

Peer Review File

Endolysosomal dysfunction in radial glia progenitor cells leads to defective cerebral angiogenesis and compromised Blood-Brain Barrier integrity



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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript is written to describe brain development abnormalities in the absence of Scarb2 function using zebrafish as an experimental model. The authors find that cerebral angiogenesis and the blood-brain barrier (BBB) are disrupted in homozygous loss-of-function *scarb2* mutant zebrafish larvae. Building on this information, the authors perform experiments that lead them to conclude that endolysosomal dysfunction in radial glial cells disrupts formation of the neurovascular unit, which is important for the formation and function of the BBB. The data reported here are potentially important because they might add to our understanding of the complex cellular interactions necessary to build the neurovascular unit.

The manuscript is nicely written and the Figures are well organized, fairly easy to interpret, and informative. The experimental approach is quite rigorous and comprehensive and statistical analyses seem appropriate. The experiments that connect Scarb2 function to endolysosomal function and Notch3 activity in radial glia are quite elegant and convincing. The methods description is detailed and thorough, ensuring that the experiments could be reproduced by others. The conclusions drawn from the individual experiments are generally well supported. My only real concern with this manuscript is the overall conclusion connecting Scarb2 function, radial glia development, and angiogenesis and the BBB. Quite simply, loss of Scarb2 function results in massive structural changes in the brain, stemming mostly from a large overproduction of neurons and depletion of radial glia and other glia subtypes. Thus, loss of BBB integrity does not strike me as specifically connected to *scarb2* mutation nor is it very surprising given the large-scale disruption of neurogenesis and gliogenesis. It certainly seems to be a stretch to conclude that this is particularly informative for understanding cerebral small vessel diseases. I'll be interested to learn of the authors' response to my hesitation on this point.

Minor points:

- Lines 238-243. The way that this is written could be taken to mean that the authors think that the deficit of *mbp*:EGFP+ oligodendrocytes might result from loss of Scarb2 functions during “additional steps during oligodendrocyte lineage progression.” However, mutant larvae have a deficit of oligodendrocyte precursor cells, which likely explains the deficit of oligodendrocytes. There is now basis, except for the scRNA-seq data, to make any conclusion about additional functions in myelination.
- The injection experiments described in lines 270-281 are not included in the Methods section.
- In Figures 1, 3, 4, and 6, what do the plots dots indicate? “n” is not specified for these data, it is only specified for the data in Figure 5.
- Figure legend 2 has a statement about error bars but there are none in the figure.

Reviewer #2 (Remarks to the Author):

The article from Bassi et al explores the relationship between the Central Nervous System (focusing on the hindbrain) and the vascular system during zebrafish embryonic development. Using a novel mutant obtained from a CRISPR/Cas-based mutagenesis screen, they describe the expression of *scarb2* in neural progenitors and its role in controlling Notch-signaling, which maintains these cells not engaged in neurogenesis. In addition, they show that the associated morphogenetic defects upon *scarb2* mutation as a result of the increase of neurogenesis, lead to vascular abnormalities.

This work beautifully demonstrates how Notch3-activity is regulated by a cellular machinery involved in the control of the cleavage of the Notch Intracellular Domain, and the ulterior effects in cell fate. However, the main role of Notch3-signaling as shown in other contexts is to control binary cell fate decisions, such as progenitor vs. differentiation. Thus, the effects observed in all cell fates upon *scarb2* mutation are secondary effects, as the result of exhausting the progenitor pool, not as something specific of the different fates. The authors clearly mention it in line 267. However, all along the manuscript they insist in the effects on different cell fates.

A very relevant observation is that morphogenetic changes due to the increase of the neuronal differentiation domain impact/collapse the vascular system, demonstrating the importance of the control of the coordination of different tissues' growth and size, although the authors do not explore it or discuss it further.

Main issues:

1. Authors show expression of *scarb2* by constructing a transgenic line with a fluorescent reporter. Since these reporters have a long half-life, they track cells that express *scarb2* and those that they expressed it but they do not anymore. It would be nice to combine in situ hybridizations for *scarb2* with the transgenic line to decipher whether i) *scarb2* is expressed in all progenitors (neuroepithelial cells and radial glia cells) as it seems, or only in a subpopulation of progenitors; ii) *scarb2* is expressed early in the vascular primordium, to rule out any cell-autonomous effect.
2. Line 140 indicates that only 25% of *scarb1*^{-/-} display intraventricular hemorrhage. Better characterization of the penetrance and expressivity of the phenotype should be provided, since it seems that several phenotypes can be obtained.
3. All along the work they use GFAP as a marker of Radial Glia Cells (RGC). However, GFAP is a marker of RGC only in the adult. During early zebrafish embryonic development GFAP is a marker of progenitors, and this is why it overlaps with Sox2. A better marker of RGC at these stages is *slc1a*/*Glast* or *fabp7a* (Belmonte-Mateos et al, 2023; Hartfuss et al., 2001; Than-Trong and Bally-Cuif, 2015). They should verify their results.
4. Following with the earlier point, there is a confusion with markers. No astrocytes as such have been described in the zebrafish embryo. Thus, no well-established astrocyte markers are available yet and *slc1a* is a marker of RGC. Extensive care with markers and statements should be put along the manuscript.
5. As mentioned before, decrease of *olig2*, *isl1*, *HuC*, etc is due to the exhaustion of progenitors upon *scarb2* mutation. Thus, upon *Gal4:UAS:scarb2* GOF experiments, only cells that express *Gal4*

should express *scarb2* (*olig2:Gal4*, *scl1:Gal4* populations), and only cells from the specific populations should be forced to differentiate. However, they seem to have many ectopic cells too. Can they quantify cells that are *Gal4* and express *scarb2* and they differentiate? Can they see whether they activate Notch-signaling in those?

6. Can they rule out whether the defects on the vasculature are due to slower cell proliferation due to the lack of “secreted factors” from the progenitor population or just a collapse due to morphogenetic changes since they claim the importance of RGC signals.

7. In *scarb1* mutants the observation of defects of the vasculature correlates with an increase of neurogenesis, although no demonstration of a direct link is provided. Can they temporally inhibit Notch, analyze vasculature defects, and wash out the Notch inhibitor and see whether they recover the neurogenic and vasculogenic phenotype? Or provide any other insight?

8. They beautifully show the effect on NICD-cleavage and that relies on Notch3. Notch3 onset of expression is around 30-34hpf, thus earlier effects should be separated from Notch3-activity. Did they explore whether DeltaD, the ligand for Notch3, is affected?

The discussion is very much biased towards the importance of cerebrovascular diseases. However, the strongest part of the article is the role of *scarb1* in Notch3-cleavage. Authors should balance their statements and observations. There is a recent paper quite relevant to their work that is not quoted (O’Brown et al, Dev Cell 2023).

Reviewer #3 (Remarks to the Author):

The authors raised several valid points as a justification for the study, namely, lack of detailed analysis of radial glial progenitors and their functional contribution to the emergence of the neurovascular unit in embryos and larvae. Therefore, the study is warranted and original.

The authors identified that *scarb2* is abundantly expressed in radial glia cells and their progeny. However, the imaging and co-localization studies definitely can be improved, as this is the primary utility of zebrafish.

