EMBO reports

Fast neurogenesis from carotid body quiescent neuroblasts accelerates adaptation to hypoxia

Verónica Sobrino, Patricia González-Rodríguez, Valentina Annese, José López-Barneo and Ricardo Pardal

Review timeline:

Submission date:	7 June 2017
Editorial Decision:	21 July 2017
Revision received:	7 November 2017
Editorial Decision:	21 November 2017
Revision received:	30 November 2017
Accepted:	13 December 2017

Editor: Martina Rembold

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. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 21 July 2017

Thank you for the submission of your manuscript to our journal. I apologize for the delay in getting back to you; it is rather difficult to find referees in the summer season, and we also give the referees more time for peer-review at this time of the year. I have taken over the handling of your manuscript from my colleague Martina since she is not in the office.

We have now received the reports from both referees, which are pasted below. As you will see, both referees acknowledge that the findings are potentially interesting. However, both also suggest a number of experiments to strengthen the study. I think that all suggestions are valid and should therefore be addressed, with the exception of the lineage tracing analyses that - while certainly most welcome - may indeed be beyond the scope of this study.

We would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further.

Regarding data quantification, please remember to specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends.

Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

Sobrino and colleagues report on the identification and characterization of a previously unreported cell stage within the rat carotid body cell hierarchy. In vivo and in vitro experiments lead the author to conclude that a subset of TH+ glomus cells, original thought to be differentiated neuron-like cells, are actually only partially mature "neuroblasts", capable of rapidly responding to hypoxia and to hypoxia-induced signals by entering cell cycle, and eventually mature into fully mature TH+ glomus cells.
The manuscript introduces the interesting concept of a "primed" cell stage to the already remarkably dynamic carotid body context, and as such represents a significance advance. However, the authors' conclusions are at times not yet as strongly backed up experimentally as may be needed.
Furthermore, the study avoids entirely the issue of how this proposed "primed neuroblast" population would be related, in lineage terms, to the resident stem cell population and to the more mature glomus cells of the CB.
The most obvious missing bit of evidence are lineage-tracing analyses of the origins and fate of this population of cells. While it is perhaps beyond the scope of this study, employing a genetic murine model to lineage-trace HNK+ cells will eventually be necessary.
There are a few specific points, which follow below.
1) The in vivo cycling dynamics of TH+ cells are difficult to fully understand. Figs.1E-G show results from a series of experiments (Fig.1D) that suggest that at all time points most BrdU+ cells are already postmitotic at the moment of analysis; only approx 10% of cells labeled with BrdU over the previous 48hrs are Ki67+ at d2 and d4, while only 1% at d6. Conversely, the bulk of the sizeable Ki67+ populations at d4 and d6 are not BrdU+, i.e. haven't yet completed G1.
Cumulative BrdU labeling during 2,4 and 6 days may help get an idea of the overall size of the proliferating population, assuming generation of TH+ cells from previously TH- cells doesn't play a major role within this time interval.
In general, I think the presented data suggest the intriguing possibility that activation of several cohorts of previously quiescent TH+ cells may take place, each of which would mostly become postmitotic right away.
2) Claims by the authors that proliferation is a prerogative of the mTH/HNK population seem unwarranted since, as clear from Fig.2D, TH+ (HNK-) cells proliferate basally to a similar (and low) extent, and increase their proliferation in response to hypoxia in a similar way (5-6x vs 7-8x for mTH/HNK cells).
3) A population identified by cytofluorimetric means deserves further characterization: the small-sized, TH+ (but HNK-) population clearly visible in Suppl. Fig.2B. These cells look like good candidates for an intermediate stage generated by HNK+ cells, on the way to a more fully mature condition. Data from the sorted HNK- cells used for the in vitro timelapse experiments should be assessed to establish whether a correlation exists between e.g. cell size at the beginning of the experiment, and eventual proliferative ability. Conversely, cells from the HNK+ population should be analyzed at the end of the timelapse for HNK expression.
Along the same lines, data presented in Fig. 5/6 should, whenever possible, be reanalyzed to assess the degree of functional maturation of the above small-bodied HNK- population. As above, I suggest cell size is evaluated as a possible indicator of heterogeneity within the sorted HNK-

population and, if the somatic size info can be retrieved for the cells assayed in the various single-cell analyses (e.g. Calcium and NAD(P)H dynamics), that it be used to stratify the analysis of TH+ cells.
4) The whole notion of the generation of TH+/HNK- cells from mTH+/HNK+ cells is very attractive, but there is no direct evidence yet for that. Sorted mTH+/HNK+ cells should be assessed, after culturing in conditions expected to drive differentiation (Hx vs Normoxia), for their ability to give rise to TH+/HNK- cells and for their ability to do so in a Hx-modulated manner.
5) A marker for mature neurons should be stained for in CB sections, alongside HNK and TH, to substantiate the claim, based on Tuj1 IF, that TH+/HNK+cells are immature. Alternatively, expression levels of selected markers could be tested on cDNA from purified populations.
6) Discussion of EM data potentially conflates coverage of the different classes of HNK+ cells; the authors show in Fig.2 a clearly HNK+/TH- population, which cannot be distinguished from the mTH/HNK+ in EM pics, since immunogold staining is done only for HNK. The authors should assess variability in cytological features observed in HNK+ cells in EM pics and comment on possible subgroups that may correspond to the two HNK+ populations. Co-staining for TH would of course be of help. Authors should not refer to HNK+ cells as mTH/HNK unless some positive identification is possible, directly or by some verified proxy.
7) With regard to EM-based claims about the relative positioning of the HNK+ subpopulation(s) relative to other landmarks (e.g. vasculature), no quantifications are shown and should be either provided (e.g. shortest distance to a vessel) or the claim otherwise substantiated (or eliminated).
8) On page 9, a claim is made that "mature (TH+) glomus cells can form synaptic contacts with mTH+/HNK+ neuroblasts (see fig4)"; it's not at all clear what this is based on, as the only identifiable cell-cell contact is the highlighted electron-dense patch in Fig.4C-1. Legend for Fig.4 lacks mention of the scale bar size.
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"40K" should be replaced in figures by an appropriate reference to [K+].

