded per slice per mouse, so pseudoreplication is not an issue. For in vivo plethysmography experiments, each mouse was an independent biological replicate.

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

This has been verified.

-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.

This has been verified.

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EDITOR COMMENTS

Reviewing Editor:

Methods Details:

Referee 1 indicated some methodological aspects that require further clarification.

We have addressed specific comments from Referee 1 below.

Ethics Concerns:

The manuscript misses a description of how anesthesia was monitored during the surgical procedures in adult mice.
This information has now been added in the text (see p. 6). The level of anesthesia was monitored throughout the procedures by testing for absence of withdrawal response due to firm paw pinch.

Comments to the Author:

The study used imaging and functional procedures to investigate the expression of 5-HT7 receptors (5-HT7R) in neurons of the parafacial region and their contribution to cellular and respiratory responses to high CO2. The authors reported that 5-HT7R mRNA is found mainly in RTN neurons that may not exhibit intrinsic chemosensitive mechanisms. It was also demonstrated that the antagonism of 5-HT7R in the RTN did not affect the CO2-induced firing response of putative chemosensitive neurons in vitro and the ventilatory response to hypercapnia in freely behaving mice. The authors conclude that 5-HT7Rs in the RTN neither mediate the chemosensitivity of neurons nor contribute to the ventilatory response to hypercapnia. This conclusion contrasts a previous study that, using a different approach, reported different results.

The manuscript is straightforward, the results are well described, and the discussion is appropriate. As highlighted by the referees, the information provided contributes to understanding the functioning and modulation of chemosensitive cells in the parafacial region. However, there is a gap between the electrophysiological and imaging data that requires authors' attention. If the expression of Htr7 was low or undetectable in most Nmb-expressing RTN neurons located ventral to the facial nucleus, it is not clear why in CO2-responsive neurons the 5-HT7R antagonist reduced the response to 5-HT. This observation parallels the referees' comments on the phenotypic characterization of the recorded neurons, the evaluation of CO2 responses in 5-HT7R-expressing neurons, and cellular targets of 5-HT7R agonists in the RTN to evoke an increase in breathing in vivo (despite the developmental-related reduction in the 5-HT7R expression). The referees also provided other important comments that, if addressed adequately, will enhance the manuscript. Other minor comments:

We thank the editor for their thoughtful handling of our manuscript and we agree that inconsistencies between Htr7 expression and 5-HT7 receptor action in RTN neurons required further clarification. Therefore, several additional sets of experiments were performed (see p. 10, 12-13).

Specifically, we repeated experiments to test effects of the 5-HT7 receptor blocker SB269970 on 5-HT responsiveness, this time minimizing the time between 5-HT exposures while ensuring the incubation time in SB269970 prior to 5HT exposure was consistent with previous work (Wu et al., 2019). These experiments were performed at both room temperature and near body temperature. Also, to test the possibility that 5-HT7 receptors regulate activity of RTN neurons indirectly by modulation of synaptic input, we characterized 5-HT responses under control conditions and in the presence of a cocktail of blockers of fast synaptic transmission (CNQX, strychnine, and gabazine). We also added additional new experiments testing effects of LP-44 in vitro, and we provided further verification that recorded neurons were RTN neurons (using a Phox2b-reporter mouse line and post hoc Phox2b immunostaining).

In short, we found under these more carefully controlled experimental conditions, that SB269970 did not blunt firing responses to 5-HT at room temperature (22°C), near body temperature (37°C) or with synaptic blockers in the bath (p. 12-13); moreover, LP-44 had only modest excitatory effects (Δ 0.4 ± 0.1 Hz) in ~37% of the RTN neurons tested (n=7/19). These results are consistent with data from molecular and histochemical analyses that a subpopulation of RTN neurons show low expression of Htr7 and support our main conclusion that 5-HT7 receptors are not required for cell autonomous CO2/H+ sensing by RTN neurons.

Please see responses below for more details.

- provide the ethical approval protocol number of all institutions.
We have now provided the ethical approval protocol number from each of our Institutions (p. 2).

- indicate the animals' sex.

In the “Animals” section of the Methods (p. 2), we now indicate that experiments were done with mice of either sex.

- describe the perfusion and histological procedures to check the microinjection sites in adult mice.

This information has now been added as a new section in the Methods (see p. 7-8). We also now include Phox2b+ staining of recorded RTN neurons and have added a section to describe the procedure for post hoc immunostaining procedure in slices (p. 6).

- please make sure that all data are represented as mean (plus minus) SD (Fig 5 indicates SEM, and panels D-F of Fig 6 miss this information) and that all statistical tests and results are described in the manuscript.

