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Resistance of glia-like central and peripheral neural stem cells to genetically-induced mitochondrial dysfunction - differential effects on neurogenesis

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Corresponding authors: Jose Lopez-Barneo and Jose I. Piruat , University of Seville

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(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.)

Editor: Barbara Pauly

1st Editorial Decision 17 July 2014

Thank you for the submission of your research manuscript to our editorial office. I have now had the opportunity to carefully read it and I have also discussed it with the other members of our editorial team and with an additional, external advisor. I am afraid that the outcome of these discussions is not a positive one, as we all agree that the manuscript is not well suited for publication in EMBO reports.

We acknowledge that by analyzing mice with dysfunctional mitochondria specifically in GFAP-positive cells you find that the generation, maintenance, and multipotency of brain neural stem cells is unaffected by mitochondrial dysfunction, but that neuronal maturation is impaired. Interestingly, astrocytes that are derived from the same cells are not affected. You then show that neuronal populations in the peripheral nervous system are unaffected by disruption of mitochondrial function, indicating that peripheral NSCs do not derive from the same GFAP+ glia lineage. We appreciate the potential interest of these findings. However, the advisor who we consulted in this case and who is an expert in neuronal development and metabolism felt that the experimental setup used in the study

does not provide conclusive and convincing evidence to support the claims put forward. For example, s/he pointed out that preexisting mitochondria would still exist and function for several weeks, which would make the analysis of early effects difficult. S/he also raised the point that the loss of SDH would cause a variety of respiration defects, including loss of ATP and redox damage, so that it is difficult to interpret the exact cellular effects of this kind of manipulation.

Based on these and several other concerns our advisor did not recommend in-depth review of the manuscript and we have therefore decided to return it to you at this point so that you can submit it elsewhere without further delay. I would like to thank you again for considering EMBO reports for publication of your work. I am sorry to have to disappoint you on this occasion, and hope that this will not prevent you from considering EMBO reports for publication of your work in the future.

Correspondence - authors 18 July 2014

This is a letter of rebuttal to your message (see below) regarding our research manuscript "Resistance of glia-like central and peripheral neural stem cells to genetically-induced mitochondrial dysfunction. Differential effects on neurogenesis", submitted to EMBO Reports (EMBOR-2014-39315V1). We have been much disappointed by your decision to reject the paper based on the opinion of an external advisor to whom you have consulted. We consider that the comments of the advisor are incorrect for the following reasons:

He/she stated that "...preexisting mitochondria would still exist and function for several weeks, which would make the analysis of early effects difficult...".

Response: We are aware that after activation of Cre recombinase complete wash-out (disappearance) of preexisting mitochondrial proteins probably takes several days. This phenomenon could have imposed a serious limitation to the interpretation of our data in the case that the GFAP-SDHD mice had not exhibited a clear phenotype. We have no direct information on the turnover of the SDHD protein during fetal life (probably much faster than in the adult), however it is obvious that mitochondrial dysfunction in GFAP+ neural progenitors was manifested soon alter the allele deletion, since after birth animals were unable to develop a normal brain, meaning that the progenitors were already affected by mitochondrial dysfunction. Affected areas in the mutated brain (dorsal cortex, hippocampus and cerebellum) did not show any obvious changes between P1 and P15, thus indicating that the damage was already fully present at birth. Moreover, at P15 (once the brains showed extreme atrophy) the peripheral nervous system (with the exception of the carotid body) was intact and in vitro experiments performed with SVZ neural stem cells demonstrated that although the progenitors were capable of forming colonies (neurospheres), these were smaller in the mutant, indicating lack of mitochondria function. In addition, when differentiated to astrocytes and neurons, these last cells died a few days later in the mutant. The inescapable conclusion of these experiments is that neural stem cells are resistant to genetic mitochondrial damage and that their neuronal progeny cannot undergo maturation. They also suggest that peripheral progenitors (with the exception of the CB) do not pass through a GFAP+ phenotype. Finally, the CB atrophy phenotype displayed by the mutant, confirms that mitochondrial dysfunction has taken place. In all these contexts, knowledge of the exact timing of mitochondria protein (SDHD) wash-out has little relevance.

"He/she also raised the point that the loss of SDHD would cause a variety of respiration defects, including loss of ATP and redox damage,

so that it is difficult to interpret the exact cellular effects of this kind of manipulation"

Response: This comment of your advisor has no sense, as the mechanism of neuronal death is not addressed in the paper (because it has no relevance for the conclusions). Nonetheless, as it is indicated in the manuscript, we have already reported which are the effects of this kind of manipulation on central and peripheral neurons (see Diaz-Castro et al., 2012). Neurons seem to die from lack of ATP production and oxidative stress.

We believe that our paper is technically sound and, on our view, well suited for EMBO Reports, as it provides novel information on an emergent topic of obvious biomedical interest. Naturally, if you do not reconsider your decision we will seek publication elsewhere. Nonetheless, we think it is our duty to respectfully manifest that your decision is based on comments that lack solid scientific justification and therefore it is precipitated.

Appreciating your kind attention to this letter.

Correspondence - editor 06 August 2014

This is just a quick note to let you know that I have discussed your rebuttal with our external advisor again and even though this expert still stands behind his/her initial concerns, I have decided that, on balance, I would indeed send the study to some additional referees to hear their opinion on it.

I will get back to you as soon as I have received their feedback, but I wanted to let you know at this point, that your study is being peer-reviewed now, but also that it might take a little longer than normally, given the summer holiday season.