Using transgenic and mutant lines, the authors clearly delineate a number of phenotypic observations, ranging from defects in central arteries of the brain and intracerebral hemorrhages, to excessive neurogenesis and reduced glial differentiation. Intriguingly, defects in Notch3 processing appear to underlie these defects, hence, potentially ascribing a non-endothelial role for Notch3 signaling during blood brain barrier formation.

It is this reviewer's opinion that the massive hemorrhages that emerge as early as 44 hpf, particularly, in the hindbrain and expanding into the ventricles (as revealed by *gata1:dsred* line), can - as has been shown by others - cause many of the defects that the authors analyzed at later stages (e.g. at 60 hpf or 5 dpf). Extravasation of blood in the hindbrain and ventricle can cause death of *olig2+* cells, *mpp+* cells, and even reduce astrocyte counts simply due to displacement of these

cells by virtue of the expansion of hematoma. The shear volume of blood occupying the brain parenchyma can, even temporarily, change the preponderance of these cells. The authors are encouraged to look at the immune and thrombosis that ensue following bleeding in the brain and address how these processes can cause damage to the cells in this milieu.

Hence, the study is compounded by the bleeding phenotype which makes it difficult to tease out the primary defects and rule out involvement of pathophysiology of hematoma expansion.

Point-by-point responses to reviewers' comments (our responses in blue)

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We thank the reviewer for recognizing the potential of our work and for providing critical inputs and evaluations. Based on the reviewer's comments we have now added a **large amount of experimental data** and have **significantly increased the statistical power of our observations**. Overall, we believe that the new experiments provided in this revised version strongly support and further expand our initial findings. We truly appreciate the reviewer's advice, which has significantly helped strengthen our manuscript.

My only real concern with this manuscript is the overall conclusion connecting Scarb2 function, radial glia development, and angiogenesis and the BBB. Quite simply, loss of Scarb2 function results in massive structural changes in the brain, stemming mostly from a large overproduction of neurons and depletion of radial glia and other glia subtypes. Thus, loss of BBB integrity does not strike me as specifically connected to *scarb2* mutation nor is it very surprising given the large-scale disruption of neurogenesis and gliogenesis.

We agree with the reviewer on the need to better elaborate on these issues in our manuscript.

Similar to mammals¹⁻⁴, CNS angiogenesis and barrierogenesis in zebrafish are tightly coordinated by the VEGF⁵⁻⁷ and Wnt signaling pathways⁸⁻¹³ and occur in a synchronized manner¹⁴. Chemical inhibition of VEGF signaling during specific stages of hindbrain development led to faulty EC migration and survival, leading to malformed CtAs¹⁵. During the same angiogenic phases, Wnt signaling is highly active in the sprouting CtA ECs, and its inhibition results in diminished *glut1* expression¹⁵ and defective CtA anastomosis¹⁰, causing intracerebral hemorrhages at later developmental stages.

In this revised version, we present new experimental data, demonstrating alterations in both the VEGF and WNT signaling pathways in *scarb2a* mutants, each responsible for different aspects of the observed phenotypes:

1. Using the *TgBAC(vegfaa:EGFP)^{pd260 16}* reporter, we show that during the initial stages of CtA sprouting and migration (~32 dpf) VegfAa expression is similarly detected in *scarb2a*⁺ RGCs both in wt and mutant embryos (Extended Data Fig. 8h-k'). These results are in accordance with the normal development of the BA and initial sprouting of the CtAs from the PHBCs

observed in *scarb2a*^{-/-} (fig. 1k). In contrast, by 60 hpf, higher levels of *VegfAa* were observed in the hindbrains of *scarb2a*^{-/-} embryos, likely produced by RGCs that adopted a neuronal fate (**new Fig. 6h-i'**; **Extended Data Fig. 8l-m'**). These results are further supported by qRT-PCR analyses on whole heads, indicating a specific two-fold increase in the levels of *VegfAa* transcript (**new Extended Data Fig. 8n-o**), which results in aberrant angiogenesis, as previously described^{5,6,8}.

2. To functionally confirm these results, we treated wt and mutant embryos with the VEGFR2 inhibitor SU5416 at ~32 hpf (right before the initial appearance of the vascular phenotypes). As seen in **new Fig. 6j-m**, we observed a strong decrease in the number of CtA anastomoses, confirming that at least part of the altered angiogenesis phenotypes seen in *scarb2a*^{-/-} are mediated by excess *VegfAa* production (most likely due to the excessive neuronal differentiation).
3. We carried out several experiments to investigate whether Wnt signaling is also altered in the hindbrains of *scarb2a*^{-/-} embryos. Wnt7a has been previously shown to be important for BBB formation in mammals¹² and zebrafish^{10,11}. Accordingly, we assessed the expression of Wnt7Aa in both GFAP⁺ and *scarb2a*⁺ sorted cells at 2 dpf and confirmed a consistent decrease in mutant brains (**new Extended Data Fig. 9a-b**).
4. We also assessed Wnt activation in the hindbrains of wt and mutant embryos using the *Tg(7xTCF-Xla.Sia:GFP)*¹⁷ reporter. As seen in **new Fig. 7a-b'**; **Extended Data Fig. 9c-d'**, we detected decreased Tcf activation (i.e., Wnt signaling downregulation) specifically in CtAs-ECs, confirming that indeed, Wnt signaling is not properly activated in the hindbrain vessels of *scarb2a* mutants.
5. To functionally corroborate these results, we treated *scarb2a* mutants with the Wnt pathway activator LiCl. This treatment, carried out at 32 dpf, resulted in significant rescue of CtA anastomoses (**new Fig. 7c-e'**; **Extended Data Fig. 9e**), Glut1 expression (**new Fig. 7c-e'**), and BBB leakiness (**new Fig. 7f-h**). These data demonstrate that Wnt secreted from RGCs and their glia derivatives (OPCs and astrocytes) are responsible for barrierogenesis in accordance with previous reports^{8-13,15,18}.
6. Finally, we now show that combined treatment with SU5416 and LiCl at 32 hpf results in complete normalization of CtAs anastomosis and vascular morphology (**new Fig. 7i-k**).

Taken together, our new data indicate that the increased neurogenesis and the exhaustion of RGCs with the consequent reduced number of glia subtypes in *scarb2a* mutants have a direct impact on the production of VEGF and Wnt7a, thereby affecting the normal establishment of the CNS vasculature, the neurovascular unit and functionality of the BBB. These results strengthen and expand our original findings showing that overexpression of wt *scarb2a* (*UAS:scarb2a*) in GFAP⁺ cells of *scarb2a* homozygous mutants fully rescues the patterning of the hindbrain vasculature (Fig. 6a-d) and restores Glut1 expression (Fig. 6e-g') by reinstating a normal neuronal vs. glia differentiation balance.

It certainly seems to be a stretch to conclude that this is particularly informative for understanding cerebral small vessel diseases. I'll be interested to learn of the authors' response to my hesitation on this point.