Referee #2:

The authors are world leaders in the study of the carotid body, and have previously made many important discoveries about this interesting site of neurogenesis. IN the current study, the authors suggest that there are two populations of glomus cells, fully differentiated and less differentiated neuroblast populations. THe study identifies markers that can be used to isolate these two populations and showed that the neuroblast population is proliferative in vivo and in vitro in response to hypoxia. THe authors then suggest that the neuroblast population exists to promote acclimatization to hypoxia. The study is pretty convincing, but there are some facets that could be improved to bolster the claims made.

1, essentially the authors have identified a novel population within the tissue, and this reviewer would argue that the focus of the paper should be around that observation. The description of "fast neurogenesis" is distracting, as there is no comparison made directly to neurogenesis from the existing stem cell population described previously by the authors.

2, the authors did not show that their experimental manipulation (hypoxia) exerts the desired effect. There is no staining for HIF stabilization, or pimidizole to show that the lowered oxygen was sufficient to stabilize the oxygen sensing pathway. Are the authors suggesting that the CB is differentially sensitive?

3, the claims of independent cell types would be strongly bolstered by purification of both populations and transcriptional profiling versus the NSCs as they have described previously. Is there a reason this cannot be done?

4, roughly half of the quantification/histograms presented do not have error bars, how reproducible are the results?

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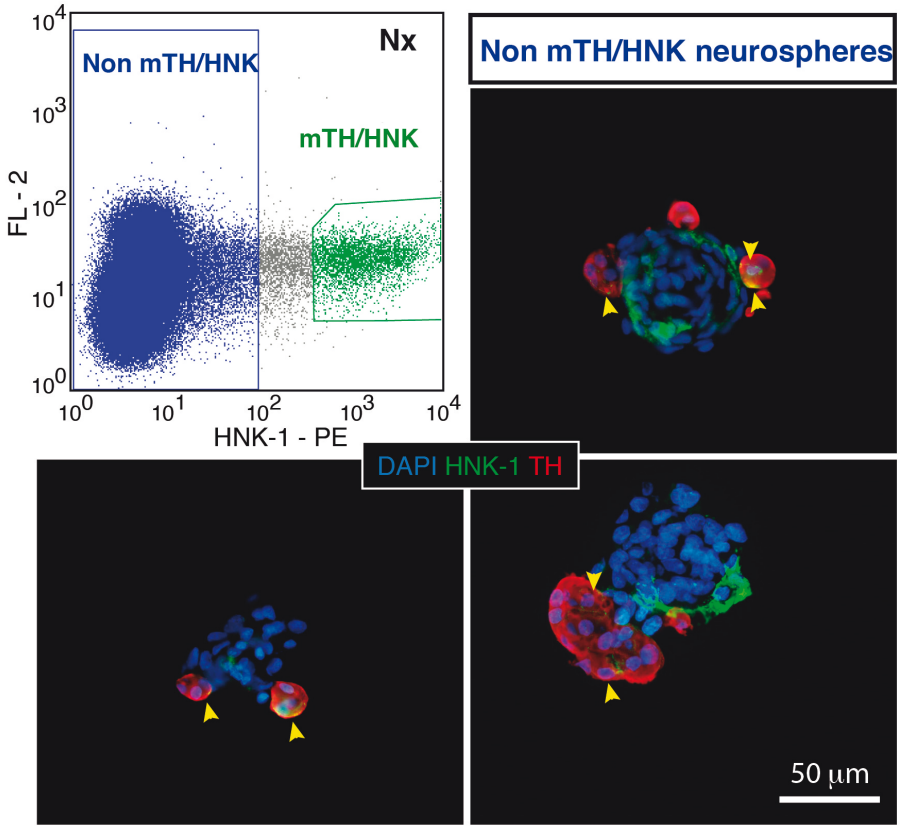
The most obvious missing bit of evidence are lineage-tracing analyses of the origins and fate of this population of cells. While it is perhaps beyond the scope of this study, employing a genetic murine model to lineage-trace HNK+ cells will eventually be necessary.

Authors: We agree with the reviewer that a precise cell fate mapping analysis would be necessary to corroborate lineage relationship between carotid body stem cells, neuroblasts and mature glomus cells. Unfortunately, mouse neuroblasts are HNK-1 negative. Hence, we cannot identify them as easily as in the rat, making really difficult the cell fate mapping studies. To address the question raised by the reviewer, we have performed neurosphere assays with multipotent stem cells in the organ (non mTH/HNK cells, to remove neuroblasts; see figure below). As shown in the figure below, these clonal neurospheres have a core of HNK-1 negative progenitors (the ones originating the colony) and some HNK-1 positive cells (green), which appeared later in the periphery. The blebs of differentiated TH+ cells (red) contain some cells with both TH (red) and HNK-1 (green) stainings (resulting in a yellowish color, arrowheads) which are most likely neuroblasts. These data obtained from clonal neurospheres suggest that all the cells (progenitors, HNK-1+ cells, HNK-1+/TH+ cells and TH+ cells) derive from a common stem cell. However, since this is not a regular cell fate mapping approach, we have decided not to include this figure in the manuscript.



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In general, I think the presented data suggest the intriguing possibility that activation of several cohorts of previously quiescent TH+ cells may take place, each of which would mostly become postmitotic right away.

Authors: We believe the referee is right in this appreciation. We do not know whether it corresponds to different cohorts or just a stepwise activation of all neuroblasts present, but truth is that the data is compatible with a quite longer G1 phase, and extremely short S, G2 and M phases. It looks like once the neuroblast is activated and enters S phase (is labeled with BrdU), it goes quickly through the rest of cell cycle and becomes postmitotic. We have tried to rephrase this part in the manuscript (see page 5) to achieve more clarity in the interpretation. In any case, it is obviously beyond the scope of this study to enter in too much detail in the proliferation dynamics of carotid body neuroblasts.