2nd Editorial Decision 27 August 2014

Thank you for the submission of your research manuscript to our editorial offices and your patience while we were waiting to hear back from the referees. We have now received the three enclosed reports on it. As you will see, the reviewers all agree on the potential interest of the manuscript, but they have also all raised a number of concerns about the study that would need to be addressed prior to publication.

Most importantly, both referees 1 and 2 feel that stronger roof is needed that SDHD is indeed lost in the proposed cells and at the proposed times. Reviewers 1 and 3 also state that the phenotypical changes and mitochondrial defects need to be analyzed in more details. Referee 2 comments on the neurosphere assays and reviewer 3 also feels that this aspect of the study needs to be strengthened by further experiments. All reviewers also point out instances in which further controls are needed to back up the results and conclusions.

From the analysis of these comments it becomes clear that publication of your manuscript in our journal can only be considered after significant revision. But given the potential interest of your study and the reviewers' constructive suggestions on how to improve it, we would like to give you the opportunity to address the referee concerns and would be willing to consider a revised manuscript with the understanding that all main issues raised by our reviewers must be fully addressed.

I should also remind you that it is EMBO reports policy to allow a single round of major revision

only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next version of the manuscript.

Revised manuscripts should be submitted within about three months, even though I can potentially extend this period if you feel that this time is insufficient for a successful revision. However, manuscripts have to be accepted six months after the invitation to revise them; they will otherwise be treated as new submissions and their novelty will be assessed again at the time of their submission.

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:

Referee #1:

The manuscript describes the interesting results observed when SdhD is conditionally deleted in brain stem cells using hGFAP-cre. This deletion produces a marked phenotype, with abnormally small brain size, relative preservation of the peripheral nervous system and hypoplastic carotid bodies. From their observations in these mice, the authors conclude that "neuronal differentiation" is impaired by mitochondrial injury, while the survival of stem cells and glia is not compromised. Unfortunately, the conclusions are not justified by the data as currently presented, because the phenotype is not presented in adequate detail, and the cellular processes that drive the phenotype are not examined. The manuscript requires additional work that may be beyond the scope of a revision. With the addition of more detail, however, it would be likely to be of great interest and suitable for publication in Embo Journal.

While the presentation of the phenotype is of interest, the manuscript does not analyze the phenotype in adequate detail. The authors should have presented more images of the brain, including sagittal images of the rostral migratory stream and cerebellum. These images should include a series of time points, perhaps P3 and P7, in addition to P15. The additional images would make it possible to examine the processes of postnatal neurogenesis and reveal the temporal pattern in which the phenotype becomes manifest. Additionally, while the authors look superficially into neuronal population changes using NeuN, they should devote some images to showing the glial population. The GFAP-GFP reported mouse that they made would be a useful tool for this purpose. It is important to look at glia since one of the conclusions is that glia are tolerant to mitochondrial injury.

The nature of the mitochondrial injury should be examined in some detail. Is there increased brain lactate? Are mitochondria present or lost? Are mitochondria depolarized? Is there premature release of cytochrome C into the cytoplasm? Is there activation of the internal apoptotic pathway? Mitochondrial stains and staining for cleaved caspase 3 could answer most of these questions and would contribute greatly to the work.

Lastly, the authors confuse GFAP expression with GFAP-lineage. The hGFAP-cre line drives recombination in a large population of brain cells. The GFAP-GFP reporter identifies only the portion of the GFAP lineage that continues to express GFAP. Thus neurons and oligodendrocytes will derive from cells that have undergone cre mediated recombination, but will be excluded from analysis using the GFAP-GFP reporter. This nuance needs to be made clear in the text and considered in the interpretation. It is understandable that SdhD antibodies may not be available that can demonstrate loss of the protein. If SdhD protein loss cannot be mapped by IHC, the authors should at least use a cre-activated lineage tracer, such as lox-STOP-lox tdTomato to show that cre-mediated recombination has occurred. For example, it is not clear if peripheral neurons are tolerant to SdhD deletion, of if this population was not targeted by the hGFAP-cre transgene. Prior reports of other investigators using the hGFAP-cre line are not optimal to answer this question, as there may be variation in the function of this transgene. Rather, the authors should include contemporaneous lineage tracing.

Some additional textual concerns:
Abstract:
"homozygous deletion of the succinate dehydrogenase subunit D gene restricted to glial fibrillary acidic protein-expressing cells"- is it GFAP expressing or GFAP lineage?
"thus highlighting their non-glial origin"- needs to be rephrased- both Schwann cells and peripheral neurons are NC origin, what data shows is that peripheral neurons either do not depend on SDHd or that they are not targeted by the cre driver.

Intro:
"Intermediate metabolism"- what does that mean? Intermediary metabolism? Energy metabolism?

Results
On page 5 "dilatation" should be changed to "dilation"
GFAP-cre line should be referred to as hGFAP-cre, to make clear that the promoter is a construct based on the human GFAP promoter sequence.
Fig 1: sagittal sections should show whole brain, or images should show the RMS and cerebellum in sagittal sections should be included.
hGFAP-cre and GFAP-GFP are not the same and this distinction is lost in the text. GFAP-GFP+ cells are only a portion of the entire population targeted by GFAP-cre. A more interesting use of GFAP-GFP would be to breed hGFAP-cre;Sdh fl/fl;GFAP-cre;Lox-STOP-Lox tdTomato mice, to look at changes in the GFAP+ (glial) and GFAP- (neuronal) portions of the hGFAP lineage.
Page 7 experiment with neurospheres does not show that neuronal differentiation is impaired, since neurons do form from SdhD-deleted stem cells. The more clearly suggested interpretation is that mature neurons require mitochondrial function for survival.
Page 9 use of "conform" is not appropriate, not sure what meaning is intended, maybe "comprise"

