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Endothelium-derived lactate is required for pericyte function and blood-brain barrier maintenance

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **24th Nov 2021**

Dear Dr Simons,

Thank you again for the submission of your manuscript (EMBOJ-2021-109890) to The EMBO Journal and in addition providing us with a preliminary revision plan. As mentioned earlier, your study has been sent to three reviewers for evaluation, and we have received reports form all of them, which I enclose below.

As you will see from their comments, the referees acknowledge the potential interest and value of your findings, although they also express major concerns. In more detail, referee #1 states substantial issues regarding the support provided for claims on an in vivo relevance of endothelial cell-derived lactate for pericyte coverage and BBB integrity (ref#1, pts.1,2,5). Further, this reviewer points to discrepancies with earlier literature, which remain unresolved (ref#1, standfirst). Referee # 2 agrees in that the physiological role of the lactate shuttle between endothelial cells and pericytes remains unclear, and direct causalities to be tested (ref#2, pts. 1,2). This expert also states that the mechanistic details of how pericyte coverage is affected remain too prematurely explored (ref#2, pts.4,5). Reviewer #3 requests consideration of cell death as an alternative contributing factor (ref#3, pt.1) and more detailed characterise junctional organisation (ref#3, pt.2).

Given the interest stated and timeliness of your findings, we are overall able to invite you to revise your manuscript experimentally to address the referees' comments, along the lines sketched in your outline. I need to stress though that we do require strong support from the referees on a revised version of the study in order to move on to publication of the work.

We do concur that an integration of mouse genetic loss-of-function data on the MCT involvement is not required for this study. However, claims made about their physiological role in the context should be toned down and adjusted accordingly.

Please feel free to contact me if you have any questions or need further input on the referee comments.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

When submitting your revised manuscript, please carefully review the instructions below and include the following items:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point response to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) It is mandatory to include a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition).
In case you have no data that requires deposition in a public database, please state so in this section. Note that the Data Availability Section is restricted to new primary data that are part of this study.
*** Note - All links should resolve to a page where the data can be accessed. ***

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can be provided as individual .xls or .csv files (including a tab describing the data). For 'blots' or microscopy, uncropped images should be submitted (using a zip archive or a single pdf per main figure if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at .

9) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc. in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: .

- Additional Tables/Datasets should be labelled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

10) When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

11) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

Thank you for the opportunity to consider your work for publication.

I look forward to your revision.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

Revision to The EMBO Journal should be submitted online within 90 days, unless an extension has been requested and approved by the editor; please click on the link below to submit the revision online before 22nd Feb 2022:

Link Not Available

Referee #1:

Using EC specific KO mice Lee et al. demonstrate that loss of Glut1 in ECs reduces brain pericyte coverage, leading to decreased BBB integrity and consequent demise. The authors suggest that this phenotype results from reduced EC production of lactate, which is required by the surrounding pericytes, and which can be rescued by oral administration of lactate. The existence of lactate shuttles whereby lactate is transferred from lactate-producing cells to lactate-consuming cells, is well established. For example, in the brain, astrocytes have been described as a major source of lactate which is delivered to neurons (as cited in the discussion). Whilst the phenotype of this Cdh5-Cre driven EC-specific Glut1 deletion model is interesting, questions relating to the lack of an EC phenotype and the contrast to the recent publication by Veys et al raises concerns. Comments are listed below:
1. A major conclusion, which rests on in vitro analyses, is that the loss of BBB integrity is not due to defects in the ECs themselves but due to the lack of EC produced lactate, affecting pericytes. The lack of cell autonomous affect is quite surprising given the in vitro analyses presented here and the in vivo analyses by Veys et al. on their mouse model with Pdgfb-Cre driven excision of Glut1 in ECs. For such a statement a more thorough investigation of whether in vivo loss of Glut1 affects ECs themselves would be required.
2. The authors assume that the pericyte defect is as a result of loss of CSF lactate levels. However, it is conceivable that the pericyte defect is as a result of the loss of glucose shuttling across the BBB via ECs. The authors should measure CSF levels of glucose following Glut1 deletion in ECs. Furthermore, potentially lowered glucose levels may explain the partial rescue of CD13 levels after oral lactate administration (Figure 8B).
3. The authors conclude that brain EC-produced lactate resulting from glucose metabolism in brain ECs, is specifically required for healthy pericyte coverage. From the data presented however it is only clear that loss of EC Glut1 reduces CSF lactate and that pericytes can utilise lactate as an energy supply. Alternative interpretations from the data however might be that the loss of CSF lactate following Glut1-deletion in ECs could result from changes in the supply of glucose eg to astrocytes resulting in reduced lactate production. An investigation of CSF glucose levels could answer this. Furthermore, given that muscle is also a major source of lactate, and that it is known that muscle derived lactate can shuttle across the BBB, the decrease in CSF lactate in EC Glut1 KO mice may result from defective EC glucose transport and metabolism elsewhere outside of the brain. Are there changes in lactate levels outside of the CSF as well?
4. The authors state that EC-pericyte proximity is required for pericytes to use lactate. This is assumed from co-culture experiments where pericytes in contact with ECs show higher FRET efficiency. However, this might also occur because the lactate concentration is higher nearer ECs where glucose is being metabolised. As a control to the data in Fig. 2 please complement the medium with lactate to distinguish between lactate concentration per se vs pericyte proximity.
5. The authors state that MCT1 and MCT5 are required for the shuttling of lactate out of brain ECs for use by pericytes. This assumption is true in the in vitro setting used but in the in vivo experiments, the inhibition of MCT1 would affect the secretion of lactate from all cells, thus affecting whole body lactate levels, and as MCT1 is required for the influx and efflux of lactate it could also be responsible for the shuttling of lactate across the BBB rather than lactate produced in the ECs themselves. This a difficult point to address without the use of further mouse models but the authors could perhaps investigate whether lactate is shuttled across the BBB by ECs following oral lactate and AZD3965 treatment. At the least the authors should modify their conclusions to be more in line with what the experiments are actually showing.

Minor points:
• Often the communication of the intended message is ill defined due to some problems with language and context. For

example, the sentence in lines 55-58- two mural cell types? This is fairly common through the text. Please also check typos such as VACAD in figure labels.

• There are some statements that are not referenced.

• Line 371 should be Figure 4E?

• It is unclear what Figure S8 is it trying to convey. A more informative legend would be helpful.