2) Claims by the authors that proliferation is a prerogative of the mTH/HNK population seem unwarranted since, as clear from Fig.2D, TH+ (HNK-) cells proliferate basally to a similar (and low) extent, and increase their proliferation in response to hypoxia in a similar way (5-6x vs 7-8x for mTH/HNK cells).

Authors: The flow cytometry analysis presented in Fig. 2C was performed with very general gates (represented as different colors in the dot plot), so an important contamination within the two TH+ populations might be expected. Moreover, preparation of cells for flow cytometry using intracellular immunocytochemistry, induces damage to the cells that could diminish the amount of HNK-1 epitope in their membranes, making contamination of the HNK-1 negative population with HNK-1+ cells a more frequent issue. That is why we decided to perform the time-lapse recording experiment (Fig. 2E and F) with much tighter sorting gates and live cells (see Fig. EV2B). In this case, contamination is much lower, and the difference in proliferation capacities between both populations is clearer.

3) A population identified by cytofluorimetric means deserves further characterization: the small-sized, TH+ (but HNK-) population clearly visible in Suppl. Fig.2B. These cells look like good candidates for an intermediate stage generated by HNK+ cells, on the way to a more fully mature condition. Data from the sorted HNK- cells used for the in vitro timelapse experiments should be assessed to establish whether a correlation exists between e.g. cell size at the beginning of the experiment, and eventual proliferative ability. Conversely, cells from the HNK+ population should be analyzed at the end of the timelapse for HNK expression.
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Authors: We are afraid we do not understand exactly which cell population the reviewer is referring to. The plot in Suppl. Fig. 2B does not really look at cell size, as Suppl. Fig. 2A does, in order to distinguish between cell populations. We actually do not see the existence of any small-sized TH+/HNK- cells. The small-sized HNK- cells ('Neg' in Suppl. Fig. 2B) are also TH-, and the small-sized TH+ cells are also HNK+ ('mTH/HNK' in Suppl. Fig. 2B).

Nevertheless, this point raised by the reviewer made us wonder about whether we could see a clear increase in neuroblast cell size and a clear decrease in HNK-1 epitope expression upon maturation in our experiments in vitro. We have performed the measurements and have included a new figure with the resulting data (see new Suppl. Fig. EV3A-B, and page 7 first paragraph).

4) The whole notion of the generation of TH+/HNK- cells from mTH+/HNK+ cells is very attractive, but there is no direct evidence yet for that. Sorted mTH+/HNK+ cells should be assessed, after culturing in conditions expected to drive differentiation (Hx vs Normoxia), for their ability to give rise to TH+/HNK- cells and for their ability to do so in a Hx-modulated manner.

Authors: We have added these new data as a new figure (Suppl. Fig. EV3C), and have introduced comments regarding these data in page 7 of the manuscript (first paragraph).

5) A marker for mature neurons should be stained for in CB sections, alongside HNK and TH, to substantiate the claim, based on Tuj1 IF, that TH+/HNK+cells are immature. Alternatively, expression levels of selected markers could be tested on cDNA from purified populations.

Authors: Although we have tried several potential markers, we have not been able to set up a clear and nice immunological staining to distinguish mature neurons and neuroblasts (neither in sections nor in single cell suspensions) other than the HNK-1 staining. However, as suggested by the reviewer, we have performed real time PCRs to distinguish both types of cells by showing differential expression of significant markers associated to their maturation state. We have included the data in new Suppl. Fig. EV5 and have commented the results on page 8 (second paragraph).

6) Discussion of EM data potentially conflates coverage of the different classes of HNK+ cells; the authors show in Fig.2 a clearly HNK+/TH- population, which cannot be distinguished from the mTH/HNK+ in EM pics, since immunogold staining is done only for HNK. The authors should assess variability in cytological features observed in HNK+ cells in EM pics and comment on possible subgroups that may correspond to the two HNK+ populations. Co-staining for TH would of course be of help. Authors should not refer to HNK+ cells as mTH/HNK unless some positive identification is possible, directly or by some verified proxy.

Authors: We thank the reviewer for this observation. It is true that we see different HNK+ cells in our EM preparations, although we did not mention them before. We see cells like the one shown in Suppl. Fig. EV4A. As indicated in page 7 (second paragraph) of the manuscript, we believe these cells correspond to the uncharacterized TH-/HNK low cells observed in cytometry dot plots (blue dots in Fig. 2C). We have not added these cells in our EM analysis. We have only used cells highly positive for HNK (with abundant gold particles all around the surface), which we believe mostly correspond to CB neuroblasts. Therefore, we feel pretty confident when referring to these cells as mTH/HNK cells, since they are not only HNK high but they also contain dense-core synaptic vesicles.

7) With regard to EM-based claims about the relative positioning of the HNK+ subpopulation(s) relative to other landmarks (e.g. vasculature), no quantifications are shown and should be either provided (e.g. shortest distance to a vessel) or the claim otherwise substantiated (or eliminated).

Authors: New quantitative data regarding the positioning of both mature and immature glomus cells have been added (see new Suppl. Fig. EV4 and comment in page 8, first paragraph).

8) On page 9, a claim is made that "mature (TH+) glomus cells can form synaptic contacts with mTH+/HNK+ neuroblasts (see fig4)"; it's not at all clear what this is based on, as the only identifiable cell-cell contact is the highlighted electron-dense patch in Fig.4C-1. Legend for Fig.4 lacks mention of the scale bar size.

Authors: We fully agree with the reviewer and have softened the sentence. Scale bar sizes have been added to Figure 4 legend.

9) All molecular mechanisms suggested to play a role in HNK+ cells maturation of proper responsiveness to hypoxia would be strengthened if it could be shown that Hx-exposed mature (i.e. TH-only) cells' conditioned medium can trigger this maturation in purified HNK+ cells.

Authors: We have tried hard to perform this experiment, without success. We believe that the molecules released from type I cells in the vicinity of neuroblasts as a spill over, become too diluted when trying to perform the experiment in vitro with isolated and purified populations. Hence, it

might be that the 'conditioned medium' does not contain the necessary concentration of 'maturation agents' when testing this paracrine communication in vitro.

