progress in neurobiology

Peer Review Overview

Manuscript Title: "Astrocyte-derived Wnt growth factors are required for endothelial bloodbrain barrier maintenance"

Received	20-Mar-2020
1st Decision	21-Apr-2020
Revision Submitted	28-Aug-2020
Accepted	18-Oct-2020

Decision Letter

Dear Dr Liebner,

Thank you for submitting your manuscript to Progress in Neurobiology.

We have completed our evaluation of your manuscript. The reviewers recommend reconsideration of your manuscript following major revision. We invite you to resubmit your manuscript after addressing the comments below. Please resubmit your revised manuscript by Jun 20, 2020.

When revising your manuscript, please consider all issues mentioned in the reviewers' comments carefully: please outline every change made in response to their comments and provide suitable rebuttals for any comments not addressed. Please note that your revised submission may need to be re-reviewed.

Progress in Neurobiology values your contribution and we look forward to receiving your revised manuscript.

Kind regards,

Aimee Kao
Associate Editor

Sabine Kastner
Editor-in-Chief
Progress in Neurobiology

Editor and Reviewer comments:

Reviewer 1

By deletion of the Evi gene specifically in astrocytes in mice (EviΔAC mice) the authors here provide evidence for a role of astrocytes derived Wnt growth factors in maintaining BBB integrity in adult mice. The EviΔAC mice develop a progressive loss of BBB properties as shown by slight edema formation in the brain accompanied by increased tracer leakage across the BBB. The data provided suggest that this is due to lower pericyte coverage of

brain microvessels, increased expression of caveolin-1 and vesicular trafficking across the BBB rather than significant junctional alterations. In ageing mice the authors finally observe astrocytic end-feet alterations based on expression and distribution of the astrocytic water channel aquaporin-4 (AQP4), concomitant with a loss of end-feet structural integrity and end-feet swelling.

Taken together the study supports a role for astrocyte derived Wnt growth factors in regulating pericyte coverage of CNS microvessels and possible autocrine Wnt/β-catenin signaling in astrocytes to maintain the BBB in adulthood.

This is an elegant study employing state-of-the-art methodology that provides evidence for a role of astrocyte derived Wnt growth factors in maintaining BBB integrity in the adult mouse by affecting different cells of the NVU. The experiments are described in a thorough manner and the data are very convincing.

Nevertheless, there is a number of issues that should be addressed prior to publication of this study.

Overall the authors should elaborate to a larger degree on the specific mouse model employed. Although briefly referred to by the authors, they should elaborate on the possibility that regional differences they observed in the brains of the EviΔAC mice may correlate to the distribution of GFAP positive and negative astrocytes. Furthermore, the authors should discuss the potential role of GFAP expression and thus deletion of the Evi gene in other cells of the CNS, e.g. neuronal progenitors (Garcia et al, Nature Neurosci, 2004).

Also the manuscript should be more precise with respect to referring to the correct age of the mice in which the respective differences have been observed, as several changes became only detectable at 40 weeks of age. Did the authors observe the described changes in male and female mice in a comparable manner?

It would furthermore be interesting to learn if the authors detected any change in the molcular composition of the extracellular matrix, which has been reported to be an essential part of the NVU in EviΔAC versus control mice.

In Figure 2B the relative expression data are normalized to CD31, an endothelial junctional molecule involved in regulating BBB permeability and published to be regulated under neuroinflammatory conditions. It seems thus an inappropriate reference for these studies. The authors should rather consider a more appropriate reference, e.g. beta actin as a reference for the expression data analysis.

It is unexpected that a lower expression of Cdh5 is not accompanied by a significantly lower expression of Cldn5 -although it seems to be expressed at a lower level based on Figure 2B. Is expression of catenins associated with VE-cadherin also not affected?

The IF stainings in Figures 3A and 3B should include a high magnification insert as the lack of change in junctional localization of the VE-cadherin and claudin-5 stainings in EviΔAC mice cannot be appreciated. The authors may also consider to include a method of quantification of the IF stainings or methodology that better highlights the reported lack of change in junctional localization of the IF signal for VE-cadherin and claudin-5 in EviΔAC mice.

With reference to Figure 3C all the authors can say is that EviΔAC mice display morphologically intact tight junctions.

Additional comments:

The first sentence in the introduction attributes the original concept of a blood-brain barrier to Ehrlich and Goldman, which is inappropriate as described in detail in the excellent review of Saunders et al. in Front. Neurosci 2014.

The reference given for the GFAP-Cre mice is not correct. Please add the proper reference.

Reviewer 2

Thank you for this opportunity to review the manuscript titled 'Astrocyte-derived Wnt Growth Factors are required for endothelial Blood-Brain Barrier Maintenance' by Dr. Sylvaine Guérit and colleagues for publication in *Progress in Neurobiology.*

The identification of Wnt-beta-catenin signaling in CNS ECs as a central player in the induction of BBB genesis during embryonic development was a major milestone in BBB research. In particular Prof. Liebner's previous findings demonstrated that inducible and conditional ablation of this signaling pathway after birth (in contrast to ablation from early embryogenesis) could disrupt barrier properties in the vasculature that already acquired barrier properties earlier. This seminal work together with later work from Prof. Nathans group established that Wnt and Norrin signaling is required for continuous maintenance of BBB properties. Moreover, blocking signaling in adults disrupts barrier properties and re-introducing signaling could restore barrier properties. Altogether, this pathway seemed to hold many promising entry points toward barrier manipulations needed for drug delivery or barrier restorative approaches.

The current manuscript therefore aimed at exploring a very important direction of the cellular source for Wnt signaling in adults and in particular the question of astrocyte contribution. I believe that their findings are very surprising and important: they report that '*loss of astrocytic Wnt release progressively impairs endfoot integrity in ageing'*. They also highlight that *'Wnt factor release from astrocytes is crucial for blood-brain barrier maintenance'*. Considering that in homeostatic conditions, astrocytes are suspected to be the main Wnt source, their finding that blocking this astrocytic signaling presents neurovascular abnormalities only in aging is the major finding in my opinion. Making the unusual case where it is important to put on stage and emphasize the negative data (in this case, the lack of very early/dramatic phenotypes), because they will open new questions such as other cellular sources of brain Wnt signaling (maybe unregulated as a compensatory mechanism) or alternatively, compensatory signaling pathways backing up the Wnt pathway.