• Figure 8: It is claimed that the oral lactate rescued the CD13 phenotype in Glut1 KO mice but the CD13 are still much lower than the WT situation (Figure 1E) so it is improved but not rescued. Does oral lactate rescue CSF lactate levels?

• The efficiency of Glut1 loss is not quantified; the Glut1 immunostaining in Figure S4 needs to be complemented by qPCR analyses on ECs isolated from brain, RNAscope, Glut1 immunoblotting, or other techniques allowing quantification.

Referee #2:

The manuscript expands on two important areas of research addressing 1) the critical intercellular relationship between endothelial cells and pericytes, and its impact on blood brain barrier (BBB) function, and 2) the biological relevance of the robust glycolytic metabolism in endothelial cells and its effect on blood barrier integrity. Overall, through their data the authors propose that lactate production and release by endothelial cells and uptake by pericytes is imperative for pericyte-vascular association and summarize that the dependency of BBB integrity on endothelial cell glycolysis is secondary at least in part to delivery of lactate to pericytes.

The authors demonstrate convincingly that endothelial, Glut1-dependent glycolysis is necessary for BBB integrity and angiogenesis in mice, as well as overall survival irrespective of age. Moreover in vitro, GLUT1-dependent glycolysis is needed for proliferation and migration of human endothelial cells, but is not required for in vitro permeability, nor does GLUT1 loss affect cadherin junction localization.

The authors then attempt to explore the mechanism of the effects of attenuated glycolysis in vivo and determine that loss of Glut1 in mouse endothelial cells impairs pericyte coverage of brain vasculature. They further demonstrate through FRET reporter system that pericytes can take up endothelial-derived lactate, and that exogenous lactate supplements pericyte metabolism, increasing ATP production and feeding various metabolite pools, such as pyruvate, citrate, aconitate, and alanine. In vitro, the intercellular transport of lactate was dependent on MCT1- and MCT5-mediated export by endothelial cells and MCT12-driven import by pericytes. This is an interesting and well-executed basic science study with a clinical relevance to the pathology of GLUT1DS, and overall, the paper is well written. The authors uncover an elegant metabolic relationship between endothelial cells and pericytes which contributes to brain vascular integrity with exciting implications. However, although the authors evidence that lactate homeostasis overall contributes to BBB integrity, the extent to which the endothelial-pericyte lactate shuttle specifically is relevant to BBB homeostasis is not as clear. Some additional concerns also exist.

1) First, although the authors demonstrated evidence for the endothelial-pericyte lactate shuttle in vitro, its relevance in vivo is not as appreciable. Although the rescue experiment with lactate treatment in mouse improved permeability relative to non-lactate treated mutant mice, they did not compare the effect of lactate rescue to WT mouse. In other words, although lactate improved permeability in mutant mice, did it substantially restore permeability to WT levels? Without including the WT control, it is hard to appreciate to what extent BBB permeability is dependent on endothelial lactate release. Judging from the fact that survival was not improved with lactate, it suggests that lactate uptake in pericytes may not be so substantially relevant to BBB integrity. To better address this, WT control would be appreciated.

2) Also, important for the lactate rescue experiment is the question of whether whole-body lactate supplementation improved BBB integrity through effects in endothelial cells (i.e. energy source to substitute glucose and counterbalance impaired glucose uptake via Glut1 loss) rather than through any effects in pericytes, a question which the authors leave unaddressed. This is relevant as Glut1 loss led to direct negative effects in endothelial cells (reduced glycolysis and ATP production) which could affect pericyte coverage through other mechanisms besides lactate release. And although the authors showed that lactate supplementation augmented OCR in pericytes and not cultured endothelial cells, it is equally important to interrogate whether Glut1-depleted endothelial cells can substitute lactate for glucose as an energy source. If so, then it provides an alternative mechanism for in vivo lactate supplementation rescue in Glut1 mutant mice.

3) The MCT1 inhibitor experiment partially phenocopied the Glut1 mutant mice in that it replicated BBB permeability defects,

supporting relevance of MCT1-mediated lactate transport to BBB integrity in vivo. However, the effect of MCT1 inhibitor on mouse survival or overall health was not addressed, which is notable since Glut1 mutant mouse died only within 2 to 3 weeks of induced Glut1 loss in endothelial cells. In other words, by not comparing MCT1 treated mice to Glut1 mutants, we cannot appreciate to what extent MCT1 inhibition phenocopies the effects of glycolysis inhibition in endothelial cells. Here, the mutant control would greatly assist in determining the extent of relevance of MCT1-mediated lactate transport to pericyte-dependent BBB permeability and animal homeostasis.

4) Additionally, left unaddressed is the mechanism of reduced pericyte coverage. Is there impaired expansion of pericytes? Reduced survival? Impaired interaction with endothelial cells?

5) By what mechanism could loss of lactate release disrupt pericyte coverage? Could it be through impaired bioenergetic homeostasis? Are pericytes dependent on endothelial-derived lactate for ATP-production? Does reduced ATP production in pericyte decrease pericyte coverage? Could other carbons sources (i.e. glucose) substitute for lactate in mouse/human brain pericytes? The authors demonstrate that lactate feeds pericytes, but they don't show whether or not pericytes are dependent on it, and if so, to what extent.

6) While I appreciate the in-depth characterization of the lactate transporters in both endothelial cells and pericytes, the loss of pericytes phenotype with MCT1 inhibition in vivo was not complemented with an MCT12 inhibition/deletion experimental design in vivo or in vitro. If lactate influx to pericytes is essential for survival, reduced lactate metabolism and subsequent cell death should be expected with MCT12 loss even when extracellular lactate is supplemented.

7) the abbreviations should have been named/denoted in the beginning of the paper.
In the results and discussion, some sentences needed clarity:
(#120 - This may be referring to the 2-DG treatment from the context of that paragraph and previous indications, while the sentence does not clarify. #412 - This sentence is not clear, how is the ketogenic diet not working?. Also, during the FRET experiment texts,Venus is repeatedly written as Venous. #240 and #284 subheadings are also not very clear and falls short in reflecting the overall working hypothesis/statement of the following results section #390 should be :have not established, a typo that changes the meaning)

8) While the authors cite Veys et al, 2020 as a another study which showed no deficit in EC migration upon Glut1-loss conflicting with data presented in this manuscript, they provide no discussion on why this may have been the case. Another discussion point worth mentioning is the lack of amelioration of lethality observed in GLUT1-ECKO with lactate supplementation in vivo as well as the seemingly lack of the lethality phenotype in the MCT1-inhibition in vivo (data not shown/mentioned).