A mistaken previous reference to SEM has been removed from the former Fig. 5 (now Fig. 9) – all data points are shown on the figure panels, represented in box-and-whiskers format, and all variance references are to SD (not SEM).

**Senior Editor:**

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

Define n with animal numbers as well as number of samples. Use SD throughout. All tables and figures must be incorporated into the manuscript.

We use SD throughout, and a previous inadvertent reference to SEM has been removed from the former Fig. 5 (now Fig. 9) – all data points are shown, and represented in box-and-whiskers format. We define n=cells and N=mice, and provide the relevant n and N values throughout. Only one cell was recorded from one slice obtained from each mouse, so concerns with pseudoreplication have been avoided.

Comments to the Author:

Please indicate how the level of anesthesia was assessed and maintained, and how animals were terminated.

We have provided information on how anesthesia was monitored (by firm toe pinch) and provided information on how they were terminated (rapid decapitation or perfusion).

Supplementary tables and figures must be incorporated into the manuscript. Material such as figures, tables, text (e.g. expanded/detailed methods or results), equations, and other material must be incorporated into the manuscript itself as part of the text or as standard figures or tables and not supplied as Supporting Information.

We have now moved all supplementary materials into the paper itself.

Please indicate the number of animals as well as the number of preparations when describing 'n' sample size.

We have provided the n, number of cells for each experiment, and the N, number of animals.

Please ensure all summary data variance is described as Standard Deviation

A previous mistaken reference to SEM has been removed from the former Fig. 5 (now Fig. 9). In all cases where variance is provided, it is given as SD or represented in box-and-whiskers format (median, 25%ile-75%ile, and range).
REFEREE COMMENTS

Referee #1:

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The manuscript is well written, the data is well presented. Although the expression of the protein/receptor may be quantitatively different from its mRNA, it is reasonable to assume that very low level of 5HT7 receptors are present in the parafacial region.

I have some comments on the manuscript, as follows:

We are grateful for the positive assessment of the manuscript, and the constructive suggestions for improvement.

Introduction

Page 6," it was recently suggested instead that the chemosensitivity of RTN neurons is largely conferred indirectly -. " I would substitute "largely" with "in part" since there is extensive and compelling literature that supports direct chemosensitive properties of RTN neurons.

This text has been revised as directed (see p. 1). Insofar as we have provided some of the evidence for direct chemosensitive properties of RTN neurons, we certainly agree there is much support for that conclusion in the literature.

To clarify: At this point in the manuscript, we were referring to the actual “suggestions” offered in Wu et al. 2019, indeed using their specific wording where they concluded that:

(Abstract) “The primary role of RTN neurons may be as relays and amplifiers of the pH response from 5-HT neurons and other chemoreceptors rather than as pH sensors themselves.” and

(Discussion) “Taken together, these results indicate that a large component of the pH response of RTN neurons is due to input from chemosensitive 5-HT neurons under these experimental conditions.” and “a substantial component of chemosensitivity of RTN neurons is not cell-autonomous.” and “our findings show that a large component of the response of Phox2b-expressing RTN neurons to pH within a narrow range expected to occur under physiological conditions in vivo is extrinsic – mediated in part by release of 5-HT in proportion to the level of acidosis.”
Methods

page 8. Single-cell molecular biology of RTN neurons. It would be interesting to provide details on how single cells were harvested for sc RT-PCR.

Additional information on the cell harvesting approach has been added to the Methods (p. 4).

"We rapidly isolated the brainstem from anesthetized mice (as above), extracted RNA..." Was the entire brainstem used for this analysis or was the ventral portion of the brainstem selected for this analysis? Please clarify and provide further details.

For this, we used the entire brainstem from the region from -5.4 mm to -7.0 mm, relative to bregma. This information is now provided in the Methods (p. 5).

Results.

Page 11. Single cell mRNA quantification reveals limited Htr7 expression in RTN neurons. There is no mention on how transcriptomics data was collected or analyzed. It would be useful to report details in the methods section, even though it is a secondary analysis of previous work published by the authors.

A single cell harvesting approach like that used here for sc-qPCR was employed to obtain cells for single cell transcriptomics. We now point out that out in the Methods section (p. 4), and explicitly refer the reader to Shi et al. (2017) for detailed methods on how the transcriptomic data were obtained and analyzed. We hope the reviewer agrees that it is not appropriate to extensively re-visit the methods from that earlier paper in this report.

The authors report a multiplex analysis for Htr7, Kcnk5, Gpr4 and NMB. Did the authors assess also the relative expression of PHOX2B? Since previous work reports the link between 5HT7 receptor and PHOX2B, these results could contribute to assess this relationship in the current study.