The manuscript is very well written. It is clear that a lot of carefully designed and well executed research work was done in order to cover many aspects of phenomena. There are several points I think need to be addressed (as pointed below). Most might be addressed in a revised text or additional analyses. I will also suggest some experiments that might make the manuscript stronger but none are essential, as I don't think they will change the conclusions. I think the manuscript merit publication in *Progress in Neurobiology.*

Specific comments:

1. The main mouse genetic tool used in this study, EviΔAC is based on '*Cre expression under the constitutively active, human glial fibrillary acidic protein (GFAP) promoter (Ba Carpenter et al., 2010)*'. The authors must address several questions related to this driver:
    a. GFAP is expressed by radial glia cells, which are known to play a role in BBB development. These cells give rise to neurons and oligodendrocytes early in development and then later shift to produce astrocytes (e.g Kristen B. et al., 2006 *MCN*). In addition the original paper (Bajenaru et al., 2002 *MCB*), indicate that the transgene express in the CNS 'by E14.5' while images in that manuscript show expression even earlier. At minimum authors should discuss the possible implications of such information on blocking Wnt ligand expression in other cell types, unless they could cite works that rejected this possibility.

b. Validation of Cre expression and function with a reporter cross (Figure S2) should be better explained. '*We also confirmed the recombination in the EviΔAC CNS tissue by the co-localization of GFAP (astrocyte intracellular protein) with b-galactosidase, which is expressed in parallel to the Cre-recombinase downstream of an IRES site (Fig. S2B)*'. If I understand this correctly, this assay does not confirm recombination of Eviff, it only shows which cell at the adult CNS is expressing the Cre. This explains why there is dotted appearances in S2B in contrast to S2A. '*Global AC-specific Cre activity and recombination was confirmed by crossing the hGFAP-Cre to the ROSA26-mTmG reporter line (Muzumdar et al., 2007), suggesting a homogeneous recombination throughout the CNS (Fig. S2A)'.* Again, this confirms CNS specific recombination but not AC specificity – I couldn't find indication for what is the labeling other then green and red of the reporter. Cre activity is clearly CNS specific but why is the green so wide spread covering the entire cortex – could this be accounted for tailing of astrocytic protrusions or might this be indications for linage tracing for all the radial glia progenies? In the GFAP-Cre+:mTmG image you could find red patterns of vasculature – does this mean that only non-ectodermal cells (like ECs) are not recombining?

c. Finally, if indeed it is possible that EviΔ is radial glia + astrocytic, does this makes the lack of dramatic phenotype even more surprising? Alternatively, if indeed EviΔ is only astrocytic, GFAP is known to be constitutively expressed only by some AC population (like hippocampal ACs) and up regulated in activated ACs (e.g cortical ACs). Would that explain the aging phenotype? Is there any indication for GFAP activation in aging?

2. '*Electron microscopy revealed significant swelling of astrocytic end feet in EviΔAC mice, suggesting a cell-autonomous role of astrocytic Wnt factors via an autocrine pathway'.* I suggest not excluding other possible options such as AC end-feet swelling as a secondary response or a cross talk with affected ECs (consider discussing).

3. *In vivo* hyper-permeability in this study in tested with whole tissue extraction of tracers. Indeed *in vitro* permeability testing further substantiates the findings. I think these are sufficient to support the conclusions. Nevertheless, evidences for hyperpermeability with microscopy could make the argument much stronger. This is relevant to use of fluorescent tracers but is even more relevant to use of HRP with EM. Figure 3C shows normal TJ ultrastructure but HRP would help solidify normal TJ function. Similarly figure 6A shows vesicles but HRP could demonstrate active vesicular transport. Suggested experiments might make the manuscript stronger but none are essential.

4. With no HRP-EM, I suggest modifying the terms transcytosis/vesicular transport into something less definitive such as elevated vesicular abundance/activity. I suggest also adding to Figure 6B quantifications of luminal/abluminal pits, as they are also good indicators for the transcytotic path. Finally, quantifications of vesicles/vessel might be better presented as vesicle/EC cytoplasm area (or membrane length).

5. Previous studies (including by the authors) used some markers to demonstrate modulation of BBB ECs by Wnt signaling LOF and GOF. I suggest analyzing their current data to show in a designated figure how do these markers change in light of the current manipulations and more important provide their hypothesis once these changes do not align with previous findings. Examples for previously used markers are Glut1 (seems up regulated?), Occludin, Cldn5, Cldn3, Plvap (MECA32). One interesting notion arises from Figure 5: one interpretation might be that AC-EC interactions that drive elevated TEER (maybe TJ function) are Wnt independent. This does not align with previous publications claiming that Wnt signaling affect TJ function/TJ protein expression.

6. The authors write that their findings are *'suggesting that the function of AC-derived Wnts could be compensated to a large extent'.* I might have missed this data but I believe it is

possible to analyze the RNA data (Figure 4) to at least test if the vasculature itself might be the source of the compensatory Wnt. Alternatively mine the data and suggest if there are other ACs producing ligands (such as SHH) unregulated. An experiment that might make the manuscript stronger but is not essential; test up-regulation of Wnt ligands in whole brains, or in tissue depleted from vasculature/ACs.

7. Ideally, all the diverse approaches presented here would have been tested in all the time points. *In vitro* ACs are from pups, ECs are from adults. BBB permeability and water content are 10-20 weeks. Other experiments are 10 or 40 weeks. I agree that in some cases it is not critical because a certain hypothesis is better tested in a certain time point. There are some points that might be considered:
    a. I suggest putting a clear labeling of age in all the figures.
    b. Make sure that the conclusions are not affected by not having a certain assay in all time points.
    c. In the discussion the authors indicate that they found no change in Mfsd2a expression but do not specify at what age was this tested. Less pericytes is aligned with some suggestions that pericyte-EC interaction control Mfsd2a expression, which leads to elevated tanscytosis.

Minor comments:

1. It would be informative to explain why *in vivo* Wnt7a/7b are mentioned/tested and in cultures there is use of Wnt3.
2. Line 713 RNA-seq misspelled.
3. Figure 2A – I understand that the brain diagram and the bright field image are shown only to demonstrate the method. Nevertheless, I suggest replacing the bright field image with a different image (the same image already appears in a previous publication – Devarj K. et al., 2016 *JCBFM*).
4. I find it very hard to understand how was the quantification done in figure 7B,C. It is not clear how volume was calculated. In addition in 7A it seems (based on DAPI) that these are not captured from similar cortical regions?
5. I recommend replacing the image representing swollen end-feet in figure 7E. After close examination I believe that if indeed this is an end-foot, it is a very irregular angle. It seems that around the end-foot there is a second basement membrane (typical of a pericyte). In addition, end-feet area (normalized to lumen area) could be quantified.
6. Figure legend 5 – E and F are replaced.
7. I suggest clarifying exactly what n=X are in all experiments. Especially what was the data used for statistical tests. e.g Figure 1 'n=5-6 animals'. How many repetitions of measurements were done for each brain? Did means of each brain was used for the statistical test? If so, does t-test is the best option vs. an a-parametric test? I couldn't find justification for the chosen test either.
8. In Figure 6I, J n=2 and 1. I am not sure how critical data part is, having 6F,G and for the low number of repetition would recommend omitting it.

Author Response Letter

Reviewer 1

By deletion of the Evi gene specifically in astrocytes in mice (EviΔAC mice) the authors here provide evidence for a role of astrocytes derived Wnt growth factors in maintaining BBB integrity in adult mice. The EviΔAC mice develop a progressive loss of BBB properties as shown by slight oedema formation in the brain accompanied by increased tracer leakage across the BBB. The data provided suggest that this is due to lower pericyte coverage of brain microvessels, increased expression of caveolin-1 and vesicular trafficking across the BBB rather than significant junctional alterations. In ageing mice the authors finally observe astrocytic endfeet alterations based on expression and distribution of the astrocytic water channel aquaporin-4 (AQP4), concomitant with a loss of end-feet structural integrity and end-feet swelling.