Referee #3:

Summary: In the manuscript, "Endothelium-derived lactate is required for normal pericyte function and the blood-brain barrier maintenance", Lee and colleagues investigate functional roles of endothelial-derived lactate - the end product of glycolytic metabolism. Endothelial cells are known to be highly glycolytic, secreting high amounts of lactate into the extracellular environment, providing the rationale for this work.

The authors report that vascular mural cells, namely pericytes, use lactate secreted from endothelial cells for energy and biomass generation. Disrupting this "metabolic communication", e.g. by inactivating the glucose transporter GLUT1 in endothelial cells, leads to pericyte dropout, blood-brain-barrier defects and pathological vascular permeability. The authors further report that supplementing mice with lactate in this setting is sufficient to mitigate some of these phenotypes.

General comment: Understanding mechanisms of metabolic communication between cell types is a timely and relevant area of research that is interesting for the journal's wide readership. Here, Lee et al. provide novel insights into how such metabolic communication can affect the development and function of the blood-brain barrier.

The manuscript is well written and uses both in vivo and (elegant) in vitro approaches to show that endothelial-derived lactate is an important metabolic substrate for pericytes. While I am generally enthusiastic about this work, some points require further investigation/clarification. These points are detailed below.

Specific points:
* The authors propose that in the absence of endothelium-derived lactate, pericytes fall off the CNS vasculature resulting in impaired BBB function. However, the increased permeability could also result from other, more simplistic effects that the authors

did not consider. For instance, changes in endothelial survival upon GLUT1 loss could also influence the integrity of the CNS vasculature. It will not be surprising if changes in the main energy-generating pathway in endothelial cells cause significant survival issues. Therefore, the authors should look at endothelial cell death in vitro and in vivo in some more detail.
* Along these lines, the junctional organization in the endothelial-specific GLUT1 knockout mice needs to be investigated. Staining retinal or brain samples for junctional markers (e.g., VE-cadherin or Claudin-5) should reveal whether a major junctional effect can be excluded in GLUT1 mutant mice.
* What happens to the pericytes following endothelial GLUT1 inactivation? Do they de-differentiate, migrate away or die off?
* The co-culture experiments of Figure 2C and the following are difficult to interpret because the glycolysis inhibitor in HBVPs might just be washed out after 48 hours post-treatment. It would be important to show that idoacetic acid treatment is active for such a long time, even the compound is no longer in the cell culture medium.
* The authors should also compare the uptake and glycolytic breakdown of glucose between endothelial cells and pericytes in vitro. Such studies might give clues why pericytes use lactate as a metabolic resource.

Additional points:
* Does endothelial GLUT1 or MCT1/5 depletion lead to compensatory changes in the expression of the other transporters?
* Line 71 and following: "Endothelial cell do not utilize lactate to any meaningful extent ...". This is a quite general and bold statement and I am not sure whether this is true. As a minimum, the authors should provide a reference for this statement.
* Figure 1A: The (10x) overview images in this panel do not really help. Hard to see pericytes at this low magnification. In addition to the high magnification (63x) images on the right, an image of intermediate magnification (e.g. 20x) would help. Are capillaries and venules affected in the same manner?
* Figure S1B,C: Higher magnification images would be beneficial to demonstrate the colocalization clearly. Do pericytes express Glut1 too?
* Figure 1C,D: How does the retinal and brain vasculature of the adult GLUT1 mutant mice look like ten days after induction with tamoxifen?
* Figure 2E: The lactate secreted by endothelial cells should be freely diffusible in the culture medium. So I am surprised to see that only pericytes close to endothelial cells show activity of the lactate reporter.
* The pericyte loss in Glut1 iECKO mice could also be caused by more indirect effects on other types of the CNS. For instance, metabolic changes in astrocytes or neurons due to impaired glucose delivery to these cells could also harm pericytes. This possibility should also be discussed.
* Figure S2E: the error bars are missing in this graph. Also, how can 2DG block glycolytic metabolism if it is not taken up GLUT1-deficient ECs? The knockdown of GLUT1 looks efficient in the immunoblots.
* Line 115 and following: It is expected that cells increase glycolysis after glucose addition. This does not necessarily show that ECs have "strong" glycolytic activity.
* Line 144 and following: I am confused about this statement. Not sure which Cre line they are referring to.

Comments from the Editor:

Thank you again for the submission of your manuscript (EMBOJ-2021-109890) to The EMBO Journal and in addition providing us with a preliminary revision plan. As mentioned earlier, your study has been sent to three reviewers for evaluation, and we have received reports form all of them, which I enclose below.

As you will see from their comments, the referees acknowledge the potential interest and value of your findings, although they also express major concerns. In more detail, referee #1 states substantial issues regarding the support provided for claims on an in vivo relevance of endothelial cell-derived lactate for pericyte coverage and BBB integrity (ref#1, pts.1,2,5). Further, this reviewer points to discrepancies with earlier literature, which remain unresolved (ref#1, standfirst). Referee # 2 agrees in that the physiological role of the lactate shuttle between endothelial cells and pericytes remains unclear, and direct causalities to be tested (ref#2, pts. 1,2). This expert also states that the mechanistic details of how pericyte coverage is affected remain too prematurely explored (ref#2, pts.4,5). Reviewer #3 requests consideration of cell death as an alternative contributing factor (ref#3, pt.1) and more detailed characterise junctional organisation (ref#3, pt.2).

Given the interest stated and timeliness of your findings, we are overall able to invite you to revise your manuscript experimentally to address the referees' comments, along the lines sketched in your outline. I need to stress though that we do require strong support from the referees on a revised version of the study in order to move on to publication of the work.

We do concur that an integration of mouse genetic loss-of-function data on the MCT involvement is not required for this study. However, claims made about their physiological role in the context should be toned down and adjusted accordingly.

Please feel free to contact me if you have any questions or need further input on the referee comments.

We thank the Editor and the Reviewers for their interest in this study and thoughtful comments that have improved this study. Reviewers comments are reproduced below followed by our replies in italics and underlined.