"40K" should be replaced in figures by an appropriate reference to [K+].

Authors: A clarifying note has been added to the corresponding legends (Figs. 6 and 7).

Referee #2:

The authors are world leaders in the study of the carotid body, and have previously made many important discoveries about this interesting site of neurogenesis. IN the current study, the authors suggest that there are two populations of glomus cells, fully differentiated and less differentiated neuroblast populations. THe study identifies markers that can be used to isolate these two populations and showed that the neuroblast population is proliferative in vivo and in vitro in response to hypoxia. THe authors then suggest that the neuroblast population exists to promote acclimatization to hypoxia. The study is pretty convincing, but there are some facets that could be improved to bolster the claims made.

1, essentially the authors have identified a novel population within the tissue, and this reviewer would argue that the focus of the paper should be around that observation. The description of "fast neurogenesis" is distracting, as there is no comparison made directly to neurogenesis from the existing stem cell population described previously by the authors.

Authors: We agree with the point raised by the reviewer. However, we think that our manuscript is already focused around the immature neuroblasts discovered in the carotid body. We like the description of fast neurogenesis from quiescent neuroblasts as one of the important points of our work. As we discuss in a whole section of Discussion ('Carotid body fast neurogenesis' section), we observe production of new neurons within hours of exposure to hypoxia, in contrast to days or weeks previously reported for stem cell-based processes, including our previous data in the carotid body. Unless it is considered absolutely necessary we would like not to remove “fast neurogenesis” from the title of the paper. This concept describes a major fact reported in the manuscript and it may also help to capture the attention of potential readers.

2, the authors did not show that their experimental manipulation (hypoxia) exerts the desired effect. There is no staining for HIF stabilization, or pimidizole to show that the lowered oxygen was sufficient to stabilize the oxygen sensing pathway. Are the authors suggesting that the CB is differentially sensitive?

Authors: The fact that experimental hypoxia is being used correctly in our experiments is routinely corroborated in our laboratory by different methods. Hematocrit is routinely measured in hypoxic animals, although it is true that short exposures to hypoxia do not affect hematocrit. Expression of TH, a hypoxia-sensitive gene, is also checked in neuronal cells exposed to hypoxia (see the clear increase of TH expression under hypoxia shown in the cytometry plots of Fig. 3A-C). To directly address the point raised by the reviewer and further confirm that hypoxia exerts the desire effect, we have tested for HIF stabilization in CB neuroblasts and have observed the presence of HIF2alpha in hypoxic cells (see new Suppl. Fig. EV6). A comment on this intrinsic sensitivity to hypoxia of CB neuroblasts has been added to the manuscript (page 10).

3, the claims of independent cell types would be strongly bolstered by purification of both populations and transcriptional profiling versus the NSCs as they have described previously. Is there a reason this cannot be done?

Authors: Unfortunately, we do not have specific markers for the isolation of purified NSCs from the CB. Hence, we were not able to perform the comparisons the reviewer suggests. However, a comparative transcriptional profiling between the two classes of neuronal cells is definitely in the pipeline of future studies in our laboratory, and will be attended in future publications. Nevertheless, following the suggestions of the reviewer we have performed quantitative PCR analyses to demonstrate that the two neuronal cell classes are also different at the level of gene expression (see text on page 8 and new Suppl. Fig. EV5).

4, roughly half of the quantification/histograms presented do not have error bars, how reproducible are the results?

Authors: The reason why all those graphs do not have error bars is because they refer to absolute numbers, and not to averages. The percentage of hypoxia-responsive cells is the number of hypoxia sensitive cells versus total number of cells recorded. There is no place for an error bar in this type of graph.

2nd Editorial Decision 21 November 2017

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see both referees are now positive about the study and request mostly minor changes to clarify text and figures. In addition, referee 1 suggests to re-analyze existing flow cytometry and cell size data and referee 2 points out that the experiment in Fig. EV7 is missing a normoxic control that should be provided.

REFEREE REPORTS

Referee #1:

Original concern: The most obvious missing bit of evidence are lineage-tracing analyses of the origins and fate of this population of cells. While it is perhaps beyond the scope of this study, employing a genetic murine model to lineage-trace HNK+ cells will eventually be necessary.

Authors: We agree with the reviewer that a precise cell fate mapping analysis would be necessary to corroborate lineage relationship between carotid body stem cells, neuroblasts and mature glomus cells. Unfortunately, mouse neuroblasts are HNK-1 negative. Hence, we cannot identify them as easily as in the rat, making really difficult the cell fate mapping studies. To address the question raised by the reviewer, we have performed neurosphere assays with multipotent stem cells in the organ (non mTH/HNK cells, to remove neuroblasts; see figure below). As shown in the figure below, these clonal neurospheres have a core of HNK-1 negative progenitors (the ones originating the colony) and some HNK-1 positive cells (green), which appeared later in the periphery. The blebs of differentiated TH+ cells (red) contain some cells with both TH (red) and HNK-1 (green) stainings (resulting in a yellowish color, arrowheads) which are most likely neuroblasts. These data obtained from clonal neurospheres suggest that all the cells (progenitors, HNK-1+ cells, HNK-1+/TH+ cells and TH+ cells) derive from a common stem cell. However, since this is not a regular cell fate mapping approach, we have decided not to include this figure in the manuscript.

Referee: I feel that the data provided in Fig. EV3 and the above neurosphere-based data are sufficient to support the authors´ suggestions regarding population-level lineage relationships, despite the limitations acknowledged by the authors.

Origional concern 1) The in vivo cycling dynamics of TH+ cells are difficult to fully understand. Figs.1E-G show results from a series of experiments (Fig.1D) that suggest that at all time points most BrdU+ cells are already postmitotic at the moment of analysis; only approx 10% of cells labeled with BrdU over the previous 48hrs are Ki67+ at d2 and d4, while only 1% at d6. Conversely, the bulk of the sizeable Ki67+ populations at d4 and d6 are not BrdU+, i.e. haven't yet completed G1. Cumulative BrdU labeling during 2,4 and 6 days may help get an idea of the overall size of the proliferating population, assuming generation of TH+ cells from previously TH- cells doesn't play a major role within this time interval. In general, I think the presented data suggest the intriguing possibility that activation of several cohorts of previously quiescent TH+ cells may take place, each of which would mostly become postmitotic right away.