Taken together the study supports a role for astrocyte derived Wnt growth factors in regulating pericyte coverage of CNS microvessels and possible autocrine Wnt/β-catenin signaling in astrocytes to maintain the BBB in adulthood.

This is an elegant study employing state-of-the-art methodology that provides evidence for a role of astrocyte derived Wnt growth factors in maintaining BBB integrity in the adult mouse by affecting different cells of the NVU. The experiments are described in a thorough manner and the data are very convincing.

Nevertheless, there is a number of issues that should be addressed prior to publication of this study.

Overall the authors should elaborate to a larger degree on the specific mouse model employed. Although briefly referred to by the authors, they should elaborate on the possibility that regional differences they observed in the brains of the EviΔAC mice may correlate to the distribution of GFAP positive and negative astrocytes. Furthermore, the authors should discuss the potential role of GFAP expression and thus deletion of the Evi gene in other cells of the

CNS, e.g. neuronal progenitors (Garcia et al, Nature Neurosci, 2004).

Response: We thank the reviewer for this mindful comment. In the revised manuscript we have added more information to the discussion regarding the specific issues with the hGFAP-Cre mouse line used. Specifically, on Page 28, line 724 we have added the following sentence:

> "Specificity of hGFAP-Cre-mediated recombination for mouse brain astrocytes
>
> Although GFAP is first detectable around 9.5dpc, its robust expression is detected in the mouse brain from 15.5dpc to birth coinciding with the differentiation of astrocytes (Fox et al., 2004; Morita et al., 1997). Therefore, the earlier expression of GFAP is related to other cell types, in particular to radial glia and neural precursor cells (Briona et al., 2015; Casper and McCarthy, 2006). Moreover, it has been shown for a comparable hGFAP-GFP line that the human GFAP promoter presents regional heterogeneity regarding its activity in the mouse brain (Moon et al., 2011). Consequently, hGFAP-mediated deletion of Evi likely takes place also in other cell types and may not be induced in all ACs homogeneously. However, at least in the zebrafish it has been shown for the hypothalamic region that Wnt signalling in radial glia and neural stem and precursor cells is not required for their proliferation and differentiation (Duncan et al., 2016). As we have not observed any gross morphological alterations in the EviΔAC mice, we came to the conclusion that the hGFAP-Cre:Evifl/fl mouse model mainly affects the differentiation and function of astrocytes. Still, it is possible that the EviΔAC mice might exhibit some defects in other cell types and brain regions that we did not investigate. Additionally, the observation that the BBB phenotype aggravated with age might be related to increased GFAP expression in ACs during ageing, which has been shown for the hippocampus of control and senescence-accelerated-prone mice (Wu et al., 2005). Therefore, adult neurogenesis in the subventricular zone (SVZ) (Garcia et al., 2004),

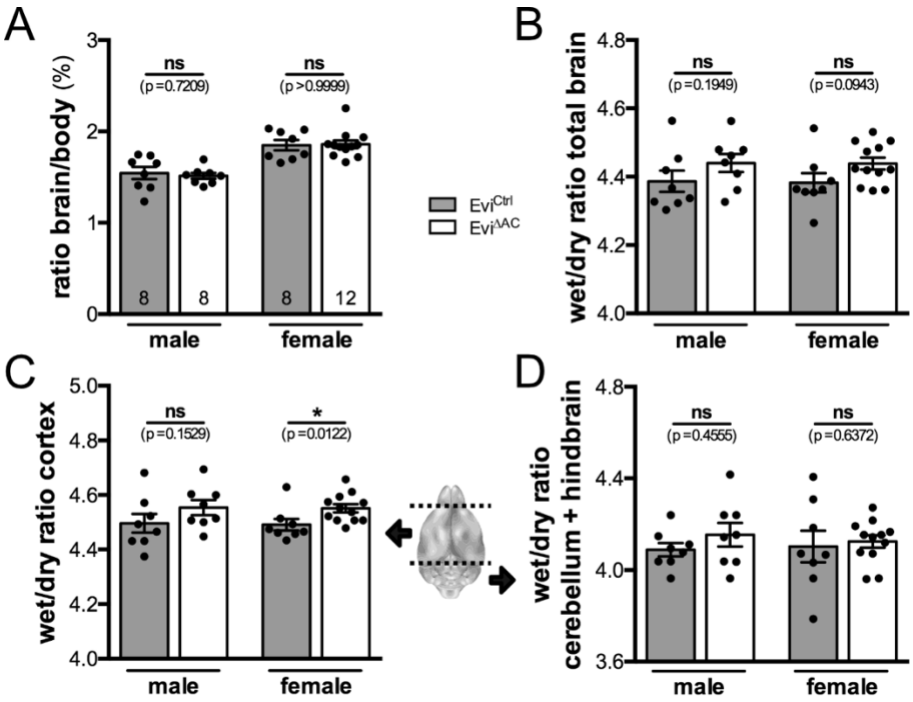
ageingrelated defects (Wu et al., 2005) or conditions of regeneration after injury (Briona et al., 2015), for which GFAP expression and Wnt/β-catenin signalling have been described might be interesting to analyse in the future."

Also the manuscript should be more precise with respect to referring to the correct age of the mice in which the respective differences have been observed, as several changes became only detectable at 40 weeks of age.

Response: We thank the reviewer for this comment. We agree that the analysis of the two different time points might create some confusion. We made an effort to clearly indicate the age of the mice for all data presented and included the age into the Results, Figures and the Material and Method sections where appropriate.

Did the authors observe the described changes in male and female mice in a comparable manner?

Response: Regarding the analysis of male and female mice, we on purpose did not separate mice by gender. However, for the analysis of brain oedema, which contains the largest group of mice, we traced back the gender of the mice (reviewer Figure 1). Both males and females showed a comparable level of brain oedema although the divided groups, due to their reduced number of biological replicates (8 ♂ for both EviCtrl EviΔAC, 12 ♀EviΔAC), showed only a significant increase in oedema formation for the female cortex (reviewer Figure 1C), whereas the male mice showed only a trend of increased oedema. It is of note that the higher brain/body ratio in females is due to their lower body weight.



Reviewer Figure 1: lack of gender-related difference regarding oedema formation in EviΔAC mice.

(A) Ratio brain/body showed no significant differences between EviCtrl and EviΔAC form the same gender (n=8 and 12 respectively). (B) Wet/dry assay of the total brain revealed comparable values in ♂ and ♀ of EviΔAC and EviCtrl mice. (C) In the cortex oedema formation is significantly increased only in ♀ of EviΔAC compared to EviCtrl mice. (D) In the cerebellum + hindbrain no significant oedema increase could be identified in either groups Mann & Whitney test.

These observations are now added to the description of the Results as „(data not shown)„ on Page 19, line 464. Moreover, we discussed the possible gender differences in the Discussion on Page 27, line 718.