Referee #1:

Using EC specific KO mice Lee et al. demonstrate that loss of Glut1 in ECs reduces brain pericyte coverage, leading to decreased BBB integrity and consequent demise. The authors suggest that this phenotype results from reduced EC production of lactate, which is required by the surrounding pericytes, and which can be rescued by oral administration of lactate. The existence of lactate shuttles whereby lactate is transferred from lactate-producing cells to lactate-consuming cells, is well established. For example, in the brain, astrocytes have been described as a major source of lactate which is delivered to neurons (as cited in the discussion). Whilst the phenotype of this Cdh5-Cre driven EC-specific Glut1 deletion model is interesting, questions relating to the lack of an EC phenotype and the contrast to the recent publication by Veys et al raises concerns. Comments are listed below:

1. A major conclusion, which rests on in vitro analyses, is that the loss of BBB integrity is not due to defects in the ECs themselves but due to the lack of EC produced lactate, affecting pericytes. The lack of

cell autonomous affect is quite surprising given the in vitro analyses presented here and the in vivo analyses by Veys et al. on their mouse model with Pdgfb-Cre driven excision of Glut1 in ECs. For such a statement a more thorough investigation of whether in vivo loss of Glut1 affects ECs themselves would be required.

A: We agreed with reviewer's comment that a possibility of a cell-autonomous effects remains. However, pericyte loss in endothelial-'specific' Glut1 deletion mice and FRET reporter assay in co-culture system clearly show the effect of non-cell-autonomous metabolic signaling through lactate shuttling. If the reviewer has a concrete experiment in mind, we would be happy to perform it if possible.

2. The authors assume that the pericyte defect is as a result of loss of CSF lactate levels. However, it is conceivable that the pericyte defect is as a result of the loss of glucose shuttling across the BBB via ECs. The authors should measure CSF levels of glucose following Glut1 deletion in ECs. Furthermore, potentially lowered glucose levels may explain the partial rescue of CD13 levels after oral lactate administration (Figure 8B).

Answer: We agree with the reviewer's concern. It is well established that the loss of glucose shuttling across the BBB leads to a progressive loss of neurons (PMID: 32404031). However, given that lactate administration rescues pericyte coverage (Figure 8A) but not CNS-dependent lethality (Figure 8E) and that pericytes use lactate for their metabolism, it is more likely that lactate loss is the main driver of pericyte defect in Glut1^{iECKO} mice. According to reviewer's suggestion, we measured CSF glucose level in Glut1^{iECKO} mice. As shown in Fig. S10, CSF glucose level in Glut1^{iECKO} mice was reduced compared to WT mice. As suggested, we expanded our discussion for the partial rescue of lactate supplement in line 413-418.

3. The authors conclude that brain EC-produced lactate resulting from glucose metabolism in brain ECs, is specifically required for healthy pericyte coverage. From the data presented however it is only clear that loss of EC Glut1 reduces CSF lactate and that pericytes can utilize lactate as an energy supply. Alternative interpretations from the data however might be that the loss of CSF lactate following Glut1-deletion in ECs could result from changes in the supply of glucose eg to astrocytes resulting in reduced lactate production. An investigation of CSF glucose levels could answer this. Furthermore, given that muscle is also a major source of lactate, and that it is known that muscle derived lactate can shuttle across the BBB, the decrease in CSF lactate in EC Glut1 KO mice may result from defective EC glucose transport and metabolism elsewhere outside of the brain. Are there changes in lactate levels outside of the CSF as well?

Answer: We agreed that measuring CSF glucose level would be necessary to reinforce our conclusion. As we stated above, we measured CSF glucose n Glut1^{iECKO} mice and found it to be decreased (Fig S10). While muscle is a well-known source of lactate, Glut1 expression is predominantly restricted to the brain ECs but is not found in muscle cells (PMID: 32059779). A publication (PMID: 15622525) reporting normal blood glucose and lactate level in Glut1-syndrome patient also supports the notion that the phenotype is a result of what's happening in the brain.

4. The authors state that EC-pericyte proximity is required for pericytes to use lactate. This is assumed from co-culture experiments where pericytes in contact with ECs show higher FRET efficiency. However, this might also occur because the lactate concentration is higher nearer ECs where glucose is being metabolised. As a control to the data in Fig. 2 please complement the medium with lactate to distinguish between lactate concentration per se vs pericyte proximity.

Answer: We certainly agree and that's what our data show. The reviewer is correct in that pericytes will take up lactate from the extracellular space. As we showed in Fig 2A, higher lactate concentration generates higher FRET response in cells. Under physiological condition, pericytes are always in close

proximity to ECs. This EC-pericyte proximity makes EC-derived lactate a more readily accessible carbon source for pericytes. As shown in Fig2E, we observed a striking increase in the FRET reporter in HBVPs in direct contact with ECs but not in HBVPs cells non in contact with the ECs. It suggests that lactate shuttling is facilitated by endothelial-pericyte proximity since that increases local lactate concentration.

5. The authors state that MCT1 and MCT5 are required for the shuttling of lactate out of brain ECs for use by pericytes. This assumption is true in the in vitro setting used but in the in vivo experiments, the inhibition of MCT1 would affect the secretion of lactate from all cells, thus affecting whole body lactate levels, and as MCT1 is required for the influx and efflux of lactate it could also be responsible for the shuttling of lactate across the BBB rather than lactate produced in the ECs themselves. This a difficult point to address without the use of further mouse models but the authors could perhaps investigate whether lactate is shuttled across the BBB by ECs following oral lactate and AZD3965 treatment. At the least the authors should modify their conclusions to be more in line with what the experiments are actually showing.

Answer: We agree with the reviewer's comment. To evaluate the role of lactate transporter (MCTs) in-vivo, we are currently generating floxed MCT mice with cell line specific inducible deletion system. However, this has a been a very slow process due to disruptions in the work of our transgenic facility due the COVID-19 pandemic. We also understand the reviewer's concern for AZD3965 treatment for using in vivo. However, as we showed in Fig6B, MCT1 is endothelial specific/enriched gene in brain cells. MCT1 also shows EC enriched expression in a recent scRNAseq data with 20 mouse organs (Tabula Muris, PMID: 30283141), suggesting the phenotype with AZD3965 treatment is mainly from the inhibition of endothelial lactate transport, but not whole body lactate level change. In agreement with te reviewer's comment and concern, we toned down our conclusion from these experiments (line 410-412).
We do like the idea of measuring lactate shuttling across the BBB following AZD3965 treatment, however it is technically a challenging experiment. This would require the use of C13-labeled glucose in vivo followed by mass-spec tracing of labeled carbons in pericyte. Given how fast the glucose flux is, and the isolation of pericytes from brain requiring multiple time-consuming steps, we believe the data would not be meaningful given the current state of the art.