Referee #2:

This is a very interesting manuscript addressing the in vivo role of succinate dehydrogenase (SDHD) in radial glial cells and their progeny in vivo. This is of key importance of the field as little is known about the mitochondrial function in neural stem cells in vivo. The authors find an initially normal development, followed by neurodegeneration at later postnatal stages with region-specific differences that they allocate to the region-specific differences in Cre recombination. They also report the normal formation of primary neurospheres (no passages - see comment below), while they observe a decrease in the proportion of neurons between 3 and 7 days of differentiation. Thus, the function in adult neural stem cells is not addressed in vivo but only in vitro. Lastly they investigate the peripheral nervous system where neurons apparently survive well, and the authors conclude probably rightly that these are not recombined, but I could not find the experimental evidence for this. Conversely, so do report recombination in the nerves, i.e. Schwann cells. Very interestingly then, they analyse the carotid body, where neurons are again affected and decreased in number, while the stem cells are present in vivo and can form neurospheres in vitro. If the authors would adequately control for when and where SDHD is truly gone, this would be a truly important contribution to the field.
1) The authors present data that SDHD function is reduced in P15 brain (Fig. 1O), but want to conclude that embryonic neural stem cells do not need SDHD. Very obviously this conclusion can only be drawn if SDHD is already successfully deleted and protein levels are reduced at embryonic stages. This must be shown to substantiate the conclusions.
2) Likewise, they need to show that SDHD is not/less reduced in the regions not affected at P15, as reporter activity is no indication for recombination of the floxed SDHD allele which may be better or worse recombined.
3) The SVZ neurosphere need to be passed to assess for self-renewal capacity and differentiation into oligodendrocytes should be assessed to see how they are affected. (Indeed the authorsmake conclusions about oligodendrocytes being susceptible to SDHD deletion but again, it is not addressed whether they are affected directly, or only indirectly as neurons are dying. Or do neurons die only because oligodendrocytes are degenerating?)
4) Decrease in the proportion of neurons is not a measure for neurons dying, as other cells may amplify. The authors must therefore count and present total numbers of bIIItubulin+ cells in the dish.

5) Ideally it would be great to use an inducible deletion of SDHD in adult neural stem cells in vivo to truly assess their role in vivo using one of the many available inducible Cre lines, but this may have to be left to future experiments.
6) Show presence of SDHD in peripheral ganglia to support the conclusion that they are not recombined.

Referee #3:

In their study "Resistance of glia-like central and peripheral neural stem cells to genetically-induced mitochondrial dysfunction. Differential effects on neurogenesis" Diaz-Castro and colleagues use a conditional mutant of the gene encoding for the membrane anchoring subunit D of succinate dehydrogenase in cells expressing Cre under the human GFAP promoter, that targets among other cell populations radial glia cells in the developing brain (GFAP-SDHD mouse). As suggested by previous data deletion of SDHD leads to mitochondrial dysfunction.
The key findings obtained using in vivo and in vitro approaches are that i) mitochondrial dysfunction in radial glia NSCs does not substantially affect the properties of NSCs, ii) differentiation into neurons and neuronal survival is impaired, resulting in extensive brain atrophy of post-natal GFAP-SDHD mice, iii) knocking out SDHD does not affect the PNS, highlighting the non-glial origin of the PNS, and that iv) the carotid body, although a part of the PNS, shows signs of atrophy indicating the glial-like cellular origin of carotid body NSCs.
Characterizing the metabolic demands of somatic stem cells is a novel and exciting topic. The experimental design is straightforward and the authors present convincing data showing in vivo and in vitro phenotypes. However, the interpretation of the results is not complete at this stage and should be improved.

1. The authors should analyze and discuss in more detail the mitochondrial deficits in the SDHD knockout cells using NSCs and their progeny in vitro (e.g., by analyzing SQR activity in these distinct cell populations; compare to Fig. 1O). There have been several studies in the past using SdhD knockout heterozygous mice, but the exact mechanism of mitochondrial dysfunction remains largely uncharacterized (e.g., Bayley 2009, Piruat 2004, Diaz-Castro 2012).

2. In this context it is also important to analyze how long pre-existing mitochondria are present in NSCs after the hGFAP driven Cre recombinase turns on at around E12. At this stage, the exact time where mitochondria dysfunction sets in remains unclear; making it difficult to understand if NSCs are indeed "resistant" to mitochondrial dysfunction, or if it just hasn't set in yet.
3. The authors state that initial proliferation and NSC function is not affected in vivo (note: the authors should show whole brain sizes at PO). In line with this, the authors show that the number of sphere forming cells is not reduced (Fig. 2B). However, they find a dramatic reduction in sphere size (Fig. 2C). This is typically considered to be a function of NSC proliferation. Thus, the authors should analyze this in more detail (using proliferation and cell death assays), as this is apparently very important for the interpretation of the results.

4. The data shown in Fig. 2F suggest that NSC can differentiate into Tuj1+ neurons. However, it is not clear if induced differentiation truly occurs at the time points analyzed (e.g., is there a significant difference between proliferating spheres, 2days, 3days? This needs to be included). When does neuronal cell death occur (the authors should analyze additional time points between 2 and 7 days)? Furthermore, glial differentiation of only 0.2% of cells (Fig. 2G) is extremely low compared to previous studies (and does not reflect the images shown in Fig. 3E where glial differentiation appears much higher; these example images should be improved to represent the quantifications).