We did not assay Phox2b by sc-qPCR because we wanted to use a more specific marker for RTN – i.e., Nmb – and there is a practical limit to the number of genes that can be assessed by this single cell qPCR method, especially when some are in low abundance (e.g., Htr7, Kcnk5).

That said, we understand the Reviewer’s interest in the link between Phox2b and Htr7, for comparison to the earlier Wu et al. 2019 work. To address this, we took the approach used by Wu et al. – i.e., a Phox2b-Cre dependent mTomato reporter expression strategy – to re-examine expression of Htr7 specifically in Phox2b-expressing parafacial neurons and in Nmb-expressing RTN neurons expressing the mTomato reporter gene (new Fig. 5). This allowed direct comparison of reporter and Htr7 expression in relation to Phox2b itself, and also to the more specific RTN neuron marker (Nmb).

As depicted in the new Fig. 5, and consistent with known expression patterns and previous results from these reporter mice, we found that there was substantial (albeit imperfect) overlap of Phox2b and mTomato expression. Importantly, numerous mTom+ cells that were Nmb-negative were observed in the immediate vicinity of the RTN, and those non-RTN neurons often expressed Htr7. This new analysis also confirmed that, within RTN neurons, Htr7 expression was most prominent in the Nmb-Hi subgroup of cells. Thus, we think this more direct comparison with the Wu et al., 2019 work reinforces the conclusion that many of the Htr7-expressing neurons in the parafacial region that are Phox2b+ and mTomato+ are not bona fide RTN neurons (i.e., they do not match the established molecular profile of RTN chemoreceptors, specifically Nmb expression).

Page 13, "At caudal levels in the neonate....". If this is referred to P12 data, I would consider the term "juvenile" or simply P12 rather than neonate.
As requested, we have re-worded neonate/neonatal throughout the manuscript when referring to P12 mice; those terms have been substituted with “young”, “early postnatal” or simply “P12”. We note that the electrophysiological recordings in vitro were obtained from P7-P12 mouse pups, and we continue to refer to those younger mice as neonates.

Page 13, At caudal levels in the neonate, strong Htr7 labeling often coincided with Phox2b+ neurons that were Nmb-negative and dorsomedially displaced from the main cluster of Nmb+ cells that comprise the RTN (Fig. 2A-C; red circles in maps, purple arrows in photomicrographs). I am a bit confused by this description. Do you mean purple/magenta circles in map and purple arrows in photomicrographs?

Thank you for pointing out this confusion. We have now corrected the color description to magenta, as correctly suggested (p. 10).

The quality of the photomicrograph seems a bit low - not sure if this is a submission issue or there is space for improvement of resolution. From these images it is hard for me to determine if Hrt7 is actually present in the NMB positive cells.

The Htr7 labeling (red dots) on the green Nmb+ cells (especially the large, bright green cells) is clearly evident in our original photomicrographs and PDFs (the red dots appear yellow in the overlays); the triple-labeled cells are indicated with white arrows. We suspect that the quality issue that was noted may have resulted from the PDF conversion process associated with the submission site. We now provide high resolution images with the resubmission (as EPS files), and we appreciate the reminder to be vigilant that the quality and resolution of these images is maintained throughout the process.

Page 14. It is mentioned in the methods that recorded cells were labelled with Lucifer Yellow. Did the authors process further the tissue collected and labeled neurons for Hrt7, NMB and Phox2b?

Thank you for the suggestion. We have now provided this additional histochemical verification in a subset of recorded RTN neurons (p. 12). We present a recording from a verified Phox2b+ cell (Fig. 9A), as well as an image of a recorded neurons that was Phox2b+ and the location of the verified Phox2b+ recorded neurons (Fig. 9B). Note that we also confirmed results by performing additional recordings from mTom+ neurons in slices from Phox2b-mTom mice (p. 12).

Did the authors attempt to record from cells that were reported to have stronger Hrt7 expression? What about the highNMB cells? The authors claim that these highNMB cells are not chemosensitive - is the lack of expression of GPR4 and Kcnk5 the only justification for reaching this conclusion? Maybe this can be discussed further in the discussion section or recordings from these cells could be presented.

As we discussed in the limitations section of the original manuscript, it is unfortunately not possible to reliably identify, a priori, the Nmb-high cells that have stronger Htr7 expression for targeted recording (see p. 17). We now reiterate at this point in the manuscript that this Nmb-high cell population fails to show an increase in Fos expression after CO2 exposure in vivo (also mentioned on p. 11, 17) which together with the absence of Gpr4/Kcnk5 expression, provides the evidence for their lack of CO2 chemosensitivity. Note that this CO2 insensitivity in vivo is despite the higher Htr7 expression in Nmb-high RTN neurons, a finding further inconsistent with the hypothesis that CO2 sensitivity is mediated by 5-HT7 receptors.