Minor points:
• Often the communication of the intended message is ill defined due to some problems with language and context. For example, the sentence in lines 55-58- two mural cell types? This is fairly common through the text. Please also check typos such as VACAD in figure labels.

Answer: Thank you. The text has been checked and corrected.

• There are some statements that are not referenced.

Answer: We corrected the referencing.

• Line 371 should be Figure 4E?

Answer: Yes. We corrected the typo.

• It is unclear what Figure S8 is it trying to convey. A more informative legend would be helpful.

Answer: It shows mass spectra and molecular weights of the parent compound and fragments. We improved the legend as suggested.

• Figure 8: It is claimed that the oral lactate rescued the CD13 phenotype in Glut1 KO mice but the CD13 are still much lower than the WT situation (Figure 1E) so it is improved but not rescued. Does oral lactate

rescue CSF lactate levels?

Answer: As suggested, we measured the CSF lactate level following lactate supplementation. As shown in Fig 8D, it is significantly increased with lactate supplement, but still lower than WT. It could be a reason for the incomplete rescue of pericyte coverage using this approach.

• The efficiency of Glut1 loss is not quantified; the Glut1 immunostaining in Figure S4 needs to be complemented by qPCR analyses on ECs isolated from brain, RNAscope, Glut1 immunoblotting, or other techniques allowing quantification.

Answer: Quantitative data reflecting the deletion efficiency was added in Fig. S4D

Referee #2:

The manuscript expands on two important areas of research addressing 1) the critical intercellular relationship between endothelial cells and pericytes, and its impact on blood brain barrier (BBB) function, and 2) the biological relevance of the robust glycolytic metabolism in endothelial cells and its effect on blood barrier integrity. Overall, through their data the authors propose that lactate production and release by endothelial cells and uptake by pericytes is imperative for pericyte-vascular association and summarize that the dependency of BBB integrity on endothelial cell glycolysis is secondary at least in part to delivery of lactate to pericytes.

The authors demonstrate convincingly that endothelial, Glut1-dependent glycolysis is necessary for BBB integrity and angiogenesis in mice, as well as overall survival irrespective of age. Moreover in vitro, GLUT1-dependent glycolysis is needed for proliferation and migration of human endothelial cells, but is not required for in vitro permeability, nor does GLUT1 loss affect cadherin junction localization.

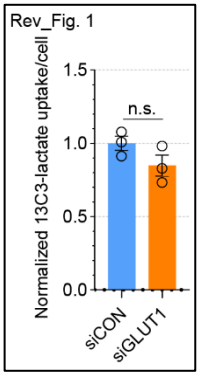
The authors then attempt to explore the mechanism of the effects of attenuated glycolysis in vivo and determine that loss of Glut1 in mouse endothelial cells impairs pericyte coverage of brain vasculature. They further demonstrate through FRET reporter system that pericytes can take up endothelial-derived lactate, and that exogenous lactate supplements pericyte metabolism, increasing ATP production and feeding various metabolite pools, such as pyruvate, citrate, aconitate, and alanine. In vitro, the intercellular transport of lactate was dependent on MCT1- and MCT5-mediated export by endothelial cells and MCT12-driven import by pericytes. This is an interesting and well-executed basic science study with a clinical relevance to the pathology of GLUT1DS, and overall, the paper is well written. The authors uncover an elegant metabolic relationship between endothelial cells and pericytes which contributes to brain vascular integrity with exciting implications. However, although the authors evidence that lactate homeostasis overall contributes to BBB integrity, the extent to which the endothelial-pericyte lactate shuttle specifically is relevant to BBB homeostasis is not as clear. Some additional concerns also exist.

1) First, although the authors demonstrated evidence for the endothelial-pericyte lactate shuttle in vitro, its relevance in vivo is not as appreciable. Although the rescue experiment with lactate treatment in mouse improved permeability relative to non-lactate treated mutant mice, they did not compare the effect of lactate rescue to WT mouse. In other words, although lactate improved permeability in mutant mice, did it substantially restore permeability to WT levels? Without including the WT control, it is hard to appreciate to what extent BBB permeability is dependent on endothelial lactate release. Judging from the fact that survival was not improved with lactate, it suggests that lactate uptake in pericytes may not be so substantially relevant to BBB integrity. To better address this, WT control would be appreciated.

Answer: As suggested, we added WT control in Fig8.

2) Also, important for the lactate rescue experiment is the question of whether whole-body lactate supplementation improved BBB integrity through effects in endothelial cells (i.e. energy source to substitute glucose and counterbalance impaired glucose uptake via Glut1 loss) rather than through any effects in pericytes, a question which the authors leave unaddressed. This is relevant as Glut1 loss led to direct negative effects in endothelial cells (reduced glycolysis and ATP production) which could affect pericyte coverage through other mechanisms besides lactate release. And although the authors showed that lactate supplementation augmented OCR in pericytes and not cultured endothelial cells, it is equally important to interrogate whether Glut1-depleted endothelial cells can substitute lactate for glucose as an energy source. If so, then it provides an alternative mechanism for in vivo lactate supplementation rescue in Glut1 mutant mice.

Answer: As suggested, we checked 13C-lactate uptake in siGlut1-treated ECs but could not find significant difference with siControl-treated ECs (Rev_Fig 1, right). However, those experiment cannot exclude the possibility that Glut1-depleted ECs can substitute other nutrients (including glutamine, FA and so on…) as an alternative energy source, which the reviewer mentioned above. Although it would be interesting to see how EC metabolism adapts in Glut1- or glucose-deficient condition, we feel this would be out of scope for this study that is focused on EC-pericyte interactions and not on EC metabolism per se. And please understand that expanding this study on EC metabolism in glut1-deficient condition would exceed the limit of figure numbers and timeline for revision (90 days).