We agree with the reviewer's comment, and in the revised version of our manuscript, we have toned down the implications of our work to the understanding of CSVD. Yet, the point we are trying to convey is the focus on non-vascular cells of the brain parenchyma as crucial players in the development of CSVD-associated phenotypes. Traditionally, CSVD research has been primarily centered in the vascular

components of the brain (ECs, vascular smooth muscle cells, and pericytes). These changes include thickening and stiffness of arterioles, decreased lumen size, reduced blood flow, a compromised BBB, and microbleeds due to mural cell and/or endothelial cell dysfunction. In contrast, the contribution of cells of the neural parenchyma to the disease and the molecular signaling pathways underlying their pathogenic interactions remain poorly understood. Moreover, although the involvement of glial cells and brain-resident immune cells of the CNS (microglia) in white matter injury is well documented, the role of these cell populations in CSVD initiation and disease progression remains unclear. We believe that our findings open a new avenue for research focusing on **dysfunctional glia as a cause/contributor of vascular abnormalities associated with CSVD rather than being solely a result of them**. This suggests that glial cells play pivotal roles not only in the development and function of the neurovascular unit (NVU) but also in diseases such as CSVD. Notably, current therapies for CSVD primarily focus on preventive measures to enhance or restore cerebral blood flow, with limited attention given to treatments targeting non-vascular cells.

Overall, we propose that *scarb2a* mutants could serve as a valuable model for elucidating the largely unexplored mechanisms by which glial cells regulate CNS angiogenesis and BBB establishment in the broad context of the developing organism. This understanding could lead to improved insights into the glial pathophysiology of CSVD and its influence on the neurovascular progression of the disease.

We hope the way these points have now been highlighted in the discussion better explains our interpretation of the results.

Minor points:

- Lines 238-243. The way that this is written could be taken to mean that the authors think that the deficit of mbp:EGFP+ oligodendrocytes might result from loss of Scarb2 functions during “additional steps during oligodendrocyte lineage progression.” However, mutant larvae have a deficit of oligodendrocyte precursor cells, which likely explains the deficit of oligodendrocytes. There is now basis, except for the scRNA-seq data, to make any conclusion about additional functions in myelination.

Based on the reviewer’s comment we realize we have not been clear enough when addressing this point in the original version of our manuscript, and we apologize for any confusion this may have caused. Indeed, as stated by the reviewer, Notch signaling has been shown to act on RGCs to keep them in a stem-like state by inhibiting a neuronal fate¹⁹, thereby preserving the pool of precursors available to generate OPCs later. This is also supported by the fact that global inhibition of Notch signaling with DAPT leads to excessive neurogenesis at the expense of gliogenesis^{20,21}. Finally, the phenotypes displayed by zebrafish *notch3* mutants²² (Extended Data Fig. 6m-r) further support this idea. We focused on the effects of impaired RGC differentiation on the different cell fates (namely astrocytes and cells of the oligodendrocyte lineage) since the lack of those cell types is important for the vascular phenotype. However, to avoid confusion, we have amended the text to clarify that as a consequence of pool exhaustion, there is no proper glia differentiation.

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All graphs are now properly described in legends

- Figure legend 2 has a statement about error bars, but there are none in the figure.
- All figure legends have been corrected

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i) Following the reviewer's suggestion, we attempted to perform fluorescent *in situ* hybridization for *scarb2a* on *Tg(scarb2a:KalTA4:UAS:EGFP)* embryos; however, we were unsuccessful. In order to address the reviewer's concern, though, we carried out whole mount *in situ* for *scarb2a* at 36 hpf and compared it with our transgenic reporter. As seen in the images provided in **new Extended Data Fig. 2e-f**, *scarb2a* is strongly expressed in the hindbrain, along the anteroposterior (AP) axis, and along the midline with stripes that converge to it. The lateral views revealed that *scarb2a* is expressed in the dorsal part of the hindbrain. Overall, this expression pattern strongly resembles that of our transgenic reporter. To further address the reviewer's comment, we also carried out whole mount *in situ* for *fabp7a*²³ at 36 hpf (**new Extended Data Fig. 2g-h**) and confirmed a highly similar expression pattern for both genes, suggesting that the stripes observed in *scarb2a* label the rhombomere centers, as previously described by Belmonte-Mateos et al²³. Altogether these data confirm the specificity of our reporter on the one hand and, to the best of our understanding, verify that *scarb2a* is expressed in RGCs. However, it's worth noting that according to Than-Trong and Bally-Cuif²⁴, *fabp7* labels both RGCs and NE progenitors at these stages, which makes it challenging for us to strictly differentiate between these two populations.

ii) Analysis of confocal images of *Tg(kdrl:TagBFP;scarb2a:KalTA4;UAS-mKate2)* at 22 hpf did not reveal any co-localization between the *kdrl* (endothelial marker) and the *scarb2a* reporter lines, indicating that *scarb2a* is not expressed in the vascular primordium (**new Extended Data Fig. 2c-c''**).

2. Line 140 indicates that only 25% of *scarb1*^{-/-} display intraventricular hemorrhage. Better characterization of the penetrance and expressivity of the phenotype should be provided, since it seems that several phenotypes can be obtained.

We thank the reviewer for this important comment. We have now expanded the phenotypic characterization of *scarb2a* mutants and have provided additional quantitation.

As seen in **new Extended Data Fig. 1c**, around 25-30% of the mutants display intraventricular hemorrhage, primarily in the hindbrain, starting at ~40 hpf. Yet, as described in the original version of our manuscript, 100% of mutant larvae present compromised BBB integrity as shown by Rhodamine dye extravasation (Fig. 1n,o) and Glut1 reduced expression (Fig. 1p-q'), regardless of whether or not this phenotype is accompanied by intracranial hemorrhage. Homozygous *larvae* die at ~10-14 dpf, independently of the presence of hemorrhage (**new Extended Data Fig. 1d**), indicating that the hemorrhage per se is not the main cause of lethality. Heterozygous animals, in contrast, reach adulthood and are viable and fertile. Finally, we confirmed that reduced specification of progenitors

towards glia cell fate is not secondary to the morphological changes derived from hemorrhagic events. As seen in **new Extended Data Fig. 3m-n**, the reduction in the number of oligodendrocytes and astrocytes was unaffected by the presence or absence of hemorrhage. Collectively these data indicate that the hemorrhage by itself does not inhibit or exacerbate the additional described phenotypes, including BBB integrity, neurogenesis vs. gliogenesis, and lethality.

3. All along the work they use GFAP as a marker of Radial Glia Cells (RGC). However, GFAP is a marker of RGC only in the adult. During early zebrafish embryonic development GFAP is a marker of progenitors, and this is why it overlaps with Sox2. A better marker of RGC at these stages is *slc1a*/*Glast* or *fabp7a* (Belmonte-Mateos et al, 2023; Hartfuss et al., 2001; Than-Trong and Bally-Cuif, 2015). They should verify their results.

Following the reviewer's suggestion, we have now performed whole mount *in situ* hybridization for *fabp7a*²³ at 36 hpf (**new Extended Data Fig. 2g,h**). We noticed that, as previously shown by Belmonte-Mateos et al²³, *fabp7a* is expressed in the hindbrain RGCs along the anteroposterior (AP) axis, along the midline and in the rhombomere centers, and its expression strongly resembles that of *scarb2a* mRNA **new Extended Data Fig. 2e,f**). However, as explained above, we found that according to Than-Trong and Bally-Cuif²⁴, *fabp7* labels both RGCs and NE progenitors at these stages, which makes it challenging for us to strictly differentiate between these two populations. Based on the reviewer's suggestion and our interpretation of the results, we decided to define the *scarb2a*+ cells as "progenitors" or "RGCs", based on the specific developmental stage. If, however, the reviewer thinks otherwise, we will be happy to adopt a more accurate nomenclature.