We believe the referee is right in this appreciation. We do not know whether it corresponds to different cohorts or just a stepwise activation of all neuroblasts present, but truth is that the data is compatible with a quite longer G1 phase, and extremely short S, G2 and M phases. It looks like once the neuroblast is activated and enters S phase (is labeled with BrdU), it goes quickly through the rest of cell cycle and becomes postmitotic. We have tried to rephrase this part in the manuscript (see page 5) to achieve more clarity in the interpretation. In any case, it is obviously beyond the scope of this study to enter in too much detail in the proliferation dynamics of carotid body neuroblasts.

Referee: It seems to me that the amended text clarifies enough the point to allow readers to at least appreciate the potential complexity of the system. I´m thus fine with the proposed changes. I just note that the proposed phrasing "exit of the cell cycle" may be best rendered as "exit from the cell cycle".

Original concern 2) Claims by the authors that proliferation is a prerogative of the mTH/HNK population seem unwarranted since, as clear from Fig.2D, TH+ (HNK-) cells proliferate basally to a similar (and low) extent, and increase their proliferation in response to hypoxia in a similar way (5-6x vs 7-8x for mTH/HNK cells).

Authors: The flow cytometry analysis presented in Fig. 2C was performed with very general gates (represented as different colors in the dot plot), so an important contamination within the two TH+ populations might be expected. Moreover, preparation of cells for flow cytometry using intracellular immunocytochemistry, induces damage to the cells that could diminish the amount of HNK-1 epitope in their membranes, making contamination of the HNK-1 negative population with HNK-1+ cells a more frequent issue. That is why we decided to perform the time-lapse recording experiment (Fig. 2E and F) with much tighter sorting gates and live cells (see Fig. EV2B). In this case, contamination is much lower, and the difference in proliferation capacities between both populations is clearer.

Referee: It is not clear to me why the authors would perform the initial characterization with admittedly unreliable gates and then proceed to refine these only for the subsequent analyses. Data shown in Fig.2D should be based on gate settings as close to those used in later experiments as possible. I believe reanalysis of the already existing cytofluorimetric data (Fig.2C) should be straightforward, and hopefully supportive of the current claims.

Original concern 3) A population identified by cytofluorimetric means deserves further characterization: the small-sized, TH+ (but HNK-) population clearly visible in Suppl. Fig.2B. These cells look like good candidates for an intermediate stage generated by HNK+ cells, on the way to a more fully mature condition. Data from the sorted HNK- cells used for the in vitro timelapse experiments should be assessed to establish whether a correlation exists between e.g. cell size at the beginning of the experiment, and eventual proliferative ability. Conversely, cells from the HNK+ population should be analyzed at the end of the timelapse for HNK expression. Along the same lines, data presented in Fig. 5/6 should, whenever possible, be reanalyzed to assess the degree of functional maturation of the above small-bodied HNK- population. As above, I suggest cell size is evaluated as a possible indicator of heterogeneity within the sorted HNK- population and, if the somatic size info can be retrieved for the cells assayed in the various single-cell analyses (e.g. Calcium and NAD(P)H dynamics), that it be used to stratify the analysis of TH+ cells.

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Nevertheless, this point raised by the reviewer made us wonder about whether we could see a clear increase in neuroblast cell size and a clear decrease in HNK-1 epitope expression upon maturation in our experiments in vitro. We have performed the measurements and have included a new figure with the resulting data (see new Suppl. Fig. EV3A-B, and page 7 first paragraph).

Referee: While the authors provided some analysis of cell size changes (see further comment below) it may be still be worth clarifying my original comment. In supplementary figure 2B, the FSC-H/TH-FITC scatter plots to the right of the main FL-2/HNK-PE scatter plot quite clearly show the

following: the "green" mTH/HNK population shows a FSC-H (hence my original mention of cell size) with a FSC value (among TH+ cells) centered somewhere around 75 (y-axis value); the "red" TH-only population seems to be distributed in two subclusters, one similar in FSC and TH-FTC values to the "green" one, and one with higher FSC values (close to 100), characterized by a broader TH-FITC distribution, evidenced as a spindle-like horizontal cloud of cells. My suggestion was that the smaller and less TH-intense "red" population may be a precursor stage to the latter one (bigger and more brightly TH+), given the overall similarity to the "green" population. Given the separation, in the main FL-2/HNK-1-PE plot, of the TH-only population from the TH/HNK one, it is unlikely that the above observation could be due to contamination by a poorly HNK-stained population. As an aside, it seems to me no mention is made anywhere in the manuscript of what the two values (78.6{plus minus}12 and 90.6{plus minus}0.7) refer to, even if it seems to me they may be the mean FSC values.
In relation to the newly added measurements of cell size changes (Fig. EV3), I assume the values plotted in EV3B refer to all cells grown out of the sorted mTH/HNK population and not only some. Since HNK-1 staining was performed and size measured, it would be feasible to re-plot size values based on whether the cells are positive or negative for HNK-1. The prediction from my comments above on cell size would be that HNK-1-negative/dim cells would be divided in two size-based populations. If anything EV2C, upper panel, already shows small and big TH-only cells, the former seemingly less intensely TH+ than the latter (only 4 cells are visible, though), and similar in size to the TH/HNK cells (middle panel, EV2C).

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Authors: We have added these new data as a new figure (Suppl. Fig. EV3C), and have introduced comments regarding these data in page 7 of the manuscript (first paragraph).

Referee: The performed experiments may have profited from a time-course analysis, but the reported data are already supportive of the authors´ claims and will suffice.

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Authors: Although we have tried several potential markers, we have not been able to set up a clear and nice immunological staining to distinguish mature neurons and neuroblasts (neither in sections nor in single cell suspensions) other than the HNK-1 staining. However, as suggested by the reviewer, we have performed real time PCRs to distinguish both types of cells by showing differential expression of significant markers associated to their maturation state. We have included the data in new Suppl. Fig. EV5 and have commented the results on page 8 (second paragraph).