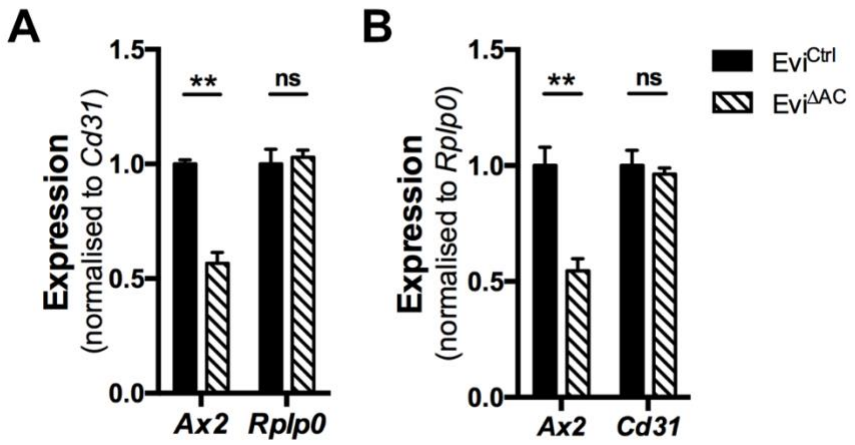
It would furthermore be interesting to learn if the authors detected any change in the molecular composition of the extracellular matrix, which has been reported to be an essential part of the NVU in EviΔAC versus control mice.

Response: We thank the reviewer for bringing up this important question. Beside the collagen IV staining that has already been provided in Figure 3A, and is now shown in higher magnification along with the junctional Cdh5 staining, we have additionally performed stainings for laminin α2 (Lama2; parenchymal, astrocytic BM), laminin α4 (Lama4; vascular constitutive) and laminin α5 (Lama5; vascular differentially regulated). Also for these three markers we could not detect any differential expression and/or localisation. The new data are shown in Figure S6. Furthermore, we describe the data on page 21 line 519 and discuss them on page 31 line 849 as follows:

> "Although endothelial differentiation genes as well as several ECM genes like Lama5 turned out to be upregulated in the EviΔAC mice, staining for Lama5 did not corroborate this on the level of protein distribution in cortical sections of 10- and 40-week-old mice (Fig. S6). Moreover, the angiogenic-like gene profile did not coincide with increased vascular density and branching in various brain regions (Fig. S3)."

In Figure 2B the relative expression data are normalised to CD31, an endothelial junctional molecule involved in regulating BBB permeability and published to be regulated under neuroinflammatory conditions. It seems thus an inappropriate reference for these studies. The authors should rather consider a more appropriate reference, e.g. beta actin as a reference for the expression data analysis.

Response: We thank the reviewer for this comment. In general, we normalised qRT-PCR data to Rplp0, which proofed in our hands not to be regulated in various experimental settings. Due to a lack of sufficient cDNA, as well as of the lack of properly aged mice, we could not repeat all the qRT-PCR analyses with Rplp0 as reference gene. However, we have already had compared Axin2 expression in MBMVs to assure that normalisation to CD31 would not introduce a bias to the results. We have now provided the data in Reviewer Figure 2.



Reviewer Figure 2: normalisation with Cd31 (A) or Rplp0 (B) does not influence the results of qRT-PCR on MBMV (40-week-old animals; n=3).

The normalisation by CD31 aimed to estimate the content of endothelial cells. Moreover, despite the fact that the FACS sorting was carried out by staining for CD31, the number of sequenced endothelial cells was comparable between EviΔAC and EviCtrl mice, suggesting that we did not introduce a bias with regard to the expression profiling of MBMV.

It is unexpected that a lower expression of Cdh5 is not accompanied by a significantly lower expression of Cldn5-although it seems to be expressed at a lower level based on Figure 2B. Is expression of catenins associated with VE-cadherin also not affected?

Response: We thank the reviewer for this comment. Indeed the expression of Cdh5 appears to be puzzling given that it appeared to be downregulated in the qRT-PCR analysis of the isolated microvessels (MBMVs) specifically in the 40w samples, whereas it turned out to be slightly, but significantly up-regulated in the FACS sorted endothelial cells. We interpret these seemingly contradictory data as a consequence of the different isolation protocols for microvessels, containing cells of the entire vascular unit, comprising at least PCs and ACs, and the FACS sorting for ECs only by CD31 that additionally takes more time until RNA can be harvested. Therefore, the RNA from other cell types than ECs might influence the relative expression of a particular gene like Cdh5. A clear conclusion of gene regulation can only be drawn if a gene is regulated the same way in both isolation techniques. If not, and this is unfortunately the case for Cdh5, it is difficult to interpret the data regarding the mRNA expression. However, the protein expression characterised by western blot analysis supports a slight, but significant downregulation of Cdh5 that in turn was not visible by immunostaining and junction quantification not revealing obvious morphological alteration (Figs. 3, S4 and S5). It should be noted that Cdh5 was shown to be less expressed in the mature as opposed to the developing BBB (Breier et al., 1996), suggesting that the lower expression in the EviΔAC mice might not necessarily relate to lower Cldn5 expression.

As mentioned by the reviewer, what we can say is that the junctions are not significantly altered regarding their morphological structure. Consequently, we have adapted the main text where necessary in order not to over-interpret the data.

Regarding the expression of catenins we have had included β-catenin in Fig.2C. Now we have analysed the MACE RNA-Seq data and could not identify any regulation for β-catenin, given that respective reads were not faithfully annotated to the β-catenin gene. Nor did we identify any regulation for γ-catenin/plakoglobin.

The IF stainings in Figures 3A and 3B should include a high magnification insert as the lack of change in junctional localisation of the VE-cadherin and claudin-5 stainings in EviΔAC mice cannot be appreciated. The authors may also consider to include a method of quantification of the IF stainings or methodology that better highlights the reported lack of change in junctional localisation of the IF signal for VE-cadherin and claudin-5 in EviΔAC mice.

Response: We thank the reviewer for this comment. We have now exchanged the images in Figures 3A and 3B with higher magnifications of the junctional stainings for VE-cadherin and claudin-5. We omitted the lower magnification, as we felt that they would not add further information to this data set. Additionally, we have now added information on the quantification of the junctional stainings. This has now been added to the Material and Methods section (Page 11, line 228), the Supplementary Figure S5, into the Results (Page 21, line 512) as well as to the Discussion section (Page 30, line 808).

With reference to Figure 3C all the authors can say is that EviΔAC mice display morphologically intact tight junctions.

Response: Yes, this is indeed the major take home message of Figure 3 and S5. This interpretation has now been added explicitly to the text in the Discussion section (Page 30, line 810).

Additional comments:

The first sentence in the introduction attributes the original concept of a blood-brain barrier to Ehrlich and Goldman, which is inappropriate as described in detail in the excellent review of Saunders et al. in Front. Neurosci 2014.

Response: We thank the reviewer for this comment. We now have changed the introduction (Page 3, line 54) as follows:

> "Since the establishment of the blood-brain barrier (BBB) concept by Max Lewandowsky (1900) and after coining the "BBB" term by Lina Stern and Raymond Gautier (1922) (reviewed by (Saunders et al., 2014)), astrocytes (ACs) at the neurovascular unit (NVU) have been considered as a major source of barrier-promoting factors (Cheslow and Alvarez, 2016; Liebner et al., 2011)."