5. In the carotid body, the number of neurospheres formed and the size of the neurospheres are similar in control NSCs and cells derived from GFAP-SdhD mice. This is an interesting finding but the authors should discuss the different phenotypes (i.e., sphere size) they found in CNS NSCs and NSCs of the carotid body.

Taken together, we believe that this is an interesting study. However, additional analyses and more careful interpretation of the results seem required to strengthen the manuscript.

Correspondence - authors 19 October 2014

I am writing to you regarding our manuscript submitted to EMBO reports (EMBOR-2014-39315V2). We are having problems with the generation of the genetically modified mouse line (GFAP-SDHD mice) necessary to do the experiments that we need to do to address the questions raised by the referees. Therefore, it will not be possible to submit a complete revised version within the time period (three months) granted. I hope that we will be able to have everything ready before 6 moths. Nonetheless, I will keep you informed of the progress of our revision.

Correspondence - authors 24 January 2015

Thanks you very much for your kind reminder. I was planning to contact EMBO reports editorial office during the next few days, as at the end of February is the deadline for submission of our revised manuscript.

The revision of our paper requires the use of genetically modified animals (GFAP-SDHD mice) and we had problems expanding a parental line (GFAP-Cre) that needed to be crossed with SDHDfloxed to generate GFAP-SDHD. A few months ago I informed EMBO Reports editorial office of these problems and the possibility that we had to postpone the resubmission of our manuscript. Please note that the expansion of a mouse line and the generation of animals usable for experiments normally takes 4-6 months. Currently, the mouse lines have been expanded and everything seems to work fine. We expect that in 2-3 months we will have all the experiments done and the paper rewritten and ready for resubmission. Therefore, I would like to ask EMBO Reports for an extension (one or two months if possible) of the time granted for resubmission. Naturally, we will resubmit the manuscript sooner if we finish all the experiments needed to address the reviewers comments and suggestions.

I hope that we have the requested extension granted. Problems with a mouse colony are common in laboratories using genetically modified animals and, sometimes, difficult to foresee and prevent.

Appreciating your attention to these matters.

Resubmission - authors' response 07 July 2015

RESPONSE TO REVIEWERS (authors comments in red color)
Ms. Number: EMBOR- 2014-39315V1
Diaz-Castro et al.,

General comments

We thank the reviewers for their insightful comments and the time spent in the evaluation of our work. All their suggestions have been taken into consideration to prepare the revised version of the manuscript. Please note that, as we informed to the editorial office in due time, resubmission of this paper has been retarded for several months due to unexpected problems with the hGFAP-Cre line

necessary to generate the experimental hGFAP-SDHD mice. The hGFAP-Cre line had to be reinitiated and expanded in our animal facility and this has delayed the execution of the experiments necessary to address the points raised by the reviewers. We apologize for the delay and hope that the reviewers and the editors will be satisfied by the revisions made.

Referee #1:
The manuscript describes the interesting results observed when SdhD is conditionally deleted in brain stem cells using hGFAP-cre. This deletion produces a marked phenotype, with abnormally small brain size, relative preservation of the peripheral nervous system and hypoplastic carotid bodies. From their observations in these mice, the authors conclude that "neuronal differentiation" is impaired by mitochondrial injury, while the survival of stem cells and glia is not compromised. Unfortunately, the conclusions are not justified by the data as currently presented, because the phenotype is not presented in adequate detail, and the cellular processes that drive the phenotype are not examined. The manuscript requires additional work that may be beyond the scope of a revision. With the addition of more detail, however, it would be likely to be of great interest and suitable for publication in Embo Journal.

While the presentation of the phenotype is of interest, the manuscript does not analyze the phenotype in adequate detail. The authors should have presented more images of the brain, including sagittal images of the rostral migratory stream and cerebellum. These images should include a series of time points, perhaps P3 and P7, in addition to P15. The additional images would make it possible to examine the processes of postnatal neurogenesis and reveal the temporal pattern in which the phenotype becomes manifest.
Authors response: The current work was not originally planned to perform a full analysis of the morphological alterations produced by deletion of the *SdhD* gene in neural precursors. Our paper was designed as a short communication in which we wanted to stress two main points: a) the fact that central and peripheral neural stem cells are unaffected by mitochondrial dysfunction; and b) the differential impact of genetic mitochondrial disruption of stem cells of glial lineage on central and peripheral neurogenesis. Honestly, we think that a detailed anatomical description of the hGFAP-SDHD mutant, although necessary for an in depth examination of the changes in the temporal pattern of postnatal neurogenesis, falls beyond the scope of the present paper. Nonetheless, we agree with the reviewer that a basic anatomical analysis is required to understand the phenotype. Therefore, following his/her recommendations we have performed new experiments to expand the brain morphology described in the paper. As suggested by the reviewer brain sagittal sections, which allow seeing at first glance the differences between the brains of normal and mutant animals, have been added to Fig. S2. Photographs of rostral (olfactory bulb) brain areas have also been added to Fig. S2. In addition, a new supplementary figure (Fig. S4) is provided, which shows coronal sections of control and mutated brains at an intermediate time point (P5).

Additionally, while the authors look superficially into neuronal population changes using NeuN, they should devote some images to showing the glial population. The GFAP-GFP reported mouse that they made would be a useful tool for this purpose. It is important to look at glia since one of the conclusions is that glia are tolerant to mitochondrial injury.
Authors response: Following the reviewer suggestion we have included a new figure (Fig. S3) in which we show glial staining (GFAP) at P0 and P15. Interestingly, in mutant animals GFAP+ glial cells seem to maintain a more immature “radial glia-like” phenotype than in controls.