4. Following with the earlier point, there is a confusion with markers. No astrocytes as such have been described in the zebrafish embryo. Thus, no well-established astrocyte markers are available yet and *slc1a* is a marker of RGC. Extensive care with markers and statements should be put along the manuscript.

We are aware that the presence of true astrocytes in zebrafish has remained controversial. However, Chen et.al.²⁵ have recently described *slc1a3b*/*Glast*+ cells in 6 dpf zebrafish hindbrain and spinal cord, that share morphological, molecular, and functional features with mammalian astrocytes. Following the reviewer's suggestion, we have amended the text and describe the cells labeled by this reporter as "*slc1a3b*+ RGCs" at 60 hpf, and as "astrocytes" in experiments carried out at 6 dpf when they have been shown to display Ca2+ signaling dynamics similar to mammalian astrocytes.

We have tried to carefully follow the literature and the different markers utilized to define each of the different cell populations. To the best of our understanding, some of the definitions, including references suggested by the reviewer, refer to the expression in adult fish brains. When searching for embryonic expression of the same markers, it appears that the definitions are much less clear. For instance, according to Than-Trong and Bally-Cuif²⁴ *blbp* (*fabp7*) labels both RGCs and NE progenitors at neural plate stages in the embryo. Most recently, Johnson et al.²⁶ utilized lineage tracing and specific *gfap*+ cell ablation experiments to demonstrate that "*subsets of interneurons, secondary motorneurons, and oligodendroglia originate from Gfap+ radial glia to support the hypothesis that Gfap+ radial glia act as a progenitor population during CNS development. Second, we show that genetic ablation of radial glia using the nitroreductase enzyme under control of gfap resulted in significant loss of the gfap+/nestin+/Sox2+ NSC population as well as later-born neurons and glia, which could not be compensated for by other progenitor populations*". Finally, Belmonte-Mateos

et.al.²³ show that progenitor cells within the rhombomeric centers are Notch-active (mostly Notch3) and display enriched expression of *fapb7a* and *slc1a2a*.

Based on the reviewer's suggestion and our interpretation of our data and the available literature, we decided to define the *scarb2a*⁺ cells as "progenitors" or "RGCs", based on the specific developmental stage. If, however, the reviewer thinks otherwise, we will be happy to adopt a more accurate nomenclature.

5. As mentioned before, decrease of *olig2*, *isl1*, *HuC*, etc is due to the exhaustion of progenitors upon *scarb2* mutation. Thus, upon Gal4:UAS:*scarb2* GOF experiments, only cells that express Gal4 should express *scarb2* (*olig2*:Gal4, *scl1*:Gal4 populations), and only cells from the specific populations should be forced to differentiate. However, they seem to have many ectopic cells too. Can they quantify cells that are Gal4 and express *scarb2* and they differentiate?

Following the reviewer's comment, we would first like to emphasize that the rescue experiments were performed by expressing *UAS-scarb2a-P2A-RFP* either in **GFAP:GAL4FF+ RGCs** or in ***scarb2a*** expressing cells (using the *scarb2a*:*KAITA4* driver), as we reasoned that this approach would better model the requirement for *scarb2a* during the process of RGC differentiation (as opposed to overexpressing the rescue construct in the differentiated progenies using *olig2*:Gal4 or *scl1*:Gal4).

As suggested by the reviewer we have now calculated the percentage of *olig2*⁺ cells that also express *scarb2a-RFP*, meaning they have properly differentiated toward the OPC lineage. As seen in **new Extended Data Fig. 5d, d', f, n, n', o**, we found that the percentage of *scarb2a*⁺/*olig2*⁺ in *Tg(olig2:EGFP;GFAP:GAL4FF; UAS-scarb2a-P2A-RFP)* and *Tg(olig2:EGFP;scarb2a:KAITA4; UAS-scarb2a-P2A-RFP)* at 60 hpf, represents 43.4 % and 44.7% respectively out of the total *olig2*⁺ cell population. In addition, we found 56.5% of cells that were *olig2*⁺/*scarb2a*⁻. This number is in line with the number of residual OPCs detected in *scarb2a* mutant hindbrains, which could hint at additional origins of oligodendrocytes, as previously reported^{27,28}. We also found 60.8% of cells that were *scarb2a*⁺/*olig2*⁻, we believe these cells give rise to astrocytes at slightly later stages.

Can they see whether they activate Notch-signaling in those?

Following the reviewer's suggestion, we now show that injection of *UAS:scarb2a-P2A-RFP* into *Tg(GFAP:Gal4FF;12NRE:EGFP);scarb2a*^{-/-} embryos significantly rescued Notch activation in RGCs, with ~80% of the cells expressing the exogenous *Scarb2* construct, displaying Notch activity. **New Extended Data Fig. 6g-l** depicts the co-localization between *scarb2a* and Notch reporter in cells of the SV zone where Notch signaling is fully absent in the mutant.

6. Can they rule out whether the defects on the vasculature are due to slower cell proliferation due to the lack of "secreted factors" from the progenitor population or just a collapse due to morphogenetic changes since they claim the importance of RGC signals.