Figure 5. Since recorded cells were labelled with lucifer yellow, it would be nice to add the location of both cells presented in figure 5A and C, below the graph.
We have now provided a map of the location of LY-filled, Phox2b+ cells (now Fig. 9B). As for these verified Phox2b+ neurons and the mTom+ cells from Phox2b-mTom mice, all other recordings were obtained in the main clusters of RTN neurons.

**Discussion**

Page 16, "Therefore, Nmb-high cells may be a distinct subset of RTN neurons that participates in other breathing functions (e.g., sighing) (Li et al., 2016), which may be particularly sensitive to 5-HT7-mediated modulation". If this is the case, did the authors observe any change in respiratory parameters following LP-44 administration? Did sigh rate increase?

Thank you for this suggestion. We have reviewed the traces and find that there are no changes in the number of sighs, i.e., it is similar before or after LP-44. This has now been noted in the manuscript (see p. 15).

**Referee #2:**

Shi and collaborators examined the potential role of the 5-HT7 receptors in CO2 chemosensitivity in the RTN. This was accomplished via an impressive array of techniques including RNASeq, scPCR, in situ hybridization via RNAscope, electrophysiology and microinjection with plethysmography. While the data on the molecular work is fairly complete, the electrophysiology data is relatively thin and the in vivo data may have additional caveats to consider.

One issue that needs addressed is the divergent ages in which the electrophysiology and in vivo breathing studies was examined. Given the authors identification of developmental changes in 5-HT7R expression, performing these two experiments at such different ages is problematic. It is understandable from a feasibility aspect why this occurred but a concern nevertheless. In addition to the age concern, the authors should comment on the recording of CO2-induced discharge at room temperature rather than closer to physiological temperature to better correlate with their in vivo studies. [break by authors]

We now address the age differences for electrophysiology and in vivo breathing studies in the limitations section (p. 17). Importantly, the slice recordings were obtained at a developmental time point when Htr7 levels are highest (and thus any 5-HT7-mediated effect should be even greater) ... and, despite this, we still saw no effect of the 5-HT7 blocker on CO2 chemosensitivity.

In addition to the age concern, the authors should comment on the recording of CO2-induced discharge at room temperature rather than closer to physiological temperature to better correlate with their in vivo studies.

We have now included some recordings at elevated temperature and find no effect of SB269970 on 5-HT responses at 37°C (p. 12). We note also that previous work has demonstrated an effect of temperature on the amplitude of RTN neuron responses to CO2/H+ but did not transform non-responsive cells to become responsive (Guyenet et al., 2005; PMID: 16192384).

While acknowledged by the authors, the inability to examine 5-HTR's role in chemosensitivity in the major expressing dorsal Nmb neurons is a major limitation. While an appropriate marker is not possible for targeted recording, it does not appear in the present study that the in vitro recordings occurred in the same region with the highest Nmb-5HT7R expression. Thus, while 5HT7R may not contribute to ventralmedial chemosensitivity, it may in these other neurons.

We agree, and have expanded our discussion of this limitation and interpretation in the revised manuscript (p. 17; also see response to Referee 1, above). We also point out, both in that limitations section and elsewhere (p. 11, 17) that there is previous evidence from Fos labeling work that the Nmb-high cells may be a distinct subpopulation of RTN neurons that are not CO2-chemosensitive.
We also note here that the previous conclusions from Wu et al., 2019 regarding 5-HT7 receptor contributions to RTN chemosensitivity made no distinctions with respect to any RTN cell subtypes – just that “a large component of the pH response of RTN neurons is due to input from chemosensitive 5-HT neurons.” Even without recording from that minor subgroup of cells with somewhat higher Nmb-5HT7R expression, our data from the major chemosensitive population of ventral RTN neurons strongly refute that general conclusion.

Vehicle controls are missing in the control data. Does repetitive application of 5-HT induce similar discharge in the absence of the SB antagonist?

The reviewer raises an important point that cellular activity can waver over the course of a long term slice-patch recording. This is a concern because in the original version of this manuscript there was ~20 min between 5-HT exposures, first alone and then in SB269970. Therefore, we re-evaluated the effects of SB269970 on the firing response to 5-HT this time with ~10 min between 5-HT exposures. Note that the incubation time in SB269970 prior to 5-HT exposure was chosen to match those of Wu et al., 2019 (PMID: 30866045). Under these conditions, we found that SB269970 had negligible effect on the firing response to 5-HT, which was well maintained.