The nature of the mitochondrial injury should be examined in some detail. Is there increased brain lactate? Are mitochondria present or lost? Are mitochondria depolarized? Is there premature release of cytochrome C into the cytoplasm? Is there activation of the internal apoptotic pathway? Mitochondrial stains and staining for cleaved caspase 3 could answer most of these questions and would contribute greatly to the work.
Authors response: The molecular effects of *SdhD* deletion on central and peripheral neurons have been studied and discussed in previous papers from our laboratory (Diaz-Castro et al., 2012; Platero-Luengo et al., 2014) using animal models more suitable for these type of studies than the hGFAP-SDHD strain. In those previous reports we show that oxidative damage and energy depletion seem to be two main causes of neuronal injury. Further analysis was not attempted because, as indicated above, the current work was designed to address other specific questions related to the glial lineage and resistance of neural stem cells to disruption of oxidative phosphorylation.

Lastly, the authors confuse GFAP expression with GFAP-lineage. The hGFAP-cre line drives recombination in a large population of brain cells. The GFAP-GFP reporter identifies only the portion of the GFAP lineage that continues to express GFAP. Thus neurons and oligodendrocytes will derive from cells that have undergone cre mediated recombination, but will be excluded from analysis using the GFAP-GFP reporter. This nuance needs to be made clear in the text and considered in the interpretation.
Authors response: We have made several modifications in the text to avoid the confusion between GFAP lineage and GFAP expression.

It is understandable that SdhD antibodies may not be available that can demonstrate loss of the protein.
Author response: As the SdhD antibodies available are not good, we have tested the loss of functional protein by direct measurement of succinate-ubiquinone oxidoreductase (SQR) activity (Fig. 1O at P15 and Fig. S1N at P0).

If SdhD protein loss cannot be mapped by IHC, the authors should at least use a cre-activated lineage tracer, such as lox-STOP-lox tdTomato to show that cre-mediated recombination has occurred. For example, it is not clear if peripheral neurons are tolerant to SdhD deletion, of if this population was not targeted by the hGFAP-cre transgene. Prior reports of other investigators using the
hGFAP-cre line are not optimal to answer this question, as there may be variation in the function of this transgene. Rather, the authors should include contemporaneous lineage tracing.
Author response: In previous work (Diaz-Castro et al., 2012) we have shown extensive neuronal death in the carotid body (CB), superior cervical ganglion (SCG) and adrenal medulla (AM) after deletion of *SdhD* in catecholaminergic cells (TH-SDHD mouse). Following the suggestion of the reviewer we have further analyzed Cre-mediated recombination in peripheral neurons using two different approaches:
a) We have performed PCR analyses showing that hGFAP-Cre mediated recombination does not occur in the SCG. A slight non-significant decrease of *SdhD* mRNA was observed in the SCG of hGFAP-SDHD mice. However, this finding can be explained by deletion of the gene in the population of glial (GFAP+) cells existing in this structure (Fig. S6A,B).
b) We have used an hGFAP-Cre LacZ reporter mouse to show the lack of Cre-mediated recombination in TH+ SCG sympathetic neurons (Fig. S6C).

Some additional textual concerns:
Abstract:
"homozygous deletion of the succinate dehydrogenase subunit D gene restricted to glial fibrillary acidic protein-expressing cells"- is it GFAP expressing or GFAP lineage?
Authors response: It is GFAP lineage. The sentence has been rewritten.

"thus highlighting their non-glial origin"- needs to be rephrased- both Schwann cells and peripheral neurons are NC origin, what data shows is that peripheral neurons either do not depend on SDHd or that they are not targeted by the cre driver.
Authors response: The sentence has been rewritten.

Intro:
"Intermediate metabolism"- what does that mean? Intermediary metabolism? Energy metabolism?
Authors response: Corrected.

Results
On page 5 "dilatation" should be changed to "dilation"
Authors response: Corrected

GFAP-cre line should be referred to as hGFAP-cre, to make clear that the promoter is a construct based on the human GFAP promoter sequence.
Authors response: The notation hGFAP-cre is now used throughout the manuscript and figures. Moreover, the name of the mutant strain has been re-named hGFAP-SDHD in order to emphasize the human origin of the GFAP promoter.

Fig 1: sagittal sections should show whole brain, or images should show the RMS and cerebellum in sagittal sections should be included.
Authors response: Sagittal sections showing the cerebellum are included in Fig. S2.

hGFAP-cre and GFAP-GFP are not the same and this distinction is lost in the text. GFAP-GFP+ cells are only a portion of the entire population targeted by GFAP-cre. A more interesting use of GFAP-GFP would be to breed hGFAP-cre;Sdh fl/fl;GFAP-cre;Lox-STOP-Lox tdTomato mice, to look at changes in the GFAP+ (glial) and GFAP- (neuronal) portions of the hGFAP lineage.
Authors response: As indicated before, corrections in the text have been done to avoid confusion between GFAP lineage and GFAP expression. LacZ reporter mice have been used to show the lack of hGFAP-Cre mediated recombination in peripheral neurons (Fig. S6C)

Page 7 experiment with neurospheres does not show that neuronal differentiation is impaired, since neurons do form from SdhD-deleted stem cells. The more clearly suggested interpretation is that mature neurons require mitochondrial function for survival.
Authors response: We fully agree with this interpretation. We have done experiments on *in vitro* neuronal differentiation at days 2, 3, 5 and 7 (Fig. 2F). The data indicates that neurons are formed but do not survive in the absence of a normal mitochondrial function (page 8, first 11 lines).