Referee: The provided data are surely enough.

Original concern 6) Discussion of EM data potentially conflates coverage of the different classes of HNK+ cells; the authors show in Fig.2 a clearly HNK+/TH- population, which cannot be distinguished from the mTH/HNK+ in EM pics, since immunogold staining is done only for HNK. The authors should assess variability in cytological features observed in HNK+ cells in EM pics and comment on possible subgroups that may correspond to the two HNK+ populations. Co-staining for TH would of course be of help. Authors should not refer to HNK+ cells as mTH/HNK unless some positive identification is possible, directly or by some verified proxy.

Authors: We thank the reviewer for this observation. It is true that we see different HNK+ cells in our EM preparations, although we did not mention them before. We see cells like the one shown in Suppl. Fig. EV4A. As indicated in page 7 (second paragraph) of the manuscript, we believe these cells correspond to the uncharacterized TH-/HNK low cells observed in cytometry dot plots (blue dots in Fig. 2C). We have not added these cells in our EM analysis. We have only used cells highly positive for HNK (with abundant gold particles all around the surface), which we believe mostly

correspond to CB neuroblasts. Therefore, we feel pretty confident when referring to these cells as mTH/HNK cells, since they are not only HNK high but they also contain dense-core synaptic vesicles.

Referee: The latter claim of dense-core synaptic vesicles being visible in HNK-high cells should be substantiated by at least pointing to examples in the EM micrographs.

Original concern 7) With regard to EM-based claims about the relative positioning of the HNK+ subpopulation(s) relative to other landmarks (e.g. vasculature), no quantifications are shown and should be either provided (e.g. shortest distance to a vessel) or the claim otherwise substantiated (or eliminated).

Authors: New quantitative data regarding the positioning of both mature and immature glomus cells have been added (see new Suppl. Fig. EV4 and comment in page 8, first paragraph).

Referee: The authors satisfactorily addressed my concern.

Original concern 8) On page 9, a claim is made that "mature (TH+) glomus cells can form synaptic contacts with mTH+/HNK+ neuroblasts (see fig4)"; it's not at all clear what this is based on, as the only identifiable cell-cell contact is the highlighted electron-dense patch in Fig.4C-1. Legend for Fig.4 lacks mention of the scale bar size.

Authors: We fully agree with the reviewer and have softened the sentence. Scale bar sizes have been added to Figure 4 legend.

Referee: I think the current version is fair enough.

Original concern 9) All molecular mechanisms suggested to play a role in HNK+ cells maturation of proper responsiveness to hypoxia would be strengthened if it could be shown that Hx-exposed mature (i.e. TH-only) cells' conditioned medium can trigger this maturation in purified HNK+ cells.

Authors: We have tried hard to perform this experiment, without success. We believe that the molecules released from type I cells in the vicinity of neuroblasts as a spill over, become too diluted when trying to perform the experiment in vitro with isolated and purified populations. Hence, it might be that the 'conditioned medium' does not contain the necessary concentration of 'maturation agents' when testing this paracrine communication in vitro.

Referee: I think the lack of these data is not critical and feel the authors´ explanation for the failure to observe any conditioned medium-induced effects is likely correct. I think a brief comment along these lines in the text may help navigate readers through data discussion.

Original concern: "40K" should be replaced in figures by an appropriate reference to [K+].

Authors: A clarifying note has been added to the corresponding legends (Figs. 6 and 7).
Referee: Thanks

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The authors have addressed most of the suggestions adequately except for these two points below:

"To directly address the point raised by the reviewer and further confirm that hypoxia exerts the desire effect, we have tested for HIF stabilization in CB neuroblasts and have observed the presence of HIF2alpha in hypoxic cells (see new Suppl. Fig. EV6). A comment on this intrinsic sensitivity to hypoxia of CB neuroblasts has been added to the manuscript (page 10)."

The authors tried to address this point, but only showed Epas1 staining under hypoxia condition, without a control, so the effect of hypoxia on tis HIF factor is unclear.

"Authors: The reason why all those graphs do not have error bars is because they refer to absolute

numbers, and not to averages. The percentage of hypoxia-responsive cells is the number of hypoxia sensitive cells versus total number of cells recorded. There is no place for an error bar in this type of graph."

I do not understand this argument. if the experiment was performed multiple independent times, they should have %s for each experiment that can be used to show the standard error of the experiment versus their results. Do all the quantifications performed in the manuscript representative of just one independent experiment? If that is the case, there should at least be replicates (replicate wells?) within the experiment, and therefore separate %s for each replicate, right?

2nd Revision - authors' response 30 November 2017

Referee #1:

Original concern: The most obvious missing bit of evidence are lineage-tracing analyses of the origins and fate of this population of cells. While it is perhaps beyond the scope of this study, employing a genetic murine model to lineage-trace HNK+ cells will eventually be necessary.

Authors: We agree with the reviewer that a precise cell fate mapping analysis would be necessary to corroborate lineage relationship between carotid body stem cells, neuroblasts and mature glomus cells. Unfortunately, mouse neuroblasts are HNK-1 negative. Hence, we cannot identify them as easily as in the rat, making really difficult the cell fate mapping studies. To address the question raised by the reviewer, we have performed neurosphere assays with multipotent stem cells in the organ (non mTH/HNK cells, to remove neuroblasts; see figure below). As shown in the figure below, these clonal neurospheres have a core of HNK-1 negative progenitors (the ones originating the colony) and some HNK-1 positive cells (green), which appeared later in the periphery. The blebs of differentiated TH+ cells (red) contain some cells with both TH (red) and HNK-1 (green) stainings (resulting in a yellowish color, arrowheads) which are most likely neuroblasts. These data obtained from clonal neurospheres suggest that all the cells (progenitors, HNK-1+ cells, HNK-1+/TH+ cells and TH+ cells) derive from a common stem cell. However, since this is not a regular cell fate mapping approach, we have decided not to include this figure in the manuscript.