The reference given for the GFAP-Cre mice is not correct. Please add the proper reference.

Response: We acknowledge the comment by the reviewer regarding the correct reference for the hGFAP-Cre line, generated by David H. Gutman. However, we are convinced that the generation of the hGFAP-Cre line is indeed described in the paper by „*Bajenaru, ..., Gutmann DH. Astrocyte-specific inactivation of the neurofibromatosis 1 gene (NF1) is insufficient for astrocytoma formation. Mol Cell Biol. 2002 Jul;22(14):5100–13*". This is also the reference that is consistently cited on repositories for these transgenic mice. In the Material and Methods section of the paper the generation of the mouse line is described as follows:

> "*GFAP-Cre-IRES-LacZ transgenic mice (GFAP-Cre mice) were generated by pronuclear injection of the purified, linearized 8-kbp GFAP-Cre-internal ribosome entry site (IRES)-lacZ DNA fragment into C57BL/6 CBA fertilized mouse eggs by using standard techniques in the Neuroscience Transgenic Facility at the Washington University School of Medicine. The transgene construct consists of the 2.2-kb fragment of the human glial fibrillary acidic protein (GFAP) promoter (Gfa2) (obtained*

from M. Brenner, National Institute of Neurological Disorders and Stroke) (9), the encephalomyocarditis virus IRES, the cDNA encoding the nucleus-targeted Cre recombinase (provided by J. Milbrandt, Washington University, St. Louis, Mo.) (26), and the cDNA encoding a nucleus-targeted LacZ, followed by the simian virus 40 polyadenylation signal (provided by A. Nagy, Mount Sinai Hospital, Toronto, Canada). The IRES-LacZ allows for cotranslation of the Cre and LacZ proteins from the same mRNA and permits indirect detection of the Cre expression pattern in vivo."

Moreover, in the introduction we have added the correct reference for the GFAP-Cre driver line to the reference of the Eviflox/flox line, Page 4, line 99:

"In order to tackle this question, we have conditionally deleted the Wnt secretion mediator *wntless* (Wls), also known as *evenness interrupted* (Evi) or Gpr177, in ACs (Bänziger et al., 2006). Cre recombinase expression was driven under the constitutively active human glial fibrillary acidic protein (hGFAP) promoter, leading to the deletion of the floxed Evi gene (Bajenaru et al., 2002; Carpenter et al., 2010)."

If doubt about the correct reference remains, we would greatly appreciate if the reviewer could provide any information on the alternative and correct reference.

Reviewer 2

Specific comments:

1. *The main mouse genetic tool used in this study, EviΔAC is based on 'Cre expression under the constitutively active, human glial fibrillary acidic protein (GFAP) promoter (Ba Carpenter et al., 2010)'. The authors must address several questions related to this driver:*
    a. *GFAP is expressed by radial glia cells, which are known to play a role in BBB development. These cells give rise to neurons and oligodendrocytes early in development and then later shift to produce astrocytes (e.g Kristen B. et al., 2006 MCN). In addition the original paper (Bajenaru et al., 2002 MCB), indicate that the transgene express in the CNS 'by E14.5' while images in that manuscript show expression even earlier. At minimum authors should discuss the possible implications of such information on blocking Wnt ligand expression in other cell types, unless they could cite works that rejected this possibility.*

Response: We thank the reviewer for this mindful comment. In the revised manuscript we have added more information to the discussion regarding the specific issues with the hGFAP-Cre mouse line used in this manuscript. As mentioned in the response to Reviewer #1, we have added the following section on Page 28, line 724:

"Specificity of hGFAP-Cre-mediated recombination for mouse brain astrocytes

Although GFAP is first detectable around 9.5dpc, its robust expression is detected in the mouse brain from 15.5dpc to birth coinciding with the differentiation of astrocytes (Fox et al., 2004; Morita et al., 1997). Therefore, the earlier expression of GFAP is related to other cell types, in particular to radial glia and neural precursor cells (Briona et al., 2015; Casper and McCarthy, 2006). Moreover, it has been shown for a comparable hGFAP-GFP line that the human GFAP promoter presents regional heterogeneity regarding its activity in the mouse brain (Moon et al., 2011). Consequently, hGFAP-mediated deletion of Evi likely takes place also in other cell types and may not be induced in all ACs homogeneously. However, at least in the zebrafish it has been shown for the hypothalamic region that Wnt signalling in radial glia and neural stem and precursor cells is not required for their proliferation and differentiation (Duncan et al., 2016). As we have not observed any gross morphological alterations in the EviΔAC mice, we came to the conclusion that the

hGFAP-Cre:Evifl/fl mouse model mainly affects the differentiation and function of astrocytes. Still, it is possible that the EviΔAC mice might exhibit some defects in other cell types and brain regions that we did not investigate. Additionally, the observation that the BBB phenotype aggravated with age might be related to increased GFAP expression in ACs during ageing, which has been shown for the hippocampus of control and senescence-accelerated-prone mice (Wu et al., 2005). Therefore, adult neurogenesis in the subventricular zone (SVZ) (Garcia et al., 2004), ageing related defects (Wu et al., 2005) or conditions of regeneration after injury (Briona et al., 2015), for which GFAP expression and Wnt/β-catenin signalling have been described might be interesting to analyse in the future."

b. *Validation of Cre expression and function with a reporter cross (Figure S2) should be better explained. 'We also confirmed the recombination in the EviΔAC CNS tissue by the co-localization of GFAP (astrocyte intracellular protein) with β-galactosidase, which is expressed in parallel to the Cre-recombinase downstream of an IRES site (Fig. S2B)'. If I understand this correctly, this assay does not confirm recombination of Evif/f, it only shows which cell at the adult CNS is expressing the Cre. This explains why there is dotted appearances in S2B in contrast to S2A.*

Response: We thank the reviewer for bringing up this issue. Indeed, the hG-FAP-Cre mouse was designed to express β-galactosidase along with the Cre recombinase (see response to Reviewer #1), as it is located behind an internal ribosomal entry side (IRES) under the same hGFAP promotor. Therefore, the reviewer is completely correct that we can only document Cre expression, rather than recombination by showing the staining for β-glalctosidase in Figure S2B. We have rephrased the sentence on Page 18, line 432 as follows:

"Although we could not directly detect recombination by staining for Evi due to the lack of suitable antibodies, we confirmed the astrocytic recombination in the EviΔAC CNS tissue by the colocalization of GFAP (astrocyte intracellular protein) with β-galactosidase, which is expressed in parallel to the Cre-recombinase downstream of an IRES site (Fig. S2B) (Bajenaru et al., 2002)."

Global AC-specific Cre activity and recombination was confirmed by crossing the hGFAP-Cre to the ROSA26-mTmG reporter line (Muzumdar et al., 2007), suggesting a homogeneous recombination throughout the CNS (Fig. S2A)'. Again, this confirms CNS specific recombination but not AC specificity – I couldn't find indication for what is the labeling other then green and red of the reporter. Cre activity is clearly CNS specific but why is the green so wide spread covering the entire cortex – could this be accounted for tailing of astrocytic protrusions or might this be indications for linage tracing for all the radial glia progenies? In the GFAP-Cre+:mTmG image you could find red patterns of vasculature – does this mean that only non-ectodermal cells (like ECs) are not recombining?