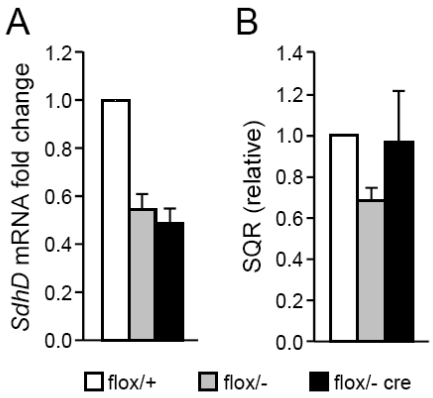
Page 9 use of "conform" is not appropriate, not sure what meaning is intended, maybe "comprise"
Authors response: We have replaced “conform” with “constitute”.

Referee #2:

This is a very interesting manuscript addressing the in vivo role of succinate dehydrogenase (SDHD) in radial glial cells and their progeny in vivo. This is of key importance of the field as little is known about the mitochondrial function in neural stem cells in vivo. The authors find an initially normal development, followed by neurodegeneration at later postnatal stages with region-specific differences that they allocate to the region-specific differences in Cre recombination. They also report the normal formation of primary neurospheres (no passages - see comment below), while they observe a decrease in the proportion of neurons between 3 and 7 days of differentiation. Thus, the function in adult neural stem cells is not addressed in vivo but only in vitro. Lastly they investigate the peripheral nervous system where neurons apparently survive well, and the authors conclude probably rightly that these are not recombined, but I could not find the experimental evidence for this.
Authors response: As indicated in the text, we have shown previously (Díaz-Castro et al., 2012) that deletion of *SdhD* in peripheral neurons cause their degeneration. Therefore, the absence of neuronal loss in peripheral structures in the hGFAP-SDHD strain is explained by a lack of hGFAP-Cre mediated recombination in their precursors. A new figure (Fig. S6) has been added to demonstrate the lack of recombination in peripheral superior cervical ganglion (SCG) neurons by two different experimental approaches (see below).

Conversely, so do report recombination in the nerves, i.e. Schwann cells. Very interestingly then, they analyse the carotid body, where neurons are again affected and decreased in number, while the stem cells are present in vivo and can form neurospheres in vitro. If the authors would adequately control for when and where SDHD is truly gone, this would be a truly important contribution to the field.
1) The authors present data that SDHD function is reduced in P15 brain (Fig. 1O), but want to conclude that embryonic neural stem cells do not need SDHD. Very obviously this conclusion can only be drawn if SDHD is already successfully deleted and protein levels are reduced at embryonic stages. This must be shown to substantiate the conclusions.
Authors response: Following the reviewer suggestion we have done analyses by PCR and by the measurement of succinate-ubiquinone oxidoreductase (SQR) activity at P0 (Fig. S1 M,N). At this stage, *SdhD* mRNA was already significantly reduced indicating that ablation of the *SdhD* gene was successfully done during embryonic life. We also observed a consistent (although not statistically significant) decrease in SQR activity indicating effective reduction in the levels of SdhD protein. A clear loss of SQR activity was seen at P15 (Fig. 1O). Independently of these direct measurements, the fact that the hGFAP-SDHD mice have a clear phenotype in early postnatal life indicates that the *SdhD* gene was effectively lost in embryonic neuronal progenitors.

2) Likewise, they need to show that SDHD is not/less reduced in the regions not affected at P15, as reporter activity is no indication for recombination of the floxed SDHD allele which may be better or worse recombined.
Authors response: We have tested that the differences of *SdhD* mRNA levels and MCII activity seen in the brains of hGFAP-SDHD mice (Fig. 1N,O) are lost when the ventral part of the brain (less affected by the mutation) is used for the analyses. We have added a sentence to indicate this observation (page 6, lines 11-13). Please see the figure below which is included only for reviewing purposes.



A. *SdhD* mRNA measured by PCR. B. Succinate/ubiquinone oxidoreductase activity. See the lack of difference between flox/- (heterozygous control) and flox/- cre (hGFAP-SDHD) mice.

3) The SVZ neurosphere need to be passed to assess for self-renewal capacity and differentiation into oligodendrocytes should be assessed to see how they are affected. (Indeed the authors make conclusions about oligodendrocytes being susceptible to SDHD deletion but again, it is not addressed whether they are affected directly, or only indirectly as neurons are dying. Or do neurons die only because oligodendrocytes are degenerating?)
Authors response: We have done the experiments suggested by the reviewer to demonstrate that self-renewal of SVZ progenitors (the number of secondary neurospheres) is unaffected in hGFAP-SDHD mice (Fig. S5 and page 7, last 4 lines). In addition, differentiation of neural stem cells to oligodendrocytes has also been studied. These data have been added to former Fig. 2 (panels H,I). These in vitro studies indicate that survival of oligodendrocytes (which similar to neurons are cells with high energy demands) also depend on an adequate oxidative metabolism (page 8, lines 4-11)

4) Decrease in the proportion of neurons is not a measure for neurons dying, as other cells may amplify. The authors must therefore count and present total numbers of bIIItubulin+ cells in the dish.
Authors response: We understand the concern of the reviewer, however in the current case we found more appropriate to count the number of Tuj1 (bIIItubulin)+ cells normalized to total number (dapi+) of cells because the size of the colonies (neurospheres) in control and mutant animals are very different.

5) Ideally it would be great to use an inducible deletion of SDHD in adult neural stem cells in vivo to truly assess their role in vivo using one of the many available inducible Cre lines, but this may have to be left to future experiments.
Authors response: This is a very interesting experiment that we plan to do in the future if we find a reliable mouse model with GFAP-driven Cre recombinase that can be activated in adulthood.