Referee: I feel that the data provided in Fig. EV3 and the above neurosphere-based data are sufficient to support the authors´ suggestions regarding population-level lineage relationships, despite the limitations acknowledged by the authors.

Origional concern 1) The in vivo cycling dynamics of TH+ cells are difficult to fully understand. Figs.1E-G show results from a series of experiments (Fig.1D) that suggest that at all time points most BrdU+ cells are already postmitotic at the moment of analysis; only approx 10% of cells labeled with BrdU over the previous 48hrs are Ki67+ at d2 and d4, while only 1% at d6. Conversely, the bulk of the sizeable Ki67+ populations at d4 and d6 are not BrdU+, i.e. haven't yet completed G1. Cumulative BrdU labeling during 2,4 and 6 days may help get an idea of the overall size of the proliferating population, assuming generation of TH+ cells from previously TH- cells doesn't play a major role within this time interval. In general, I think the presented data suggest the intriguing possibility that activation of several cohorts of previously quiescent TH+ cells may take place, each of which would mostly become postmitotic right away.

We believe the referee is right in this appreciation. We do not know whether it corresponds to different cohorts or just a stepwise activation of all neuroblasts present, but truth is that the data is compatible with a quite longer G1 phase, and extremely short S, G2 and M phases. It looks like once the neuroblast is activated and enters S phase (is labeled with BrdU), it goes quickly through the rest of cell cycle and becomes postmitotic. We have tried to rephrase this part in the manuscript (see page 5) to achieve more clarity in the interpretation. In any case, it is obviously beyond the scope of this study to enter in too much detail in the proliferation dynamics of carotid body neuroblasts.

Referee: It seems to me that the amended text clarifies enough the point to allow readers to at least appreciate the potential complexity of the system. I´m thus fine with the proposed changes. I just note that the proposed phrasing "exit of the cell cycle" may be best rendered as "exit from the cell cycle".

Authors: The recommended change has been performed (see page 5, first paragraph).

Original concern 2) Claims by the authors that proliferation is a prerogative of the mTH/HNK population seem unwarranted since, as clear from Fig.2D, TH+ (HNK-) cells proliferate basally to a similar (and low) extent, and increase their proliferation in response to hypoxia in a similar way (5-6x vs 7-8x for mTH/HNK cells).

Authors: The flow cytometry analysis presented in Fig. 2C was performed with very general gates (represented as different colors in the dot plot), so an important contamination within the two TH+ populations might be expected. Moreover, preparation of cells for flow cytometry using intracellular immunocytochemistry, induces damage to the cells that could diminish the amount of HNK-1 epitope in their membranes, making contamination of the HNK-1 negative population with HNK-1+ cells a more frequent issue. That is why we decided to perform the time-lapse recording experiment (Fig. 2E and F) with much tighter sorting gates and live cells (see Fig. EV2B). In this case, contamination is much lower, and the difference in proliferation capacities between both populations is clearer.

Referee: It is not clear to me why the authors would perform the initial characterization with admittedly unreliable gates and then proceed to refine these only for the subsequent analyses. Data shown in Fig.2D should be based on gate settings as close to those used in later experiments as possible. I believe reanalysis of the already existing cytofluorimetric data (Fig.2C) should be straightforward, and hopefully supportive of the current claims.

Authors: We agree again with the reviewer in this observation, and there is always the possibility to completely remove this first analysis. However, we added these data to the paper as a first indication of the proliferation capacity of TH+/HNK-1+ cells, not really as the definitive prove, which is achieved later in the work. We have tried to use similar gates to the ones used later to re-analyze the data, but this is not possible because the cytometry plot in Fig. 2C represents a TH staining with fixed cells, while later we worked with live cells with no staining for TH. Moreover, we still think we have neuroblasts that have lost HNK-1 expression during tissue dispersion, contaminating the mature cell population. This contamination might be more obvious in hypoxia, as neuroblasts down regulate HNK-1 expression as they start the process of maturation. As plots in Fig. 2C,D are the preliminary data that initiated the "story" we would like to leave them in the manuscript.

Original concern 3) A population identified by cytofluorimetric means deserves further characterization: the small-sized, TH+ (but HNK-) population clearly visible in Suppl. Fig.2B. These cells look like good candidates for an intermediate stage generated by HNK+ cells, on the way to a more fully mature condition. Data from the sorted HNK- cells used for the in vitro timelapse experiments should be assessed to establish whether a correlation exists between e.g. cell size at the beginning of the experiment, and eventual proliferative ability. Conversely, cells from the HNK+ population should be analyzed at the end of the timelapse for HNK expression. Along the same lines, data presented in Fig. 5/6 should, whenever possible, be reanalyzed to assess the degree of functional maturation of the above small-bodied HNK- population. As above, I suggest cell size is evaluated as a possible indicator of heterogeneity within the sorted HNK- population and, if the somatic size info can be retrieved for the cells assayed in the various single-cell analyses (e.g. Calcium and NAD(P)H dynamics), that it be used to stratify the analysis of TH+ cells.

Authors: We are afraid we do not understand exactly which cell population the reviewer is referring to. The plot in Suppl. Fig. 2B does not really look at cell size, as Suppl. Fig. 2A does, in order to distinguish between cell populations. We actually do not see the existence of any small-sized TH+/HNK- cells. The small-sized HNK- cells ('Neg' in Suppl. Fig. 2B) are also TH-, and the small-sized TH+ cells are also HNK+ ('mTH/HNK' in Suppl. Fig. 2B).
Nevertheless, this point raised by the reviewer made us wonder about whether we could see a clear increase in neuroblast cell size and a clear decrease in HNK-1 epitope expression upon maturation in our experiments in vitro. We have performed the measurements and have included a new figure with the resulting data (see new Suppl. Fig. EV3A-B, and page 7 first paragraph).