Response: We thank the reviewer for this important comment. As Cre activation downstream of the hGFAP promoter and recombination is an irreversible event, the analysis that we performed on the ROSA26-mTmG mice at the experimental endpoint, indicates the sum of all recombined cells. This might explain why the entire brain in the adult mouse appears GFP+. Moreover, GFP in recombined cells is located in the cytoplasm and hence the signal is rather diffuse instead of distinct. We now have clearly indicated the staining for GFAP(white), GFP and DAPI in Figure S2A showing GFAP+/GFP+ astrocytes. It should be noted, that specifically the GFAP staining, due to the characteristics of the antibody, faithfully works only at the tissue borders of this particular thick section due to penetration issues. In Figure S2B the anti-β-galactosidase staining indicates those cells that have expressed the Cre recombinase at the endpoint of the experiment. We now have added an inset in Figure S2B showing a GFAP+/β-gal+ astrocyte. In the revised manuscript we have added more information to the discussion regarding the specific issues with the GFAP-Cre mouse line

used. Specifically, on Page 28, line 724 we have added the following section "Specificity of hGFAP-Cre-mediated recombination for mouse brain astrocytes", as already provided in the response to Reviewer #1 and #2, comment 1a.

c. *Finally, if indeed it is possible that EviΔ is radial glia + astrocytic, does this makes the lack of dramatic phenotype even more surprising? Alternatively, if indeed EviΔ is only astrocytic, GFAP is known to be constitutively expressed only by some AC population (like hippocampal ACs) and up regulated in activated ACs (e.g cortical ACs). Would that explain the ageing phenotype? Is there any indication for GFAP activation in ageing?*

Response: We thank the reviewer for this mindful comment. In the revised manuscript we have added more information to the discussion regarding the ageand site-specific GFAP expression that would explain the phenotype of the EviΔAC mice. Specifically, on Page 28, line 741 we have added the section as mentioned for the previous point of the reviewer.

2. *'Electron microscopy revealed significant swelling of astrocytic end feet in EviΔAC mice, suggesting a cell-autonomous role of astrocytic Wnt factors via an autocrine pathway'. I suggest not excluding other possible options such as AC end-feet swelling as a secondary response or a cross talk with affected ECs (consider discussing).*

Response: We thank the reviewer for this comment. Indeed we never intended to exclude any of the possible options, contributing to the endfoot phenotype that we observed in the EviΔAC mice. In order to clarify this, we have added the following sentence on Page 4, line 116:

> "Moreover, electron microscopy revealed significant swelling of astrocytic end feet in EviΔAC mice, suggesting reciprocal effects between ECs and ACs or a potential cell-autonomous role of astrocytic Wnt factors via an autocrine pathway."

Also, we have discussed this topic more extensively on Page 34, line 924:

> "Ultimately, our findings may support the interpretation that the endfeet phenotype in astrocytes results from the reciprocal interactions between the cells of the NVU specifically, between ECs, PCs and ACs. Moreover, ACs may require autocrine Wnt signals to maintain end-feet integrity and consequently support BBB function in ECs at the NVU. This interpretation is supported by the observation that ACs express almost the entire set of frizzled receptors and Lrp co-receptors (Fig. S1) and that the Wnt target gene Axin2 and Nkd1 were downregulated in ACs from EviΔAC mice *in vitro* (Fig. 7F and G)."

3. *In vivo hyper-permeability in this study is tested with whole tissue extraction of tracers. Indeed in vitro permeability testing further substantiates the findings. I think these are sufficient to support the conclusions. Nevertheless, evidence for hyper-permeability with microscopy could make the argument much stronger. This is relevant to use of fluorescent tracers but is even more relevant to use of HRP with EM. Figure 3C shows normal TJ ultrastructure but HRP would help solidify normal TJ function. Similarly figure 6A shows vesicles but HRP could demonstrate active vesicular transport. Suggested experiments might make the manuscript stronger but none are essential.*

Response: We thank the reviewer for these comments and suggestions. We indeed would have liked to perform the HRP EM tracer experiment, but we currently have no ethic approval for the electron dense tracer. Unfortunately, due to the SARS-CoV2 pandemic, reviewing and approval times dramatically increased and we would not have been able to complete the experiments in time. However, we have performed a permeability assay using the fluorescent tracer Alexa Fluor® 555 Cadaverine (~1kDa) and documented leakage by IF

microscopy. This experiment confirmed the increased permeability in EviΔAC mice, which is shown in Figure 1J of the revised manuscript and described on Page 19, line 472:

> "In addition to the quantitative analysis of the total extravasated tracer in the supernatant of the homogenised brain, we qualitatively analysed tracer extravasation via IF microscopy upon intravenously injected Alexa Fluor® 555 Cadaverine (~1kDa; A555-Cad), which was allowed to circulate for 5min prior to sacrification of the animal. Qualitative analysis revealed increased leakage of A555-Cad in EviΔAC mice compared to EviCtrl mice at 10- (data not shown) and 40-weeks of age, evidenced by the accumulation of the tracer in cortical neurons (Figure 1J)."

4. *With no HRP-EM, I suggest modifying the terms transcytosis/ vesicular transport into something less definitive such as elevated vesicular abundance/activity. I suggest also adding to Figure 6B quantifications of luminal/abluminal pits, as they are also good indicators for the transcytotic path. Finally, quantifications of vesicles/vessel might be better presented as vesicle/EC cytoplasm area (or membrane length).*

Response: We thank the reviewer for these comments and suggestions. As requested, we have change the terms „transcytosis & vesicular transport" into alternative and less definitive terms such as „elevated vesicular abundance & increased vesiculation". Moreover, we have quantified the luminal/abluminal pits and added the data to Figure 6B-E of the revised manuscript. The quantitative total vesicles are now presented as „total vesicle count (per µm2 of EC cytoplasm area), as well as „luminal vesicle count (per µm of luminal EC mbne)" and „abluminal vesicle count (per µm of abluminal EC mbne)" (Figure 6D-E).

