nature portfolio

Peer Review File

Open Access This file is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. In the cases where the authors are anonymous, such as is the case for the reports of anonymous peer reviewers, author attribution should be to 'Anonymous Referee' followed by a clear attribution to the source work. The images or other third party material in this file are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The Study by Lim et al., investigates the role of brain endothelial Piezo1 ion channels in functional hyperemia using laser Doppler and laser speckle imaging to whisker stimulation. This being a logical follow up study to their recently published work showing functional expression of Piezo1 channels in brain and retina endothelial cells. Their results utilize pharmacology and genetic approaches to manipulate Piezo1 function and suggest that EC Piezo1 suppresses functional hyperemia and increases the rate of recovery after the stimulation. The use of gain of function Piezo mutant and brain specific EC cre line to address this question is clever. Overall, the results of this study are important and interesting, however, I have some concerns that should be addressed prior to publication.

1. I do not understand why there is only an effect of Piezo 1 enhancement on FH and not on baseline CBF. Wouldn't you expect Piezo1 activity at baseline given that there is constant and oscillatory flow through the vascular network in vivo? Is the extra ~10-20 % increase that is seen in functional hyperemia really necessary to activate Piezo1?

2. Can the authors be certain that baseline flow be quantitatively compared between mice with laser contrast imaging using their index (Fig. S1)?. I think it would be important to verify this, especially given possible differences in the thinned skull thickness between animals.

3. A key finding of the paper is that the recovery from FH is faster. Although laser speckle imaging is valid, it does not directly measure dilation. It would be nice to verify that the post stimulus return to baseline of arteriole dilation is indeed faster, as suggested. If the authors have 2P imaging capabilities this would be straightforward to access.

4. I am slightly concerned about the variability between cre control mice as can be seen in the CO_2 experiments. Slco1c1-Cre- control mice used in the 2 different groups appear quite different from each other. For example, the tau of the recovery is 504s in Fig.5e and 74.2s in Fig. 5i (which is even faster than the GOF mouse).

I do see it is noted on L319 of discussion that Cre-negative controls exhibited different maximal hyperemic responses and kinetics – which is attributed to likely being due to background differences. Although interesting, if true these differences in genetic background appear to exceed the effect of the Piezo manipulations for CO_2 experiments. This would be something would benefit from being properly characterized and included in the results. I wonder whether Cre+ controls with the same background could be included to help solidify the robustness of this data set.

5. What is the rational for why there is no effect on the rise of the CO_2 hyperemia?

6. Although interesting that there are some behavioral deficits in the Piezo1 GOF mouse, the link with functional hyperemia is purely correlative and whether it is the result of diminished FH or something else is unknown, and I think this should be made clear.

7. I'm not a behavior expert, but the data in the figure seems odd. For example, in fig 6.c and d it appears the average DI is at 50%, suggesting that the controls don't discriminate to begin with, and the mice failed to learn the task. This questions whether making a comparison between groups is valid to begin with. The pie charts are misleading, as more than half the control mice in fig. c prefer the familiar object location to start with. The cumulative scatter plots, however, do look a bit more convincing.

8. Given the behavioral effects, I wonder if neuronal activity is impacted in the GOF mouse? If so how does this affect the interpretation of the FH results?

9. Do the authors have any data indicating the levels of tamoxifen induced recombination in the cdh5 and slco1c1 creERT2 mice using their tamoxifen feeding protocol?

Minor:
L75 "It is unknown, however, whether hyperemia-associated forces themselves affect CBF" – Unknown is a bit too strong wording – e.g. what about the myogenic response?
I couldn't find any info on the CO2 experiments in the methods.
Vehicle controls would be nice for Yoda experiments fig1

Reviewer #2 (Remarks to the Author):

In this study, Lim et al. propose a mechanism by which endothelial cell Piezo 1 ion channels contribute to the recovery of cerebral blood flow following a hyperemic response. The authors used gain of function and knockout Piezo 1 transgenic mice to demonstrate that the enhanced/loss of channel activity, changes the waveform of the functional hyperemia response. Specifically, increased activity blunts FH and accelerates recovery, whereas loss of function increases the FH response. The use of brain endothelial cells-specific mice in this study is impressive, and overall, the data supports their hypothesis, making the Piezo 1 ion channel an integral player in the process of CBF regulation. The authors also demonstrate a role for Piezo1 in reversing CBF following hypercapnia. Interestingly, the recovery rate does not affect the response magnitude here, suggesting different mechanisms involved. Finally, the authors provide evidence that GOF mice also show deficits in memory, suggesting that the impact of channel function can alter cognition.