3) The MCT1 inhibitor experiment partially phenocopied the Glut1 mutant mice in that it replicated BBB permeability defects, supporting relevance of MCT1-mediated lactate transport to BBB integrity in vivo. However, the effect of MCT1 inhibitor on mouse survival or overall health was not addressed, which is notable since Glut1 mutant mouse died only within 2 to 3 weeks of induced Glut1 loss in endothelial cells. In other words, by not comparing MCT1 treated mice to Glut1 mutants, we cannot appreciate to what extent MCT1 inhibition phenocopies the effects of glycolysis inhibition in endothelial cells. Here, the mutant control would greatly assist in determining the extent of relevance of MCT1-mediated lactate transport to pericyte-dependent BBB permeability and animal homeostasis.

Answer: Whilst Glut1 the predominant glucose transporter in ECs, there are other MCT members with significant expression in ECs (Fig. 6A). In addition, the MCT1 inhibitor (AZD3965) is currently under clinical trials indicating it was designed with minimal toxicity. We believe this is why MCT1 inhibitor-treated mice display a relatively mild phenotype compared to Glut1^{iECKO} mice. MCT1 inhibition did not show lethality; this is now mentioned in the text (lines 324-325) as suggested.

4-5) Additionally, left unaddressed is the mechanism of reduced pericyte coverage. Is there impaired expansion of pericytes? Reduced survival? Impaired interaction with endothelial cells? By what mechanism could loss of lactate release disrupt pericyte coverage? Could it be through impaired bioenergetic homeostasis? Are pericytes dependent on endothelial-derived lactate for ATP-production? Does reduced ATP production in pericyte decrease pericyte coverage? Could other carbons sources (i.e. glucose) substitute for lactate in mouse/human brain pericytes? The authors demonstrate that lactate feeds pericytes, but they don't show whether or not pericytes are dependent on it, and if so, to what extent.

Answer: We agree that the mechanistic details of how pericyte coverage is affected is important. Our data suggest that the lack of endothelial lactate results in reduced energy generation by pericytes and, likely, other metabolic abnormalities. For instance, we confirmed that lactate is one of major source of ATP production (Fig 3D), TCA metabolites and some amino acids (Fig 5) in pericytes. However, we are not

sure whether it is possible to address this by studying metabolism in vivo in vivo due a number of technical issues including extremely fast turnover of TCA metabolism. As suggested , we checked apoptotic marker and found apoptotic pericytes in Glut1^{iECKO} mice indicating that impaired pericyte survival leads to reduced pericyte coverage (Fig. 1G).

6) While I appreciate the in-depth characterization of the lactate transporters in both endothelial cells and pericytes, the loss of pericytes phenotype with MCT1 inhibition in vivo was not complemented with an MCT12 inhibition/deletion experimental design in vivo or in vitro. If lactate influx to pericytes is essential for survival, reduced lactate metabolism and subsequent cell death should be expected with MCT12 loss even when extracellular lactate is supplemented.

Answer: We certainly agree. Given that there are no MCT12 inhibitors, an MCT12 floxed animal is necessary to carry out this experiment. Unfortunately, no such mice exist. While we are trying to generate them, the process has been very slow due to pandemic-induced disruptions. Our in vitro data clearly shows that MCT12 knockdown inhibits lactate uptake in pericyte (Fig. 7F) and also lactate shuttling in coculture condition (Fig. 7G-H).

7) the abbreviations should have been named/denoted in the beginning of the paper.
In the results and discussion, some sentences needed clarity:
(#120 - This may be referring to the 2-DG treatment from the context of that paragraph and previous indications, while the sentence does not clarify. #412 - This sentence is not clear, how is the ketogenic diet not working?. Also, during the FRET experiment texts,Venus is repeatedly written as Venous. #240 and #284 subheadings are also not very clear and falls short in reflecting the overall working hypothesis/statement of the following results section #390 should be :have not established, a typo that changes the meaning)

Answer: We added an abbreviation section and removed typos.

8) While the authors cite Veys et al, 2020 and another study which showed no deficit in EC migration upon Glut1-loss conflicting with data presented in this manuscript, they provide no discussion on why this may have been the case. Another discussion point worth mentioning is the lack of amelioration of lethality observed in GLUT1-ECKO with lactate supplementation in vivo as well as the seemingly lack of the lethality phenotype in the MCT1-inhibition in vivo (data not shown/mentioned).

Answer: As we already mentioned in our manuscript, there are conflicting result regarding the migration of Glut1 deficient ECs. Veys et al. used a chemical inhibitor (BAY-876) while we used Glut1 specific siRNA. We believe the discrepancy of results here might be due to nonspecific effects of BAY-876. Retinal vasculature has been the 'gold standard' for assessing endothelial behavior. In agreement with our in vitro data with siRNA, analysis of retinal vasculature in Glut1^{iECKO} mice displayed delayed angiogenic outgrowth (supple Fig 5A-B) and reduced number of filopodia in tip ECs (data not shown in the manuscript). However, the conflict regarding EC migration is a bit out of the scope of this study, so we just briefly mentioned in line 144-150.
While lactate supplementation significantly increased CSF lactate level, it did not restore the CSF lactate level to normal range. The lower lactate level might be the reason of incomplete pericyte coverage rescue in the lactate supplementation experiment. In addition, it is known that CSF glucose level is lower than normal range in GLUT1 syndrome patients. In agreement with this clinical observation, CSF glucose level in Glut1^{iECKO} mice is also lower than WT control (Fig. S10). Given that insufficient CSF glucose leads to the loss of neuronal cells in Glut1 deficient animals (PMID: 25730668), lactate supplementation alone does not rescue the lethality of Glut1^{iECKO} phenotype as the latter is likely due to neuronal defects. As suggested we expanded our discussion for lactate supplementation experiment (line 413-418).

Referee #3:

Summary: In the manuscript, "Endothelium-derived lactate is required for normal pericyte function and the blood-brain barrier maintenance", Lee and colleagues investigate functional roles of endothelial-derived lactate - the end product of glycolytic metabolism. Endothelial cells are known to be highly glycolytic, secreting high amounts of lactate into the extracellular environment, providing the rationale for this work.

The authors report that vascular mural cells, namely pericytes, use lactate secreted from endothelial cells for energy and biomass generation. Disrupting this "metabolic communication", e.g. by inactivating the glucose transporter GLUT1 in endothelial cells, leads to pericyte dropout, blood-brain-barrier defects and pathological vascular permeability. The authors further report that supplementing mice with lactate in this setting is sufficient to mitigate some of these phenotypes.