5. *Previous studies (including by the authors) used some markers to demonstrate modulation of BBB ECs by Wnt signaling LOF and GOF. I suggest analyzing their current data to show in a designated figure how do these markers change in light of the current manipulations and more important provide their hypothesis once these changes do not align with previous findings. Examples for previously used markers are Glut1 (seems up regulated?), Occludin, Cldn5, Cldn3, Plvap (MECA32).*

Response: We thank the reviewer for this comment. In order to analyse the expression of BBB-relevant Wnt downstream targets, we have performed qRT-PCR from forebrain microvessel preparations, as well as MACE RNA-Seq from forebrain CD31 FACS-sorted ECs. In ECs we do not see substantial regulation of Wnt targets in the MACE dataset, which is derived from 10w old mice. In the analysis of the microvessels by qRT-PCR we have detected down-regulation of Wnt target genes such as Nkd1 in 10w and Nkd1 and Axin2 in 40w old mice (Figure 2B). In general, one needs to keep in mind that the down-regulation of Axin2 and Nkd1 in microvessels by qRT-PCR, might in part be derived from other cellular sources such as astrocytes, for which we also show that Wnt signaling is affected in EviΔAC mice (Fig. F, G). Although Glut-1 appears to be regulated in this analysis on the mRNA level, we could not detect differences in protein expression and/or distribution by Glut-1 staining (Figure S4C). The same is true for the classical junction markers such as Cldn5, Cdh5, Occln or Tjp1 (Fig. 2, 3, 4, 5, 6, S4, S5 and S7). In the previous publications by us and others the demonstration of the role of the Wnt signalling pathways for the BBB properties in the EC were approached from the other side. Indeed, in the endothelial GOF or LOF models, the β-catenin pathway is activated or inhibited directly in the EC, independently of the presence of the Wnt ligands that could come from any NVU or circulating cells (see for example Benz et al. Elife. 2019; 8:204. for a recent publication). The same is true for all the publications revealing the receptors involved for the activation of this pathway in ECs (Zhou et al. J Clin Invest. 2014; 124(9):3825–46.). In this study we abolished the secretion of Wnts by the deletion of the Evi gene only in the ACs. Then it is not surprising that the phenotype observed is less severe than the previous approaches as others cells could secrete Wnts or

Ndp and therefore, could activate the receptor machinery which is still present at the EC membrane.

Moreover, in the EviΔAC animals, not only the canonical Wnts are not secreted anymore, but also the factors activating the PCP and Ca2+ signalling pathways. Interestingly, it has been shown in *C. elegans* that deletion of single Wnt ligands could have opposite effects regarding the ageing of the animals (Lezzerini & Budovskaya Ageing Cell 2014; 13(1):8–18) Along the same line it has been described that Wnt5a could inhibit the effects of Wnt3a, a well known activator of the β-catenin pathway (Sato et al, EMBO J, 2010, PMID19910923). Therefore, we cannot exclude that the absence of astrocytic release of all Wnts may have led to less activation of both, canonical and non canonical pathways. Consequently, we are analysing a condition in which a loss-of-function of both flavours of the Wnt pathway was induced instead of a specific LOF for the canonical pathway.

We have added these points to the discussion of the revised manuscript, Page 31, line 837.

> *One interesting notion arises from Figure 5: one interpretation might be that AC-EC interactions that drive elevated TEER (maybe TJ function) are Wnt independent. This does not align with previous publications claiming that Wnt signaling affect TJ function/TJ protein expression.*

Response: We thank the reviewer for this interesting comment. Indeed, the finding that ACs incapable of releasing Wnt growth factors do not interfere with the junctional tightening function of ACs in a co-culture system with brain ECs, may suggest that ACs at least in the in vitro setting do not tighten the paracellular barrier via Wnts. To our knowledge it has not been shown explicitly that ACs in vitro influence the junctional function by releasing Wnt growth factors. Therefore, our data are not contradictory to previous publications. Specifically, it has been shown that ACs may not directly regulate junctional integrity and tightness of the endothelial monolayer in MBMECs in vitro (Hamm et al. Cell Tissue Res 2004, 315(2):157– 66.). Nevertheless, our data may open novel explanations for the interaction between ECs and ACs. However, their investigation is not the focus and beyond the scope of this manuscript.

6. *The authors write that their findings are 'suggesting that the function of AC-derived Wnts could be compensated to a large extent'. I might have missed this data but I believe it is possible to analyze the RNA data (Figure 4) to at least test if the vasculature itself might be the source of the compensatory Wnt. Alternatively mine the data and suggest if there are other ACs producing ligands (such as SHH) unregulated. An experiment that might make the manuscript stronger but is not essential; test up-regulation of Wnt ligands in whole brains, or in tissue depleted from vasculature/ACs.*

Response: We thank the reviewer for bringing up this topic. We have now included data for reviewing purposed, based on further analysis of the RNA-Seq data set showing that at least ECs are not the source of compensatory Wnts or Wnt pathway-related genes (Reviewer Figure 3A). This role however could be taken over by the pericytes/vascular smooth muscle cells and/or the fibroblasts, as they have been described to express these factors using a single cell sequencing approach (Vanlandewijk et al. 2018 Nature 554, 475–480).

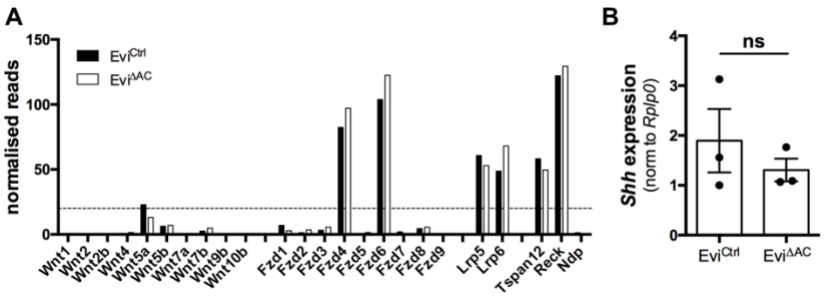


Figure Reviewer 3: (A) MACE analysis for Wnt pathway components of CD31+ FACS-sorted brain ECs. Dashed line indicates the threshold of 20 reads, for reliable gene expression. (B) qRT-PCR for Shh from cultured (P1) brain ACs derived from either EviCtrl or EviΔAC mice.

Moreover, ACs from EviΔAC animals do not show regulation of Shh (Reviewer Figure 3B), Ndp (Figure 7H) or other genes that potentially may contribute to the compensation of the abolished Wnt release. Still, it might be sufficient to maintain the junctional tightness with those genes at baseline expression. Finally, the action of Wnt ligands can be counteracted by inhibitors such as Dkks and Sfrps. Therefore, another hypothesis could be that inhibitors (also potentially secreted by different CNS cell types) are regulated in the EviΔAC animals, thereby maintaining the equilibrium between activation and inhibition of the Wnt pathways in ECs. We do believe, as the reviewer also noticed, that understanding these cross regulations and compensations, especially regarding the cellular source, is crucial but beyond the scope of this current study and will be subject to future investigations.

7. *Ideally, all the diverse approaches presented here would have been tested in all the time points. In vitro ACs are from pups, ECs are from adults. BBB permeability and water content are 10-20 weeks. Other experiments are 10 or 40 weeks. I agree that in some cases it is not critical because a certain hypothesis is better tested in a certain time point. There are some points that might be considered:*

    a. *I suggest putting a clear labeling of age in all the figures.*

Response: As suggested by the reviewer, we have added the correct aged to the results and in the figures where they were missing in the original submission.