A few comments are listed below.
Fig.1 was created from an acute window and a 60-second stimulation. Many protocols average a series of consecutive WS runs to reduce variations. Based on the information provided, this does not seem to be the case, with each run shown corresponding to a single stimulus. How do the average waveforms compare? This comment is relevant to all the WS runs in the manuscript if not averaged.

What were the actual MAP values (as opposed to deltas) during the recordings with Yoda1?

Before experiments were started, a minimum 7-day "washout period" was established. How many days after tamoxifen treatment were the studies conducted? Was there consistency in the timing for all experiments?

The rationale for looking at the structural changes is not clear unless there is an expectation that the GOF mice experience a stimulus (e.g., ischemia) that would lead to changes in vascular density. Is this the case? It is a bit unclear how much information is gained from these images. In addition, more details are needed in the methods sections regarding the acquisition and analysis of the data shown in the images in Fig 2j-k. Which cortical layer do images/analysis correspond to? Was this consistent across the acquisition? Same for the hippocampus, was there a specific region acquired/analyzed?

Fig.3. The data is compelling; however, to better assess the differences observed in d,e,h, the entire waveform needs to be shown (as in Fig 5, for example). Also, at times, it is unclear if the WS was averaged or not. Fig 3 d/e refers to "amplitudes of hyperemic responses to three consecutive WS"… were the waveforms of three runs averaged and compared? The protocol for the WS needs to be clarify as it seems different between figures.

Fig.4: Was the phenotype of the brain EC-specific mouse different from the global GOF mouse?

Was the anesthesia for the hypercapnia experiments the same as with the WS? a-chloralose and urethane?

Discussion
Line 286. What is meant by "most ECs (~85-95%) reside in the capillary bed"

The notion that Piezo1 in EC acts as a sensor of hyperemia and provides feedback to return CBF to baseline is interesting; if this were the case for an efficient system, at some point the channel function must override vasodilatory mechanisms. At what point of the FH response is the short-circuit of the EC hyperpolarization taking place?

The section discussing NO-Ca^{2+} and how hemodynamic forces engaged Piezo1-induced Ca^{2+} changes leading to EC depolarization is interesting but unclear. If shear-induced forces lead to the generation of NO via Ca^{2+}, etc… what determines the end of the vasodilatory signaling? Differences in the ion channel expression composition in ECs from different vascular segments, in the cell-cell interaction, and in the levels of Ca^{2+} in the ECs?

Line 314. The authors state that Piezo1 is crucial for CBF recovery "after" hyperemia. However, enhancement (GOF)/absence (KO) of channel function did seem to alter the magnitude of the response (Fig 3d,e). Are the channels involved throughout the hyperemic response? Under physiological conditions, would these channels have a rapid closing? A schematic illustrating the potential sequence of events driving EC Ca^{2+}-mediated vasodilations vs vasoconstrictions would clarify these concepts/pathways.

Intriguingly, the CO_2 hyperemic response did not show a significant change in the magnitude of the response, albeit changes in the recovery. What explains this? Or why are there such pronounced differences with different stimuli (WS, CO_2), considering the putative hemodynamic forces driving activation of the channels may be the same?

The methods section needs further details to help other investigators consider the study's limitations. When appropriate, specify acute cranial window vs thin skull. Given the impact of intracranial pressure, acute inflammation, and other factors on the preparation and potential data,

this information needs to be clearly defined in the methods section. A limitation section is encouraged.

Sometimes Vm or VM is used.