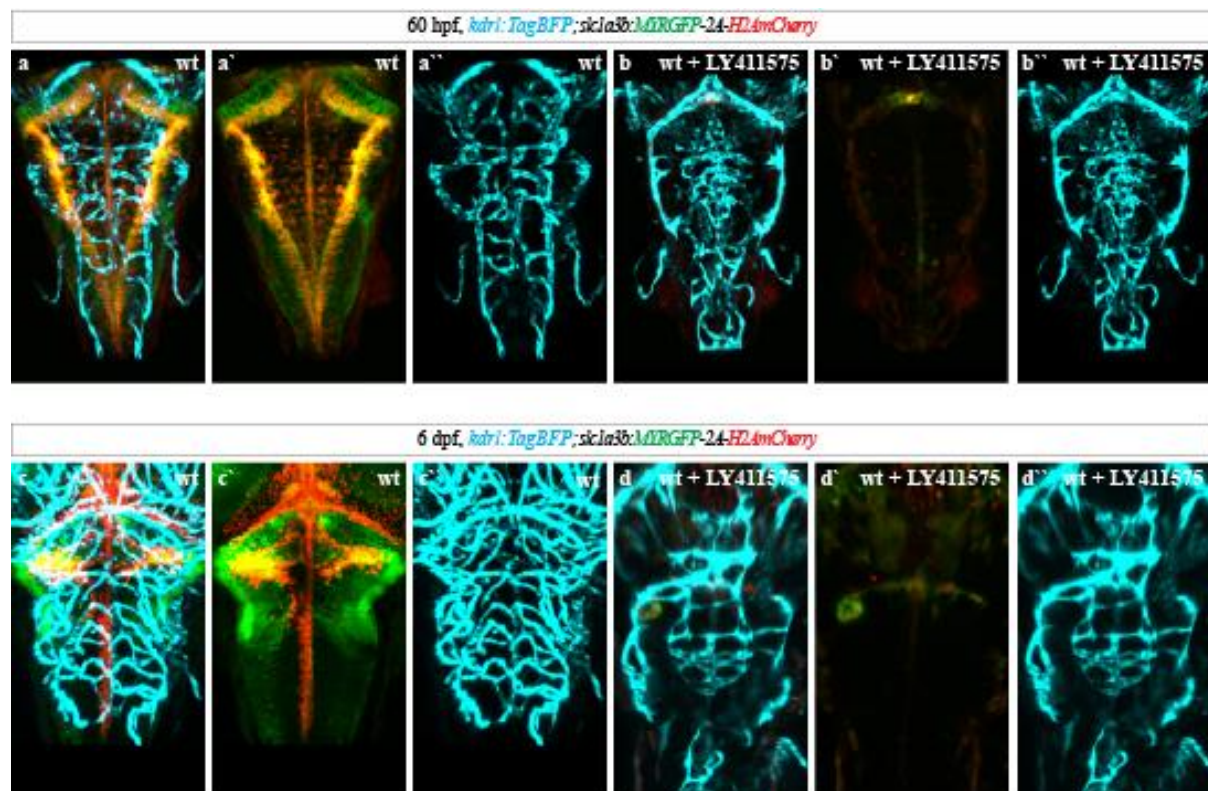
As explained above, we now provide new experimental data and quantitative analyses demonstrating that the vascular phenotypes are caused by exhaustion of the RGC pool and the impaired RGC differentiation towards glia lineage rather than the reverse. Both in terms of the timing of appearance of the phenotypes, and our ability to rescue them through manipulation of radial glia cells, but not of ECs, we reinforce and expand our original findings. In addition, this revised version contains extensive amounts of data showing how the defective RGC differentiation and the altered neuronal vs. glia balance results in aberrant *VegfA* and *Wnt* signaling, which ultimately leads to the observed vascular phenotypes.

Regarding EC proliferation, we have assessed the number of CtA-ECs using the *Tg(kdr1:NLS-mCherry)* reporter and found that it is, in fact, slightly increased (and not slowed down) in *scarb2a* mutants at 60 hpf (**new Extended Data Fig 1h-j**), due to increased production of VegfAa by the precociously differentiated HuC⁺ neurons. These data are in accordance with previous reports showing a specific role for VegfAa in controlling CtA-EC numbers^{5,29}.

7. In *scarb1* mutants the observation of defects of the vasculature correlates with an increase of neurogenesis, although no demonstration of a direct link is provided. Can they temporally inhibit Notch, analyze vasculature defects, and wash out the Notch inhibitor and see whether they recover the neurogenic and vasculogenic phenotype? Or provide any other insight?

As explained above, we believe that the new experimental data added to the manuscript, describing specific alteration in VegfAa and Wnt signaling pathways emanating from RGCs and their progenies, and functionally demonstrating their involvement in eliciting the vascular phenotypes, strongly supports the link between imbalanced neurogenesis/gliogenesis and the vascular abnormalities observed in *scarb2a* mutants.

We also performed the experiment suggested by the reviewer- we inhibited Notch signaling between 30 hpf to 48 hpf (the time window corresponding to the onset of Notch3 expression and CtA sprouting³⁰) using the LY411575 Notch inhibitor. As seen in the image attached below, Notch depletion resulted in a complete lack of *slc1a3b*⁺ cells (b'') and the vascular defects were reminiscent of those displayed by *scarb2a* and *notch3^{fh332/fh332}* mutants at 60 hpf (b''). Yet, removal of the chemical treatment did not rescue either the vascular or the gliogenic phenotypes by 6 dpf (d-d''), confirming the need for balanced Vegf and Wnt signaling early in development in order to establish a proper CNS plexus and a functional BBB.



8. They beautifully show the effect on NICD-cleavage and that relies on Notch3. Notch3 onset of expression is around 30-34hpf, thus earlier effects should be separated from Notch3-activity. Did they explore whether DeltaD, the ligand for Notch3, is affected?

We would like to point out that throughout all our experiments and the many different reporters utilized in this study, we never detected defects in any of the cell populations before 32 hpf, strongly supporting the idea that all the phenotypes are due to Notch3 alteration. In addition, as seen in Extended Data Fig. 7i-p, neither of the Notch1 domains (ICD or ECD) was able to rescue the phenotypes as opposed to N3ICD.

Since our data support the idea that the inhibition of Notch signaling takes place primarily within the receiving cell, we focused our analyses on Notch3 itself. In the future, it will be interesting to investigate whether and how the impaired production of N3ICD affects the DeltaD ligand.

The discussion is very much biased towards the importance of cerebrovascular diseases. However, the strongest part of the article is the role of *scarb1* in Notch3-cleavage. Authors should balance their statements and observations. There is a recent paper quite relevant to their work that is not quoted (O’Brown et al, Dev Cell 2023).

We agree with the reviewer's comment, and in the revised version of our manuscript, we have toned down the implications of our work to the understanding of CSVD. Yet, the point we are trying to convey is the focus on non-vascular cells of the brain parenchyma as crucial players in the development of CSVD-associated phenotypes. Traditionally CSVD research has been primarily centered in the vascular components of the brain (ECs, vascular smooth muscle cells and pericytes). These changes include thickening and stiffness of arterioles, decreased lumen size, reduced blood flow, a compromised BBB, and microbleeds due to mural cell and/or endothelial cell dysfunction. In contrast, the contribution of cells of the neural parenchyma to the disease and the molecular signaling pathways underlying their pathogenic interactions remain poorly understood. Moreover, although the involvement of glial cells and brain-resident immune cells of the CNS (microglia) in white matter injury is well documented, the role of these cell populations in CSVD initiation and disease progression remains unclear. We believe that our findings open a new avenue for research focusing on **dysfunctional glia as a cause/contributor of vascular abnormalities associated with CSVD rather than being solely a result of them**. This suggests that glial cells play pivotal roles not only in the development and function of the neurovascular unit (NVU) but also in diseases such as CSVD. Notably, current therapies for CSVD primarily focus on preventive measures to enhance or restore cerebral blood flow, with limited attention given to treatments targeting non-vascular cells.

Overall, we propose that *scarb2a* mutants could serve as a valuable model for elucidating the largely unexplored mechanisms by which glial cells regulate CNS angiogenesis and BBB establishment in the broad context of the developing organism. This understanding could lead to improved insights into the glial pathophysiology of CSVD and its influence on the neurovascular progression of the disease.

We have edited the discussion according to the reviewer’s suggestion and have included the new reference.

We hope the way these points have now been highlighted in the discussion better explains our interpretation of the results.

Reviewer #3 (Remarks to the Author):

The authors raised several valid points as a justification for the study, namely, lack of detailed analysis of radial glial progenitors and their functional contribution to the emergence of the neurovascular unit in embryos and larvae. Therefore, the study is warranted and original.

The authors identified that *scarb2* is abundantly expressed in radial glia cells and their progeny. However, the imaging and co-localization studies definitely can be improved, as this is the primary utility of zebrafish.