General comment: Understanding mechanisms of metabolic communication between cell types is a timely and relevant area of research that is interesting for the journal's wide readership. Here, Lee et al. provide novel insights into how such metabolic communication can affect the development and function of the blood-brain barrier.

The manuscript is well written and uses both in vivo and (elegant) in vitro approaches to show that endothelial-derived lactate is an important metabolic substrate for pericytes. While I am generally enthusiastic about this work, some points require further investigation/clarification. These points are detailed below.

Specific points:
* The authors propose that in the absence of endothelium-derived lactate, pericytes fall off the CNS vasculature resulting in impaired BBB function. However, the increased permeability could also result from other, more simplistic effects that the authors did not consider. For instance, changes in endothelial survival upon GLUT1 loss could also influence the integrity of the CNS vasculature. It will not be surprising if changes in the main energy-generating pathway in endothelial cells cause significant survival issues. Therefore, the authors should look at endothelial cell death in vitro and in vivo in some more detail.

Answer: We appreciate the reviewer's comment. As shown in Supple Fig 3A, Glut1-deficiency inhibit EC proliferation in highly proliferating ECs. However, ECs in adult stages enter quiescent stage and stop proliferation (PMID: 34474596). As fully adult mice were used in this study (2 months-old mice), the phenotype of Glut1^{iECKO} mice might not be due to the proliferation issue. In addition, we could not find any difference in brain vascular density between Glut1^{iECKO} and WT (Fig 1C,G,I and J)), suggesting it is not from EC survival issue.

* Along these lines, the junctional organization in the endothelial-specific GLUT1 knockout mice needs to be investigated. Staining retinal or brain samples for junctional markers (e.g., VE-cadherin or Claudin-5) should reveal whether a major junctional effect can be excluded in GLUT1 mutant mice. What happens to the pericytes following endothelial GLUT1 inactivation? Do they de-differentiate, migrate away or die off?

Answer: Our in-vitro permeability data indicates that the loss of Glut1 in ECs does not affect EC permeability (supple Fig 7). However, when pericyte coverage is impaired in Glut1^{iECKO} mice, we found the gap junction (Cldn5) expression in EC is reduced (Fig. 1F) indicating that the loss of pericyte leads to altered BBB permeability. As suggested, we checked apoptotic marker in the brain of Glut1^{iECKO} mice and found apoptotic pericytes indicating pericytes undergo apoptotic cell death in Glut1^{iECKO} mice (Fig 1G).

* The co-culture experiments of Figure 2C and the following are difficult to interpret because the glycolysis inhibitor in HBVPs might just be washed out after 48 hours post-treatment. It would be important to show that idoacetic acid treatment is active for such a long time, even the compound is no longer in the cell culture medium.

Answer: We are sorry for the ambiguous description of this experiment. We used the glycolysis inhibitor just for the validation of the FRET system in pericytes. This was done to minimize the basal lactate production via glycolysis and to check the lactate dose-dependent response of the FRET reporter. For the coculture experiment itself, cells were not treated with the glycolysis inhibitor.

* The authors should also compare the uptake and glycolytic breakdown of glucose between endothelial cells and pericytes in vitro. Such studies might give clues why pericytes use lactate as a metabolic resource.

Answer: Carbon tracing in ECs is already established in other paper (PMID: 28659379). Instead, we compared glucose metabolism between pericytes and smooth muscle cells in Fig7I.

Additional points:
* Does endothelial GLUT1 or MCT1/5 depletion lead to compensatory changes in the expression of the other transporters?

Answer: Given that other 14 family GLUT members are either not expressed in ECs or expressed at very low levels (Supple Fig1A) and our results that show that glucose uptake is significantly inhibited in siGlut1-treated ECs (Supple Fig2 B and D), we do not expect any significant compensatory changes in response to the loss of GLUT1. Likewise, siMCT1 and MCT5 knockdown significantly reduced lactate uptake in pericytes (Fig 6F and H), so we expect that the effect from compensatory changes might not be significant.

* Line 71 and following: "Endothelial cell do not utilize lactate to any meaningful extent ...". This is a quite general and bold statement and I am not sure whether this is true. As a minimum, the authors should provide a reference for this statement.

Answer: Thank you. We agree with reviewer's comment and have modified the text accordingly.

* Figure 1A: The (10x) overview images in this panel do not really help. Hard to see pericytes at this low magnification. In addition to the high magnification (63x) images on the right, an image of intermediate magnification (e.g. 20x) would help. Are capillaries and venules affected in the same manner?

Answer: As suggested, we replaced 10X images with 20X. The pericyte coverage in artery and capillary is also decreased in Glut1^{iECKO} mice. (Fig1A)

* Figure S1B,C: Higher magnification images would be beneficial to demonstrate the colocalization clearly. Do pericytes express Glut1 too?

Answer: Many scRNAseq database also indicate that GLUT1 is EC-specific glucose transporter in brain vasculature. (PMID: 32059779 and PMID: 29443965)

* Figure 1C,D: How does the retinal and brain vasculature of the adult GLUT1 mutant mice look like ten days after induction with tamoxifen?

Answer: We could not find any differences in vascular density or morphology when we delete GLUT1 in adult stages (Fig. 1C, D, I and J), but only the change in pericyte coverage. However, when we delete GLUT1 during postnatal development (P1 and P2), we could see reduced vascular outgrowth in the retinal vasculature (Supple Fig. 5)

* Figure 2E: The lactate secreted by endothelial cells should be freely diffusible in the culture medium. So I am surprised to see that only pericytes close to endothelial cells show activity of the lactate reporter.

Answer: We agree with reviewer's comment. This may reflect higher local concentration due to some sequestration process or be due to an extracellular communication between MCT1 and MCT5.

* The pericyte loss in Glut1 iECKO mice could also be caused by more indirect effects on other types of the CNS. For instance, metabolic changes in astrocytes or neurons due to impaired glucose delivery to these cells could also harm pericytes. This possibility should also be discussed.

Answer: As shown in supple Fig 10, CSF glucose level is also lowered in Glut1^{iECKO} mice. Yet lactate supplementation only rescued the pericyte coverage, but not the lethality, suggesting that the latter is largely driven by neuron-toxic effects of low brain glucose levels. As suggested, we expanded our discussion in line 413-418.

* Figure S2E: the error bars are missing in this graph. Also, how can 2DG block glycolytic metabolism if it is not taken up GLUT1-deficient ECs? The knockdown of GLUT1 looks efficient in the immunoblots.