Reviewer #3 (Remarks to the Author):

In their manuscript “Mechano-feedback control of brain blood flow”, Lim et al. introduce endothelial PIEZO1 as a built-in brake on functional hyperemia. They show convincingly that endothelial PIEZO1 gain-of-function alters the magnitude and dynamics of blood-flow response. They also show behavioral differences in endothelial PIEZO1 GOF mice. Their discovery of a functional hyperemia “brake” is an important advance for the field. However, there are some concerns that need to be addressed.

Major concerns:

1. The biggest concerns surround the behavioral testing. The first concern is the claim that neurovascular coupling deficits in endothelial PIEZO1 GOF mice are directly responsible for cognitive deficits. While the authors show differences in NOR, there is no direct evidence that this is due to the NVC changes shown in the rest of the manuscript. Additionally:
• The NOL test seems not to have worked as expected, as the control groups have DI% of ~50 (the exact numbers are not presented). Given this, perhaps the NOL assay should be removed from the study.
• The pie charts for both NOR and NOL (and the associated very low p values) are misleading as they bin data into “discriminative” (DI% >50) and “non-discriminative” (DI% <50)… but only data points = 50 are actually “non-discriminative”; data points below 50 show a preference for the familiar object/location.
• Given that the authors found a significant motor coordination deficit in GOF mice, how can we be sure that this does not affect the exploration of the different objects in the NOR assay?
Given the racial implications of suggesting a link between cognitive deficits and an allele found in high frequency in African Americans, it is crucial that these cognition experiments be exceptionally rigorous. Because the NOL test did not seem to work in control mice, it would be nice to use a second test of memory/learning in its place (regardless of what the results are). It is also important to be sure that the NOR test is properly powered.

2. The other central issue is a lack of discussion or experimentation regarding which part of the vascular tree this PIEZO1-medated NVC brake is occurring. While the hypothesis is explained clearly, this discussion is lacking. What is the rough contribution of arterioles and capillaries to the CBF dynamics picked up by the Doppler imaging? Would you expect that ex vivo PIEZO1 GOF arterioles would have decreased dilation response to both capillary and arteriole K+ stimulation?

There are several other minor issues:

1. Please report actual p values throughout the manuscript, rather than just p<0.05, etc.
2. It would be helpful to discuss the logistics of the cortical Yoda1 application, including what vessels are exposed to the aCSF. Why was cortical application chosen over iv or ip (which would presumably

have less Yoda1 action on other brain cells)?
3. It is a bit confusing to report that PIEZO1 GOF FH was "reduced by X%" when the units of measurement are % themselves. It would be clearer to just state the max ΔCBF (%) for each genotype group.
4. Figure 2b labeling is confusing—it seems like it is the key to 2c (which does not have a key).
5. The n in the data in Figure 2i is very uneven, and it seems as if some of the data is missing from the right graph, as the GOF dataset should presumably have 2.5x more data points than the control (n=17 vs 43)?
6. The authors looked for changes in neurons, astrocytes, and microglia numbers and SMA+ vessel length. It seems strange to neglect pericytes coverage given the subject matter.
7. In Figure S2, the NeuN staining in hippocampus appears to be vascular
8. In Figure 3, it would be helpful to see the trace of the downstroke (as shown in 3h for Yoda1) for the GOF and KO mice.
9. In supplemental figures 6-7, please report n of recordings. Also, for consistency, each data point should be one animal. It does not seem right to use each recording as a data point for upstroke and each mouse as a point for downstroke analyses.
10. In Figures 3 and 4, it is confusing that the data presentation of Slco1c1-CreERT2 GOF line as well as of the KO line are quite different from that presented in Figure 2 for the Cdh5-CreERT2 GOF line. It would be helpful to have the same graph types for each line of mice to allow the reader to make comparisons across different metrics. For instance, the area under the curve data for the Slco1c1 line (4c and 4e, right) seem to be from one representative mouse rather than showing all the data. Relatedly, what is the purpose of showing of the 3 stimulations separately across time (eg in Fig 3d)? Do you expect any differences between these values across time?
11. Is the n in Fig 4g sufficient?
12. It would be helpful to offer a hypothesis as to why PIEZO1 KO mice would exhibit increased FH in response to whisker stimulation (Fig 3d) but not CO_2 (Fig 5g).
13. For the data presented in Figure 6, The methods' equation for DI% says that NO and FO is "the time spent or the frequency of exploring". Was duration or frequency used to calculate DI%?
14. In the discussion, the authors acknowledge that the control cohorts of the GOF and KO mice are very different, perhaps due to strain differences. It would be helpful for this to also be earlier in the text to avoid confusion while looking at the figures.
15. The discussion calls PIEZO1 "the key, but not only" brake for hyperemia, but the only trace data from KO mice shows them recovering completely, just slightly more slowly (after CO_2-induced hyperemia). No trace at all is shown for KO mice in somatosensory experiment. Further, there are no statistics on the ΔCBF for KO mice (Fig 3d), only for the downstroke of tau. Thus, it seems as if data as presented do not support PIEZO1 being the key brake on hyperemia. That said, because there are not really any known brakes for hyperemia, any effect in this regard is interesting.
16. The discussion (lines 340-342) makes it sound as if there are no studies on mechanical force sensing and CSF dynamics, however Piezo1 has been shown to be involved in CSF flow regulation (PMID: 37917195).