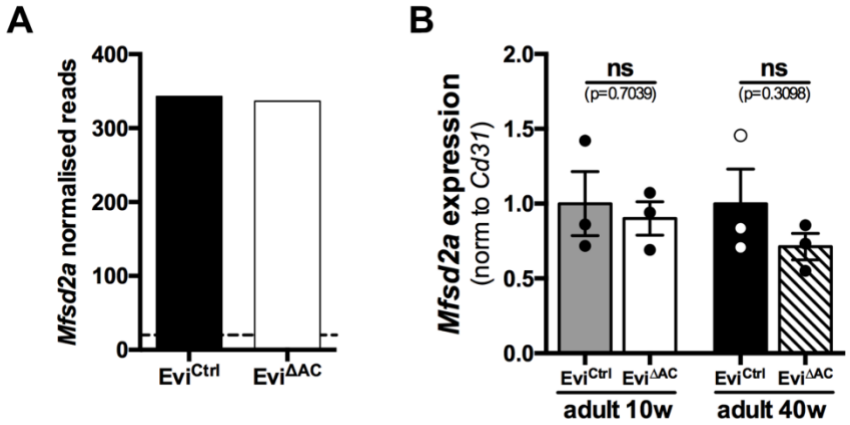
    b. *Make sure that the conclusions are not affected by not having a certain assay in all time points.*

Response: As the reviewer acknowledged, some experiments cannot be done at each age. This is particularly true for all the cell culture experiments for which the age at the isolation is of great influence regarding the yield and the purity of the primary cultures. Regarding the in vivo/ex vivo experiments most of them were done at both ages except for the wet/dry assay, the FTSC/TxR extravasation quantification and the RNA-Seq that have been done on young adults (10-20w). Still, in vivo permeability assays have been performed at both adult ages (10 & 40w) with the injection of Alexa555-Cadaverin and revealed an increased leakage of this tracer. Due to time constraints and due to the application of the 3R rule, we decided to not perform the wet/dry experiments with 40-week old animals.

    c. *In the discussion the authors indicate that they found no change in Mfsd2a expression but do not specify at what age was this tested. Less pericytes is aligned with some suggestions that pericyte-EC interaction control Mfsd2a expression, which leads to elevated tanscytosis.*

Response: We thank the reviewer for this mindful comment. We now have added some more information to the sentence on Page 32, line 887: „Interestingly, the canonical Wnt pathway was recently shown to influence the lipid metabolism together with Myc in the context of hepatocellular carcinoma, in which Wnt/Myc increased the fraction of poly-

unsaturated fatty acyl groups in a Ras-dependent manner (Yao et al., 2018). Although we could not identify an altered regulation of Mfsd2a expression in EviΔAC mice (data not shown), it will be interesting to address the interaction of Wnt and lipid metabolism in brain ECs in future investigations.“ Moreover, we decided to share the data with the reviewers in Reviewer Figure 4.



Reviewer Figure 4: (A) MACE analysis for Mfsd2a in CD31+ FACS-sorted brain ECs. Dashed line indicates the threshold of 20 reads, for reliable gene expression. (B) qRT-PCR for Mfsd2a on RNA derived from MBMVs isolated from 10 and 40-week-old EviCtrl or EviΔAC mice.

PC analysis has been carried out by CD13 staining and microscopic analysis of individual brain regions, whereas the Mfsd2a was analysed in cerebral ECs on its RNA level by MACE RNA-Seq and qRT-PCR. Therefore, we might not be able to identify Mfsd2a alterations in individual sections of the vasculature in which PC numbers are reduced in EviΔAC mice. Although this is indeed an interesting aspect, we feel that this would be beyond the scope of the current manuscript. Also, we now briefly discuss the potential importance of Cav-1 and Mfsd2a regulation in the vasculature in the context of neurovascular coupling, which has recently been shown by Chow et al. (Nature 2020; 579(7797):106–10). Page 34 line 941.

Minor comments:

1. *It would be informative to explain why in vivo Wnt7a/7b are mentioned/tested and in cultures there is use of Wnt3.*

Response: Wnt7a/7b are known to be the physiological ligands at the BBB in vivo. Unfortunately, Wnt7 do not go into solution when produced from cellular sources and also recombinant Wnt7 are not active in our hands. Consequently, only a direct co-culture of the Wnt7-producing cell would result in an endothelial β-catenin activation (Eubelen et al. Science. American Association for the Advancement of Science; 2018; 361(6403):eaat1178.). Therefore, and to stimulate brain ECs, we have used rmWnt3a, which has been proven to induce β-catenin signaling in brain ECs in vitro. Hence, the use of Wnt3a is only due to technical reasons.

2. *Line 713 RNA-seq misspelled.*

Response: Has been corrected.

3. *Figure 2A – I understand that the brain diagram and the bright field image are shown only to demonstrate the method. Nevertheless, I suggest replacing the bright field image with a different image (the same image already appears in a previous publication Devraj K. et al., 2016 JCBFM).*

Response: The image has been replaced.

4. *I find it very hard to understand how was the quantification done in figure 7B,C. It is not clear how volume was calculated. In addition in 7A it seems (based on DAPI) that these are not captured from similar cortical regions?*

Response: The quantification has been re-phrased in the Material and Methods section and the representative images in Figure 7A have been replaced as well.

5. *I recommend replacing the image representing swollen end-feet in figure 7E. After close examination I believe that if indeed this is an end-foot, it is a very irregular angle. It seems that around the end-foot there is a second basement membrane (typical of a pericyte). In addition, end-feet area (normalized to lumen area) could be quantified.*

Response: The image has been replaced. As we already have a quantification of the AQP4 volume by IF, we used the electron microscopy only for qualitative information rather than for a quantitative approach.

6. *Figure legend 5 – E and F are replaced.*

Response: The legend of Figure 5 E-F has been corrected.

7. *I suggest clarifying exactly what n=X are in all experiments. Especially what was the data used for statistical tests. e.g Figure 1 'n=5-6 animals'. How many repetitions of measurements were done for each brain? Did means of each brain was used for the statistical test? If so, does t-test is the best option vs. an a-parametric test? I couldn't find justification for the chosen test either.*

Response: The measurement of the fluorescence amount in the homogenised tissue was done once for each of the 6 biological replicates (i.e. animals) of both EviCtrl and EviΔAC. As the blood contains excess amounts of the FTSC and TxR tracers, a minor issue during the perfusion or the collection of some tissue has led to outliers that we had to exclude for the analysis (1 brain for EviΔAC and 1 spinal cord sample for EviCtrl). Therefore, this is why it is indicated n=5-6 animals in the legend.

Regarding the parametric versus non-parametric statistical tests, the regions with p value less than 0.05 with the t-test are also significantly regulated according to the Mann and Whitney test. As the parametric tests have a prerequisite of equal variance and this is the case with our data this is why we have chosen this test.

We carefully went through all the statistics of this manuscript and not only through the *in vivo* permeability assay. Unfortunately, we found 2 mistakes that we corrected. Indeed, c-fos in the LSN of young animals (Fig 8B) and qRT-PCR for Cdh5 in vessel fragments of young adults (Fig 2C) were initially indicated as significantly regulated even though their p values are 0.0531 and 0.0557, respectively. We therefore removed in the revised manuscript the corresponding asterisk to be in accordance with the official convention for significant p values.

8. *In Figure 6I, J n=2 and 1. I am not sure how critical data part is, having 6F,G and for the low number of repetition would recommend omitting it.*

Response: The Figure 6 has been reorganised and the luminal and abluminal vesicle quantification has been added (Fig. 6B-E), while the westen blots in Fig. 6I and 6J of the

original submission have been deleted in the revised manuscript as recommended by the reviewer.