Answer: As mentioned in the updated figure legend, higher concentration of 2-DG (100mM) was used to block glycolysis while glucose was added at 5mM.

* Line 115 and following: It is expected that cells increase glycolysis after glucose addition. This does not necessarily show that ECs have "strong" glycolytic activity.

Answer: We revised the sentence as suggested.

* Line 144 and following: I am confused about this statement. Not sure which Cre line they are refer

Answer: Thank you. Corrected.

Dear Dr Simons,

Thank you for submitting your revised manuscript (EMBOJ-2021-109890R) to The EMBO Journal. Your amended study was sent back to the three referees for re-evaluation, and we have received comments from all of them, which I enclose below. As you will see, the referees stated that the issues raised earlier have been adequately addressed and they are now broadly in favour of publication, pending minor revision.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal.

Please consider the remaining points of referees #1 and #3 carefully, and address these by either adding complementary data or introducing caveats where appropriate.

In addition, we need you to take care of a number of issues related to formatting and data representation as detailed below, which should be addressed at re-submission.

Please contact me at any time if you have additional questions related to below points.

As you might have noted on our web page, every paper at the EMBO Journal now includes a 'Synopsis', displayed on the html and freely accessible to all readers. The synopsis includes a 'model' figure as well as 2-5 one-short-sentence bullet points that summarize the article. I would appreciate if you could provide this figure and the bullet points.

Thank you for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to your final revision.

Again, please contact me at any time if you need any help or have further questions.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck PhD
Senior Editor
The EMBO Journal

Formatting changes required for the revised version of the manuscript:

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>> Remove the abbreviations list from the manuscript and introduce abbreviations in the running text at first appearance.

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>> Add a ToC on the first page of the 'Appendix' file; rename figures to "Appendix Figure S1" etc. .

>> Dataset EV legends: please recheck that the movie plays and rename to "Movie EV1"; it needs its legend zipped.

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Referee #1:

The revised Lee et al. study is improved and the authors have dealt with the criticisms in a reasonable manner. The issue with the contrasting data to Veys et al is also resolved. Possibly, the short term study referred to as data not shown (line 433) could be shown but this is an editorial decision. I have no further comments.

Referee #2:

Through their study, Lee et al. demonstrate that reduced lactate production and export by endothelial cells (EC) diminishes pericyte coverage of the brain vasculature and compromises BBB permeability, which findings support an intricate dependency in pericytes for EC derived lactate. As previous stated in the first review, these findings are exciting as they indicate an elegant metabolic interrelationship that supports the homeostasis of brain vasculature.

As for the earlier concerns, the authors kindly provided various helpful and satisfactory responses

Regarding point 1, that the in vivo lactate study lacked a WT reference, the authors provided WT controls, by which the reviewer can now appreciate that for some specimens, lactate supplementation restored pericyte coverage and BBB permeability to WT levels, reinforcing the relevance of in vivo exogenous lactate supply to pericyte-mediated BBB integrity (fig 8 B, C). The partial restoration in the majority of specimens could perhaps be explained by the finding that the lactate supplementation couldn't fully restore CFS lactate levels (fig 8 D). Moreover, by bringing attention to the observation that CSF glucose levels are significantly depleted in Glut1iECKO mutants, the authors also help answer why lactate supplementation could not rescue lethality despite improving BBB integrity.

As for the answer to point 2, it is appreciated that the authors investigated the uptake of lactate in GLUT1 knockdown ECs, demonstrating no elevated lactate uptake that could compensate for energy deficits in GLUT1-deficient ECs. This reinforces the premise that the in vivo lactate rescue was through supplementation in pericytes rather than through rescue of ATP homeostasis in Glut1-deficient ECs.

For point 3, the answer is well taken that other MCT transporters exist in ECs that could compensate for MCT1 inhibition, thus explaining the milder phenotype in AZD3965 treated mice.

For points 4 and 5, the limitations in the current technology for studying in vivo the mechanisms related to dysmetabolism at a cellular resolution are understandable, however, perhaps bulk RNA sequencing of isolated pericytes or single-cell RNA sequencing of dissociated brain from Glut1iECKO mutants would help elucidate candidate molecular pathways involved. However, given the work already done and the scope of the study, this is merely a suggestion for future studies and is not required for this manuscript.

Referee #3:

In their manuscript "Endothelium-derived lactate is required for normal pericyte function and the blood-brain barrier maintenance", Lee and colleagues provide additional data and explanations, which address the majority of my previous comments.

The only remaining issue is the potential cell death phenotype in endothelial cells of the GLUT1 mutants. This aspect is only indirectly covered in the revised manuscript text and the images in Figure I also do not really help to resolve this point. The authors report that the cleaved Caspase 3-positive cells in the GLUT1 mutant are pericytes. However, at the given magnification and resolution, it is not really possible to distinguish whether the signal comes from pericytes, endpothelial cells or both. Higher magnification images should clarify this point.

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As suggested, we included in-vivo data in the revised manuscript. (Appendix Figure S3 G and H).

Referee #2:

Through their study, Lee et al. demonstrate that reduced lactate production and export by endothelial cells (EC) diminishes pericyte coverage of the brain vasculature and compromises BBB permeability, which findings support an intricate dependency in pericytes for EC derived lactate. As previous stated in the first review, these findings are exciting as they indicate an elegant metabolic interrelationship that supports the homeostasis of brain vasculature.

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We replaced the confocal images with higher magnification. To distinguish endothelial cells which have elongated nuclei , we included DAPI staining in the revised manuscript.

Dear Dr Simons,

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended work and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

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Kind regards,

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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 $\chi 2$ 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?	For in vivo experiment, we tried to have more than 3 sample sizes. For in-vitro experiment, we tried to have maximum number of sample size with lowest expenses.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	YES
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	NA
For animal studies, include a statement about randomization even if no randomization was used.	No randomation was used
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.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	YES, Method for quantification is stated in method section.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	NA

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

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

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
https://osp.od.nih.gov/biosafety-biosecurity-and-emerging-biotechnology/
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

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).	YES, tthey are included in Methods section
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	YES, tthey are included in Methods section

* 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.	YES, tthey are included in Methods section
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	YES, tthey are included in Methods section
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.	NA
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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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.	NA
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.	NA

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	YES, tthey are included in Methods section
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).	NA
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).	NA
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.	NA

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.	NA