6) Show presence of SDHD in peripheral ganglia to support the conclusion that they are not recombined.
Authors response: Unfortunately, the available antibodies against SdhD are not good. We have analyzed Cre-mediated recombination in peripheral neurons using two different approaches:
a) We have performed PCR analyses showing that hGFAP-Cre mediated recombination does not occur in the SCG. A slight non-significant decrease of *SdhD* mRNA was observed in the SCG of hGFAP-SDHD mice. However, this finding can be explained by the deletion of the gene in the population of glial (GFAP+) cells existing in this structure (Fig. S6A,B).

b) We have used an hGFAP-Cre LacZ reporter mouse to show the lack of Cre-mediated recombination in TH+ SCG sympathetic neurons (Fig. S6C) (page 8 last line and page 9 first 6 lines).

Referee #3:

In their study "Resistance of glia-like central and peripheral neural stem cells to genetically-induced mitochondrial dysfunction. Differential effects on neurogenesis" Diaz-Castro and colleagues use a conditional mutant of the gene encoding for the membrane anchoring subunit D of succinate dehydrogenase in cells expressing Cre under the human GFAP promoter, that targets among other cell populations radial glia cells in the developing brain (GFAP-SDHD mouse). As suggested by previous data deletion of SDHD leads to mitochondrial dysfunction.
The key findings obtained using in vivo and in vitro approaches are that i) mitochondrial dysfunction in radial glia NSCs does not substantially affect the properties of NSCs, ii) differentiation into neurons and neuronal survival is impaired, resulting in extensive brain atrophy of post-natal GFAP-SDHD mice, iii) knocking out SDHD does not affect the PNS, highlighting the non-glial origin of the PNS, and that iv) the carotid body, although a part of the PNS, shows signs of atrophy indicating the glial-like cellular origin of carotid body NSCs.
Characterizing the metabolic demands of somatic stem cells is a novel and exciting topic. The experimental design is straightforward and the authors present convincing data showing in vivo and in vitro phenotypes. However, the interpretation of the results is not complete at this stage and should be improved.

1. The authors should analyze and discuss in more detail the mitochondrial deficits in the SDHD knockout cells using NSCs and their progeny in vitro (e.g., by analyzing SQR activity in these distinct cell populations; compare to Fig. 1O). There have been several studies in the past using SdhD knockout heterozygous mice, but the exact mechanism of mitochondrial dysfunction remains largely uncharacterized (e.g., Bayley 2009, Piruat 2004, Diaz-Castro 2012).
Authors response: Our previous work on TH-SDHD mouse (see Diaz-Castro et al., 2012; Platero-Luengo et al., 2014) has suggested that oxidative damage and energy depletion seem to be two main causes of neuronal injury. In addition, cell lines with reductions in succinate-ubiquinone oxidoreductase (SQR) activity caused by mutations in SdhB or SdhC show signs of oxidative damage and apoptosis (see Guzy et al., Mol. Cell. Biol. 28:718 –731, 2008; Ishii et al., Mitochondrion 11:155–165, 2011). These phenomena can explain the neuronal/oligodendrocyte death observed in our hGFAP-SDHD mouse model. Further analysis was not attempted because the current work was designed to address other specific questions related to the glial lineage and resistance of neural stem cells to disruption of oxidative phosphorylation.

2. In this context it is also important to analyze how long pre-existing mitochondria are present in NSCs after the hGFAP driven Cre recombinase turns on at around E12. At this stage, the exact time where mitochondria dysfunction sets in remains unclear; making it difficult to understand if NSCs are indeed "resistant" to mitochondrial dysfunction, or if it just hasn't set in yet.
Authors response: Following the reviewer suggestion we have performed analyses by PCR and by the measurement of succinate-ubiquinone oxidoreductase (SQR) activity at P0 (Fig. S1 M,N). At this stage, *SdhD* mRNA was already significantly reduced indicating that ablation of the *SdhD* gene was successfully done during embryonic life. We also observed a consistent (although not statistically significant) decrease of SQR activity indicating effective reduction in the levels of the SdhD protein. A clear loss of SQR activity was seen at P15 (Fig. 1O). Independently of these direct measurements, the fact that the hGFAP-SDHD mice have a clear phenotype in early postnatal life indicates that in these animals the *SdhD* gene was effectively lost in neuronal progenitors. Moreover, we have shown deletion of the *SdhD* gene in striatal and cortical GFAP+ cells (Fig. 1 P-S) and almost complete disappearance of *SdhD* mRNA in SVZ neurospheres (Fig. 2D). Taken together, these data indicate that recombination is taking place in hGFAP+ neural stem cells and that they are indeed resistant to mitochondrial dysfunction.

3. The authors state that initial proliferation and NSC function is not affected in vivo (note: the authors should show whole brain sizes at PO).
Authors response: Photographs of whole brain at P0 have been added to Fig. S1 (panels A,B).

In line with this, the authors show that the number of sphere forming cells is not reduced (Fig. 2B). However, they find a dramatic reduction in sphere size (Fig. 2C). This is typically considered to be a function of NSC proliferation. Thus, the authors should analyze this in more detail (using proliferation and cell death assays), as this is apparently very important for the interpretation of the results.
Authors response: We have done secondary neurospheres to show that self-renewal is not affected in SdhD-deficient neural stem cells (Fig. S5). Proliferation of transit amplifying progenitors in neurospheres is reduced in SdhD-deficient cells probably because at this stage high levels of ATP production by oxidative phosphorylation are required to maintain the fast proliferation rate. This point is now discussed on page 10 (last 4 lines) and page 11 (lines 1-3).