Referee: While the authors provided some analysis of cell size changes (see further comment below)

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Authors: We now fully understand the reviewer's observation and we thank him/her for highlighting this interesting point. It is true that the referred population in Fig. EV2B (upper right panel) is very clear and suggestive. This small population could be explained in two different ways: 1) they are neuroblasts that have lost HNK-1 expression during tissue dispersion, which is a general concern (losing surface epitopes) in enzymatically dispersed cells; 2) Consistent with the reviewer's comment, these are intermediate cells between HNK-1+ neuroblasts and mature larger TH+ cells. In both cases this population could actually account for the basal proliferation seen in the mature cell compartment (discussed in the previous point). In any case, the existence of this population does not interfere with our main observation, which is the presence of immature HNK-1+ neuroblasts with the capacity to give rise to fast neurogenesis in response to hypoxia. Nevertheless, we recognize the potential significance of this population and have introduced a gate in Fig. EV2 and a comment in its legend to highlight it.

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Authors: We thank the reviewer for this observation. It is true that we see different HNK+ cells in our EM preparations, although we did not mention them before. We see cells like the one shown in Suppl. Fig. EV4A. As indicated in page 7 (second paragraph) of the manuscript, we believe these cells correspond to the uncharacterized TH-/HNK low cells observed in cytometry dot plots (blue dots in Fig. 2C). We have not added these cells in our EM analysis. We have only used cells highly positive for HNK (with abundant gold particles all around the surface), which we believe mostly correspond to CB neuroblasts. Therefore, we feel pretty confident when referring to these cells as mTH/HNK cells, since they are not only HNK high but they also contain dense-core synaptic vesicles.

Referee: The latter claim of dense-core synaptic vesicles being visible in HNK-high cells should be substantiated by at least pointing to examples in the EM micrographs.

Authors: Appropriate arrows have been added to Fig. 4C1. The meaning of these arrows is indicated in the text (page 8, first paragraph).

Original concern 7) With regard to EM-based claims about the relative positioning of the HNK+ subpopulation(s) relative to other landmarks (e.g. vasculature), no quantifications are shown and should be either provided (e.g. shortest distance to a vessel) or the claim otherwise substantiated (or eliminated).

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Authors: A brief comment has been added at the end of Results section.

Original concern: "40K" should be replaced in figures by an appropriate reference to [K+].

Authors: A clarifying note has been added to the corresponding legends (Figs. 6 and 7).
Referee: Thanks

Referee #2:

The authors have addressed most of the suggestions adequately except for these two points below:

"To directly address the point raised by the reviewer and further confirm that hypoxia exerts the desire effect, we have tested for HIF stabilization in CB neuroblasts and have observed the presence of HIF2alpha in hypoxic cells (see new Suppl. Fig. EV6). A comment on this intrinsic sensitivity to hypoxia of CB neuroblasts has been added to the manuscript (page 10)."

The authors tried to address this point, but only showed Epas1 staining under hypoxia condition, without a control, so the effect of hypoxia on tis HIF factor is unclear.

We thank the reviewer for the observation. We have added new pictures to Fig. EV3 (originally EV7) in normoxic conditions, to highlight the clear stabilization of Epas1 in hypoxia.

"Authors: The reason why all those graphs do not have error bars is because they refer to absolute numbers, and not to averages. The percentage of hypoxia-responsive cells is the number of hypoxia sensitive cells versus total number of cells recorded. There is no place for an error bar in this type of graph."

I do not understand this argument. if the experiment was performed multiple independent times, they should have %s for each experiment that can be used to show the standard error of the experiment versus their results. Do all the quantifications performed in the manuscript representative of just one independent experiment? If that is the case, there should at least be replicates (replicate wells?) within the experiment, and therefore separate %s for each replicate, right?

Time-lapse recordings and electrophysiological measurements are technically very challenging, so they have been performed once or twice, but starting from multiple animals and using hundreds of cells for the measurements, to make sure our results were robust and significant. That is why these results are represented as absolute numbers in graphs. We have now provided number of cells and animals in all figure legends, and have described the type of graph for each case, indicating whether it is a graph representing the sum of cells studied or the average with SEM.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Ricardo Pardal
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-44598V2

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Given the nature of this research, the effect size could be hardly pre-specified. Hence, once evidence on the differences between cell types was obtained, the sample size was increased until such effect reached statistical significance.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	In an atempt to fullfil the 3R (reduce, replace, refine) rule for animal research, the number of animals used was kept at the minimun as far as the results reached statistical significance or were absolutely convincing.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animals were excluded for analysis as far as their aspect was healthy and normal. In this type of research, no pre-establishment of inclusion/exclusion criteria is needed.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Allocation of animals in groups requested no randomization procedure, since all animals were singenic wild-type animals.
For animal studies, include a statement about randomization even if no randomization was used.	No randomization applies. The only pseudo-randomization was considered for allocation of similar numer of males and females in each group.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	When possible, most studies were undertaken with blinding of the investigator. For example, for electrophysiological recordings the investigator performing the recordings was blind for the cell type under recording.
4.b. For animal studies, include a statement about blinding even if no blinding was done	For animal studies no blinding was necessary since all animals were singenic wild type animals.
5. For every figure, are statistical tests justified as appropriate?	Yes. This issue is described in material and methods section.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	In order to apply the apropiate statistical test, normal distribution was assessed by Shapiro-wilk test.
Is there an estimate of variation within each group of data?	Yes, Standard error of the mean is reported.
Is the variance similar between the groups that are being statistically compared?	Yes, a Levene test for homogeneity of variance was applied for all the data with normal distribution.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
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http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

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http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

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http://figshare.com

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http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Primary antibodies and the dilution factors applied are indicated in Materials and Methods. Validation of these antibodies are reported in the respective technical data sheets.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N/A

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Experiments were performed using 7-11 week-old Wistar rats (Harlan). Male and female animals were used in every experiment and no differences were found between them. Rats were housed and treated according to the animal care guidelines of the European Community Council (2010/63/EU). The Animal Research Committee at the University of Seville approved all procedures.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Rats were housed and treated according to the animal care guidelines of the European Community Council (2010/63/EU). The Animal Research Committee at the University of Seville approved all procedures.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Confirmed

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No