Using transgenic and mutant lines, the authors clearly delineate a number of phenotypic observations, ranging from defects in central arteries of the brain and intracerebral hemorrhages, to excessive neurogenesis and reduced glial differentiation. Intriguingly, defects in Notch3 processing appear to underlie these defects, hence, potentially ascribing a non-endothelial role for Notch3 signaling during blood brain barrier formation.

We thank the reviewer for recognizing the potential of our work and for providing critical inputs and evaluations. Based on the reviewer's comments we have now added a **large amount of experimental data** and have **significantly increased the statistical power of our observations**. Overall, we believe that the new experiments provided in this revised version strongly support and further expand our initial findings. We truly appreciate the reviewer's advice that has significantly helped strengthen our manuscript.

It is this reviewer's opinion that the massive hemorrhages that emerge as early as 44 hpf, particularly, in the hindbrain and expanding into the ventricles (as revealed by *gata1:dsred* line), can - as has been shown by others - cause many of the defects that the authors analyzed at later stages (e.g. at 60 hpf or 5 dpf). Extravasation of blood in the hindbrain and ventricle can cause death of *olig2+* cells, *mpp+* cells, and even reduce astrocyte counts simply due to displacement of these cells by virtue of the expansion of hematoma. The sheer volume of blood occupying the brain parenchyma can, even temporarily, change the preponderance of these cells. The authors are encouraged to look at the immune and thrombosis that ensue following bleeding in the brain and address how these processes can cause damage to the cells in this milieu.

Hence, the study is compounded by the bleeding phenotype which makes it difficult to tease out the primary defects and rule out involvement of pathophysiology of hematoma expansion. We agree with the reviewer on the need to better elaborate on these issues in our manuscript.

Similar to mammals¹⁻⁴, CNS angiogenesis and barrierogenesis in zebrafish are tightly coordinated by the VEGF⁵⁻⁷ and Wnt signaling pathways⁸⁻¹³ and occur in a synchronized manner¹⁴. Chemical inhibition of VEGF signaling during specific stages of hindbrain development led to faulty EC migration and survival, leading to malformed CtAs¹⁵. During the same angiogenic phases, Wnt signaling is highly active in the sprouting CtA ECs, and its inhibition results in diminished *glut1* expression¹⁵ and defective CtA anastomosis¹⁰, causing intracerebral hemorrhages at later developmental stages.

In this revised version, we present new experimental data, demonstrating alterations in both the VEGF and WNT signaling pathways in *scarb2a* mutants, each responsible for different aspects of the observed phenotypes:

1. Using the *TgBAC(vegfaa:EGFP)^{pd260 16}* reporter, we show that during the initial stages of CtA sprouting and migration (~32 dpf) *VegfAa* expression is similarly detected in *scarb2a*⁺ RGCs both in wt and mutant embryos (Extended Data Fig. 8h-k). These results are in accordance with the normal development of the BA and initial sprouting of the CtAs from the PHBCs observed in *scarb2a*^{-/-} (fig. 1k). In contrast, by 60 hpf, higher levels of *VegfAa* were observed in

the hindbrains of *scarb2a*^{-/-} embryos, likely produced by RGCs that adopted a neuronal fate (**new Fig. 6h-i'**; **Extended Data Fig. 8l-m'**). These results are further supported by qRT-PCR analyses on whole heads, indicating a specific two-fold increase in the levels of VegfAa transcript (**new Extended Data Fig. 8n-o**), which results in aberrant angiogenesis, as previously described^{5,6,8}.

2. To functionally confirm these results, we treated wt and mutant embryos with the VEGFR2 inhibitor SU5416 at ~32 hpf (right before the initial appearance of the vascular phenotypes). As seen in **new Fig. 6j-m**, we observed a strong decrease in the number of CtA anastomoses, confirming that at least part of the altered angiogenesis phenotypes seen in *scarb2a*^{-/-} are mediated by excess VegfAa production due to the excessive neuronal differentiation.
3. We carried out several experiments to investigate whether Wnt signaling is also altered in the hindbrains of *scarb2a*^{-/-} embryos. Wnt7a has been previously shown to be important for BBB formation in mammals¹² and zebrafish^{10,11}. Accordingly, we assessed the expression of Wnt7Aa in both GFAP⁺ and *scarb2a*⁺ sorted cells at 2 dpf and confirmed a consistent decrease in mutant brains (**new Extended Data Fig. 9a-b**).
4. We also assessed Wnt activation in the hindbrains of wt and mutant embryos using the *Tg(7xTCF-Xla:Sia:GFP)*¹⁷ reporter. As seen in **new Fig. 7a-b'**; **Extended Data Fig. 9c-d'**, we detected decreased Tcf activation (i.e., Wnt signaling downregulation) specifically in CtAs-ECs, confirming that indeed, Wnt signaling is not properly activated in the hindbrain vessels of *scarb2a* mutants.
5. To functionally corroborate these results, we treated *scarb2a* mutants with the Wnt pathway activator LiCl. This treatment, carried out at 32 dpf, resulted in significant rescue of CtA anastomoses (**new Fig. 7c-e'**; **Extended Data Fig. 9e**), Glut1 expression (**new Fig. 7c-e'**), and BBB leakiness (**new Fig. 7f-h**). These data demonstrate that Wnt secreted from RGCs and their glia derivatives (OPCs and astrocytes) are responsible for barrierogenesis in accordance with previous reports^{8-13,15,18}.
6. Finally, we now show that combined treatment with SU5416 and LiCl at 32 hpf results in complete normalization of CtAs anastomosis and vascular morphology (**new Fig. 7i-k**).

Taken together, our new data indicate that the increased neurogenesis and the exhaustion of RGCs with the consequent reduced number of glia subtypes in *scarb2a* mutants have a direct impact on the production of VEGF and Wnt7a, thereby affecting the normal establishment of the CNS vasculature, the neurovascular unit and functionality of the BBB. These results strengthen and expand our original findings showing that overexpression of wt *scarb2a* (*UAS:scarb2a*) in GFAP⁺ cells of *scarb2a* homozygous mutants fully rescues the patterning of the hindbrain vasculature (Fig. 6a-d) and restores Glut1 expression (Fig. 6e-g') by reinstating a normal neuronal vs. glia differentiation balance.

While we agree with the reviewer that it will be interesting to look at the potential involvement of immune cells following the hemorrhage, this will entitle the generation of additional transgenic lines and mutants and an extensive amount of work that we believe falls beyond the scope of our manuscript.

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

I am satisfied with the revised manuscript. Thanks to the authors for their efforts.

Reviewer #4 (Remarks to the Author):

General comments:

This novel work shows that the depletion of scavenger receptor B2 (Scarb2) in neural progenitor cells leads to impaired neurovascular unit (NVU) formation in zebrafish embryos. According to the results, NVU's impairment would ultimately be due to the Scarb2 mutation altering the endolysosomal processing, disrupting Notch3 signaling. In line with previous studies, the impairment in Notch3 signaling would consequently alter the balance of progenitors vs. neuronal/glia cells. In the second version of this manuscript, the authors have added convincing data showing how the disruption of such balance adversely impacts Wnt and VEGF signaling, leading to an altered formation of the neurovascular unit.

The broad context of the study is effectively introduced, clearly outlining the focus of the work and presenting the most significant findings. The study is well-organized and clearly articulated.

The confocal images in the results section are clear and compelling, leaving no ambiguity regarding the assumptions and conclusions drawn by the authors.