4. The data shown in Fig. 2F suggest that NSC can differentiate into Tuj1+ neurons. However, it is not clear if induced differentiation truly occurs at the time points analyzed (e.g., is there a significant difference between proliferating spheres, 2days, 3days? This needs to be included). When does neuronal cell death occur (the authors should analyze additional time points between 2 and 7 days)?
Authors response: Following the indication of the reviewer we have done experiments to study neuronal differentiation at 2, 3, 5 and 7 days (Fig. 2 E,F). We have also included in the figure data on the differentiation of oligodendrocytes (Fig. 2 H,I). Page 8 lines 1-11.

Furthermore, glial differentiation of only 0.2% of cells (Fig. 2G) is extremely low compared to previous studies (and does not reflect the images shown in Fig. 3E where glial differentiation appears much higher; these example images should be improved to represent the quantifications).
Authors response: We made a mistake in the previous figure, as we normalized the data to 1 instead of 100%. The values in the ordinate of Fig. 2G have been corrected.

5. In the carotid body, the number of neurospheres formed and the size of the neurospheres are similar in control NSCs and cells derived from GFAP-SdhD mice. This is an interesting finding but the authors should discuss the different phenotypes (i.e., sphere size) they found in CNS NSCs and NSCs of the carotid body.
Authors response. The growth of carotid body neurospheres is slower (and therefore less energy demanding) in comparison with the growth of SVZ neurospheres. This point is now discussed on page 10 (last 4 lines), page 11 (lines 1-3).

Taken together, we believe that this is an interesting study. However, additional analyses and more careful interpretation of the results seem required to strengthen the manuscript.

3rd Editorial Decision 11 August 2015

Many thanks for submitting your revised manuscript to our editorial office and for your patience while we were waiting to hear back from two of the original reviewers who were asked to assess the revised version.

As you will see, the referees now in principle support publication of the study in our journal. Nevertheless, referee 1 still raises some minor issues with regard to the quality of some of the images and the interpretation of some of the results that I would like you to address.

Please do not worry about length restrictions too much as I think an insightful discussion of these points is more important than publishing a short paper in this case. Please also highlight these changes in the final version of the text, so that it will be easier for us to identify them.

Expanded view figures: We are in the process of updating the way in which we display additional/supplementary information. In essence, all supplementary figures are now called Expanded View Figures and should be labeled and referenced as Figure EV1, Figure EV2 etc. in the main text of the manuscript. The legends for the EV figures should be incorporated in the main body of the text after the legends for the main figures. Please modify your additional figures accordingly.

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:

Referee #1:

The authors have addressed most of the issues in my critique. While I continue to hold that the manuscript would be enhanced by a more detailed presentation of the phenotype, I can accept the authors' contention that such a detailed presentation is beyond the scope of a short report. If the authors' goal is to show that CNS stem cells and astrocytes tolerate mitochondrial dysfunction while neurons and oligodendrocytes do not, additional intermediate time points are not needed. One remaining issue in the presentation of the phenotype is the quality of some of the images. Most images are excellent, however, Figure S4 is dark and overly blue-tinted, and the images are smaller than ideal. Fixing this issue should not be overly burdensome and will enhance the quality of the paper.

Some other issues that need to be addressed:
1)The finding that the hGFAP-SDHD mice have persistent radial glia at P15 is intriguing, but it raises issues that need comment in the text. The finding would seem to indicate impaired differentiation of radial glial cells, that become stuck in a state typical of the prenatal brain. In the present text, the authors state on P11 "However, it seems that defective mitochondrial metabolism did not interfere with the process of differentiation itself." This comment needs to be squared with the observation of persistent radial glia.

2)The finding that the ventral brain is relatively preserved, and that there is preserved MCII activity in the ventral brain should be clearly attributed to the distribution of cre-mediated recombination, which in hGFAP-cre mice spares neural cells in this region. If the authors doubt that differential cre activity is the issue, they should examine a cre reporter on a lox-STOP-lox configuration, as previously suggested. If the authors agree that the ventral sparing is due to the choice of cre line, they should add the interpretation to their discussion. The point does not disrupt their conclusions, but it is potentially distracting.

2 typos on P4:
"encoding for the membrane anchoring subunit" (the word "for" should be removed)
"model has permitted us to experimentally examine" (split infinitive, please fix)

With attention to these issues, the manuscript can be a significant contribution that will advance understanding of metabolism during brain development and should be published.

Referee #2:

The authors have now submitted a revised version. They have sufficiently addressed our previous concerns and improved the manuscript. The data will be of interest to the field and will initiate new investigations.

2nd Revision - authors' response 20 August 2015

Thanks for your email message of August 11, 2015 in which you informed us of the potential acceptance of our manuscript by Diaz-Castro et al. entitled “Resistance of glia-like

central and peripheral neural stem cells to genetically-induced mitochondrial dysfunction. Differential effects on neurogenesis" (EMBOR-2014-40982V1). Following the indications in your letter we are submitting the following files:

- Manuscript file (Word).
All modifications suggested by reviewer 1 have been included and appear highlighted in red.
Statistics section on Material and Methods has been expanded (highlighted in red).
Supplemental figures are cited as Fig EV. Expanded view figure legends have been added at the end of the text (highlighted in red)

- Expanded View file (Word) with Expanded Material and Methods and References

- Expanded view figures (1 to 6) (Tiff). As requested by reviewer 1 we have made an improved version of figure EV4, in which, following the style in the other figures, the brains of control and mutant animals at P5 are compared.

Please let me know if we have to submit any additional material. Appreciating the attention given to our work.

4th Editorial Decision 21 August 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