The microinjection sites provided in Fig 6G (especially on right side) are larger than only the ventral RTN, and likely the any injectate will include adjacent regions. Such possibility should be addressed.

We now clarify that the tip of the injection cannula was located within the RTN (p. 13). In addition, we have now amplified our Discussion of the caveat that the respiratory stimulation observed with 5-HT7 agonist application may have been mediated by actions of the injectate on nearby cells or adjacent regions (p. 17).

**Minor-**

The concentration of Ketoflex in the methods is given for a rat rather than a mouse.

We apologize for this error, which has now been corrected to provide the concentration of ketoflex used for mice (p. 6).
Dear Dr Bayliss,

Re: JP-RP-2022-282279R1 "5-HT7 receptors expressed in the mouse parafacial region are not required for respiratory chemosensitivity" by Yingtang Shi, Cleyton R Sobrinho, Josep Soto-Perez, Brenda M Milla, Daniel S Stornetta, Ruth Stornetta, Ana C Takakura, Daniel K Mulkey, Thiago S Moreira, and Douglas A. Bayliss

Thank you for submitting your revised Research Article 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.

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

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If the Statistical Summary Document has errors please describe what is incorrect?:
The values are not reported consistently across all cells. Moreover, cells D20 and E20 show the same values.

Comments to the Author:
The authors adequately addressed the referees and my comments, providing further clarifications and additional experiments. I also have no other scientific comments. However, there are still minor issues to check:

1. Tables 1 and 2 are missing in the manuscript.

2. Is the Ketoflex dose correct: 1%, 0.002 ml (2 µl?)/mouse?

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The reviewing editor has a few minor issues that need correcting.

REFEREE COMMENTS

Referee #1:

Thanks for addressing my comments and concerns, and for including additional results that support your findings. I don't have any other concerns.

Referee #2:

The authors have adequately addressed all of my previous concerns, including adding new data and clarifying text. I have no further comments and compliment the authors on such a study.

END OF COMMENTS

1st Confidential Review 24-Feb-2022
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The values are not reported consistently across all cells. Moreover, cells D20 and E20 show the same values.

We have corrected the duplication in cells D20 and C20 of the SSD XLS file, and now also provide a more consistent presentation across cells.

Comments to the Author:
The authors adequately addressed the referees and my comments, providing further clarifications and additional experiments. I also have no other scientific comments. However, there are still minor issues to check:

We thank the editors and reviewers for their careful assessment of the manuscript, and for their helpful suggestions for improvement. We have addressed the remaining minor issues.

1. Tables 1 and 2 are missing in the manuscript.
   We have now included these Tables in the manuscript itself (p. 24).
2. Is the Ketoflex dose correct: 1%, 0.002 ml (2 µl)/mouse?
   Thank you for catching this error – the volume has been corrected to 0.2 ml (p. 6)
3. Please, revise the SSD document and report the values consistently. Moreover, cells D20 and E20 show the same values.
   We have corrected the duplication in cells D20 and C20 of the SSD XLS file, and now also provide a more consistent presentation across cells.

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

Please report actual p values in text and figures. Do not use symbols or 'ns'. In the figure and table legends, please state the statistical test used in figures and tables.

We have replaced the NS annotation in Fig. 9 panels with the actual P values, and have indicated the relevant statistical tests associated with reported P values in both the figures and text.

The reviewing editor noted mistakes in the statistical summary document. Please correct.

We have addressed comments from the reviewing editor regarding the SSD file (please see above).

Comments to the Author:
The reviewing editor has a few minor issues that need correcting.

We have addressed comments from the reviewing editor (please see above).

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REFEREE COMMENTS

Referee #1:
Thanks for addressing my comments and concerns, and for including additional results that support your findings. I don't have any other concerns.
We are grateful for the careful review of the manuscript and constructive suggestions for improvement.

Referee #2:
The authors have adequately addressed all of my previous concerns, including adding new data and clarifying text. I have no further comments and compliment the authors on such a study.

We are grateful for this positive assessment of the manuscript and the constructive suggestions for improvement.
Dear Dr Bayliss,

Re: JP-RP-2022-282279R2 "5-HT7 receptors expressed in the mouse parafacial region are not required for respiratory chemosensitivity" by Yingtang Shi, Cleyton R Sobrinho, Joseph Soto-Perez, Brenda M Milla, Daniel S Stornetta, Ruth Stornetta, Ana C Takakura, Daniel K Mulkey, Thiago S Moreira, and Douglas A. Bayliss

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Reviewing Editor:

I have no additional comments and congratulate the authors for this work.

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