The diagrams are detailed yet comprehensible, effectively illustrating the mechanisms under study. The second version of the manuscript contains quantitative graphs with a sufficient number of experimental replicates ensuring robustness.

Importantly, in the second version of this study, the authors have thoroughly addressed the previous reviewers' comments, conducting the necessary experiments to address their concerns and suggestions.

New Figures 6 and 7, as well as Extended Figures 7, 8, and 9, demonstrate the significant efforts the authors have made to collect new data following functional experiments. These additions solidify the author's claims on the link between *scarb2* mutation and Notch, Wnt, and Vegf signaling alterations and their implication in NVU formation.

The discussion section is well-structured and written in clear, precise language. The authors thoughtfully speculate on potential pathological scenarios in which their novel findings might be relevant and have toned down some of the conclusions following the reviewer's suggestions.

The Materials and Methods section is clear and detailed enough for experimental reproducibility. Authors have added experimental procedures that were missing responding to previous reviewer's concerns.

Main comments:

Despite the substantial and convincing work the authors have undertaken to address the questions raised in the initial version, the second version of the manuscript still lacks a crucial piece of data essential for the cohesion of the study. Specifically, the authors have not demonstrated the expression of *scarb2* in RGc progenitors.

This issue was previously highlighted by reviewers 2 and 3. According to the authors, attempts to perform fluorescent in situ hybridization (FISH) experiments for the RGc marker *fabp7a* on the Scarb2 reporter were unsuccessful. Although the authors have conducted alternative experiments, such as new whole-mount chromogenic ISH of *scarb2* and *fabp7a* separately (Extended Data Figure 2) showing similar expression patterns, these results do not respond the primary concern.

However, there are alternative options for the authors to consider, which might help bypass the challenges associated with fluorescent in situ hybridization. One such option is to use a Fabp7a antibody (Millipore #ABN14), which has been previously demonstrated to work in the hindbrain of zebrafish embryos (Belmonte-Mateos et al., 2023). This approach would involve performing a simple ICC for Fabp7a on Scarb2 reporter embryos while maintaining the endogenous fluorescence of the reporter (EGFP). Combining the Fabp7a primary antibody with a Far-Red secondary antibody (to avoid overlapping with EGFP) and incorporating DAPI, could help the authors definitively determine whether Scarb2+ cells are Fabp7a+.

When addressing reviewer comments, the authors have expressed concerns about whether *fabp7a* (*blbp*) is a specific marker for RGc in zebrafish embryos based on Than-Trong and Bally-Cuif (2015). According to this classic review, zebrafish embryonic progenitor cells expressing Blbp could be either neuroepithelial cells (NEc) or RGc. This observation is true. However, Belmonte-Mateos et al. (2023) demonstrate that the *fabp7a*+ cell population in hindbrain rhombomere centers overlaps with the *slc1a2a* (*glt-1*) marker, which Than-Trong and Bally-Cuif (2015) identifies as a marker for RGc when coexpressed with Blbp.

Belmonte-Mateos et al. (2023), along with older studies such as Esain et al. (2010), also demonstrate that the *fabp7a*+ cell population in hindbrain rhombomere centers coexpress *glula* (*gs*) at later stages of embryonic development, which is also a marker of RGc when coexpressed with Blbp according to Than-Trong and Bally-Cuif (2015).

Therefore, if the authors can demonstrate that the *scarb2*+ cells are the same as the *fabp7a*+ cell population in the hindbrain, such piece of evidence would be strong enough to claim hindbrain progenitors expressing *scarb2* are RGc.

Minor comments:

Results -Line 127

It may be difficult for a non-vasculature expert reader to understand what they are looking at in figure 1L-M, showing fli1 reporter transgenic line combined with a gata1 reporter considering: 1) both transgenic lines appear for the first time in the text with no further disclosure or explanation of what they're driving the reporter's expression to, and 2) the vascular system has been evaluated using the kdrl reporter in previous panels of the same figure. Therefore, it would be beneficial for the comprehension of the figure, to briefly describe what these transgenic embryos are labeling. As a suggestion:

We then evaluated the emergence of hemorrhagic bleeding by time-lapse imaging Tg(fli1:EGFP;gata1a:DsRed);scarb2a^{-/-} embryos between 40-60 hpf, *labeling intersegmental blood vessels in green and erythrocytes in red.*

Results -Line 361

Similarly, neither vegfaa nor the transgenic line TgBAC(vegfaa:EGFP)pd260 has been introduced before. Briefly mentioning what is this reporter labeling would be beneficial for the reader's understanding of the figure.

Results -Line 372

There is a preposition lacking: "[...] is mediated by excess *of* VegfAa production[...]"

Discussion- Line 412

Results show impaired production of a specific factor in the pathway, or impaired activation of the pathway. But not production of the signaling pathway.

As a suggestion for a more accurate sentence:

This disruption directly influences the production of *VEGFA and Wnt7a*

This disruption directly influences the *activation* VEGF and Wnt *signaling pathways*”.

Discussion- Line 441

The abbreviation γ -Sec, standing for γ -Secretase appears for the first time without being introduced before. The other 8 times γ -Secretase is mentioned, appears in full. Authors could either introduce the abbreviation earlier or change the abbreviation in line 441 to the full name.

Discussion- Line 459

Similar to the minor comment in line 361

VEGFA acronym for vascular endothelial growth factor A, is introduced for the first time in line 459, in the discussion section. However, it appears 7 times earlier in the study.

It would be beneficial for the understanding of the result sections involving VegfAa if it was introduced earlier, such as in line 34 (Abstract) or at least in line 87 (introduction).

Discussion- Line 463

Suggestion: toning down the strong claim “fully correspond with” to “align with”.

Figure 7a-b'

The panels in this main figure lack the wt vs. mutant genotype labels. Figure 7 seems to be split from Extended Data Fig. 9c-d' which includes the genotype reference. For a better understanding of Figure 7, the genotype labels should be included.

Moreover, in Extended Data Fig. 9c-d', adding a square to indicate where the transverse section in Figure 7 was taken would be beneficial for better locating this region along the anteroposterior axis.

Reviewer #4 (Remarks to the Author)

General comments:

This novel work shows that the depletion of scavenger receptor B2 (Scarb2) in neural progenitor cells leads to impaired neurovascular unit (NVU) formation in zebrafish embryos. According to the results, NVU's impairment would ultimately be due to the Scarb2 mutation altering the endolysosomal processing, disrupting Notch3 signaling. In line with previous studies, the impairment in Notch3 signaling would consequently alter the balance of progenitors vs. neuronal/glial cells. In the second version of this manuscript, the authors have added convincing data showing how the disruption of such balance adversely impacts Wnt and VEGF signaling, leading to an altered formation of the neurovascular unit. The broad context of the study is effectively introduced, clearly outlining the focus of the work and presenting the most significant findings. The study is well-organized and clearly articulated. The confocal images in the results section are clear and compelling, leaving no ambiguity regarding the assumptions and conclusions drawn by the authors. The diagrams are detailed yet comprehensible, effectively illustrating the mechanisms under study. The second version of the manuscript contains quantitative graphs with a sufficient number of experimental replicates ensuring robustness. Importantly, in the second version of this study, the authors have thoroughly addressed the previous reviewers' comments, conducting the necessary experiments to address their concerns and suggestions. New Figures 6 and 7, as well as Extended Figures 7, 8, and 9, demonstrate the significant efforts the authors have made to collect new data following functional experiments. These additions solidify the author's claims on the link between *scarb2* mutation and Notch, Wnt, and Vegf signaling alterations and their implication in NVU formation. The discussion section is well-structured and written in clear, precise language. The authors thoughtfully speculate on potential pathological scenarios in which their novel findings might be relevant and have toned down some of the conclusions following the reviewer's suggestions. The Materials and Methods section is clear and detailed enough for experimental reproducibility. Authors have added experimental procedures that were missing responding to previous reviewer's concerns.

Main comments:

Despite the substantial and convincing work the authors have undertaken to address the questions raised in the initial version, the second version of the manuscript still lacks a crucial piece of data essential for the cohesion of the study. Specifically, the authors have not demonstrated the expression of *scarb2* in RGc progenitors. This issue was previously highlighted by reviewers 2 and 3. According to the authors, attempts to perform fluorescent in situ hybridization (FISH) experiments for the RGc marker *fabp7a* on the *Scarb2* reporter were unsuccessful. Although the authors have conducted alternative experiments, such as new whole-mount chromogenic ISH of *scarb2* and *fabp7a* separately (Extended Data Figure 2) showing similar expression patterns, these results do not respond the primary concern.

However, there are alternative options for the authors to consider, which might help bypass the challenges associated with fluorescent in situ hybridization. One such option is to use a *Fabp7a* antibody (Millipore #ABN14), which has been previously demonstrated to work in the hindbrain of zebrafish embryos (Belmonte-Mateos et al., 2023). This approach would involve performing a simple ICC for *Fabp7a* on *Scarb2* reporter embryos while maintaining the endogenous fluorescence of the reporter (EGFP). Combining the *Fabp7a* primary antibody with a Far-Red secondary antibody (to avoid overlapping with EGFP) and

incorporating DAPI, could help the authors definitively determine whether *Scarb2*⁺ cells are *Fabp7a*⁺. When addressing reviewer comments, the authors have expressed concerns about whether *fabp7a* (*blbp*) is a specific marker for RGc in zebrafish embryos based on Than-Trong and Bally-Cuif (2015). According to this classic review, zebrafish embryonic progenitor cells expressing *Blbp* could be either neuroepithelial cells (NEc) or RGc. This observation is true. However, Belmonte-Mateos et al. (2023) demonstrate that the *fabp7a*⁺ cell population in hindbrain rhombomere centers overlaps with the *slc1a2a* (*glt-1*) marker, which Than-Trong and Bally-Cuif (2015) identifies as a marker for RGc when coexpressed with *Blbp*. Belmonte-Mateos et al. (2023), along with older studies such as Esain et al. (2010), also demonstrate that the *fabp7a*⁺ cell population in hindbrain rhombomere centers coexpress *glula* (*gs*) at later stages of embryonic development, which is also a marker of RGc when coexpressed with *Blbp* according to Than-Trong and Bally-Cuif (2015).

Therefore, if the authors can demonstrate that the *scarb2*⁺ cells are the same as the *fabp7a*⁺ cell population in the hindbrain, such piece of evidence would be strong enough to claim hindbrain progenitors expressing *scarb2* are RGc.

Following the reviewer's suggestion, we have performed immunostaining on *TgBAC(scarb2a:KaITa4;UAS:EGFP)* embryos at 36 and 48 hpf, using the *Fabp7a* antibody recommended by the reviewer. As seen in new Extended Data Fig. 2e-f, we detected *scarb2a* (green) expression in a subset of *Fabp7a*⁺ cells (red) at 30 and 48 hpf (co-localization channel is depicted in yellow). As per the reviewer's definition, based on Belmonte-Mateos et al.,¹ we conclude that *scarb2a* is expressed in a subset of RGcs.

Minor comments:

Results -Line 127

It may be difficult for a non-vasculature expert reader to understand what they are looking at in figure 1L-M, showing *fli1* reporter transgenic line combined with a *gata1* reporter considering: 1) both transgenic lines appear for the first time in the text with no further disclosure or explanation of what they're driving the reporter's expression to, and 2) the vascular system has been evaluated using the *kdrl* reporter in previous panels of the same figure. Therefore, it would be beneficial for the comprehension of the figure, to briefly describe what these transgenic embryos are labeling. As a suggestion:

We then evaluated the emergence of hemorrhagic bleeding by time-lapse imaging *Tg(fli1:EGFP;gata1a:DsRed);scarb2a*^{-/-} embryos between 40-60 hpf, *labeling intersegmental blood vessels in green and erythrocytes in red.*

We appreciate the reviewer's suggestion and have amended the text accordingly.

Results -Line 361

Similarly, neither *vegfaa* nor the transgenic line *TgBAC(vegfaa:EGFP)pd260* has been introduced before. Briefly mentioning what is this reporter labeling would be beneficial for the reader's understanding of the figure.

We appreciate the reviewer's suggestion and have amended the text accordingly.

Results -Line 372

There is a preposition lacking: "[...] is mediated by excess *of* *VegfAa* production[...]"

Corrected.

Discussion- Line 412

Results show impaired production of a specific factor in the pathway, or impaired activation of the pathway. But not production of the signaling pathway.

As a suggestion for a more accurate sentence:

This disruption directly influences the production of *VEGFA and Wnt7a*

This disruption directly influences the *activation* VEGF and Wnt *signaling pathways*”.

We have amended the sentence as follows: “This disruption affects the normal activation of the VEGF and Wnt signaling pathways, thereby impairing the normal development of the CNS vasculature.”

Discussion- Line 441

The abbreviation γ -Sec, standing for γ -Secretase appears for the first time without being introduced before. The other 8 times γ -Secretase is mentioned, appears in full. Authors could either introduce the abbreviation earlier or change the abbreviation in line 441 to the full name.

γ -Secretase is now mentioned always as a full name to avoid confusion.

Discussion- Line 459

Similar to the minor comment in line 361

VEGFA acronym for vascular endothelial growth factor A, is introduced for the first time in line 459, in the discussion section. However, it appears 7 times earlier in the study. It would be beneficial for the understanding of the result sections involving VegfAa if it was introduced earlier, such as in line 34 (Abstract) or at least in line 87 (introduction).

Following the reviewer's suggestion, we describe VEGF-vascular endothelial growth factor in the introduction and the Vegfaa isoform in the Results section.

Discussion- Line 463

Suggestion: toning down the strong claim “fully correspond with” to “align with”.

Corrected.

Figure 7a-b’

The panels in this main figure lack the wt vs. mutant genotype labels. Figure 7 seems to be split from Extended Data Fig. 9c-d’ which includes the genotype reference. For a better understanding of Figure 7, the genotype labels should be included.

Corrected.

Moreover, in Extended Data Fig. 9c-d’, adding a square to indicate where the transverse section in Figure 7 was taken would be beneficial for better locating this region along the anteroposterior axis.

Corrected.

REVIEWERS' COMMENTS

Reviewer #4 (Remarks to the Author):

All my comments and questions have been successfully addressed.
No further comments.