REVIEWER COMMENTS

REVIEWER #1

The Study by Lim et al., investigates the role of brain endothelial Piezo1 ion channels in functional hyperemia using laser Doppler and laser speckle imaging to whisker stimulation. This being a logical follow up study to their recently published work showing functional expression of Piezo1 channels in brain and retina endothelial cells. Their results utilize pharmacology and genetic approaches to manipulate Piezo1 function and suggest that EC Piezo1 suppresses functional hyperemia and increases the rate of recovery after the stimulation. The use of gain of function Piezo mutant and brain specific EC cre line to address this question is clever. Overall, the results of this study are important and interesting, however, I have some concerns that should be addressed prior to publication.

We thank the reviewer for their positive remarks and laudatory comments. Please, find below our point-by-point responses to the concerns raised.

1. I do not understand why there is only an effect of Piezo 1 enhancement on FH and not on baseline CBF. Wouldn't you expect Piezo1 activity at baseline given that there is constant and oscillatory flow through the vascular network in vivo? Is the extra ~10-20 % increase that is seen in functional hyperemia really necessary to activate Piezo1?

The question is of major interest to us. If we were to speculate as to why there is only a clear impact for Piezo1 enhancement on FH but no overt change in baseline blood flow, a possible explanation could be that increases in flow and pressure during cardiac cycles and the oscillatory flow through vascular networks are too transient, and therefore fail to engage Piezo1 sufficiently for baseline CBF to alter. Related to this speculation, we are actively pursuing blood flow pulsatility analyses and how this could be affected when Piezo1 activity is enhanced, but this work is at a very early stage. These studies also raise questions beyond the scope of this study about slower hemodynamics such as vasomotion, which will be perhaps the focus of future studies. Another possibility is that the extent to which Piezo1 activity is enhanced (minutes after Yoda1 application, or a couple of weeks after induction in GOF mice with tamoxifen) might be well sufficient to cripple FH, but not strong and sustained enough to alter baseline CBF. We appreciate the importance of this question, and therefore we included these possibilities in the revised manuscript Discussion section:

"While Piezo1 enhancement profoundly affected FH, there was no overt impact on baseline CBF. This could reflect insufficient forces (i.e., transient oscillatory hemodynamics) to trigger a mechano-feedback mechanism under baseline conditions. It could also be attributed to the extent of Piezo1 engagement (minutes after Yoda1, or days/weeks after induction in GOF mice) being sufficient to cripple FH, but not strong and sustained enough to alter baseline CBF. These possibilities await future investigations."

2. Can the authors be certain that baseline flow be quantitatively compared between mice with laser contrast imaging using their index (Fig. S1)? I think it would be important to verify this, especially given possible differences in the thinned skull thickness between animals.

We thank the reviewer for the question. In the present study, we follow the guidelines to achieve reliable and repeatable measurements using LSCI [1,2]. Briefly, we ensure that the imaging parameters are in the optimal range, allowing high precision of the measurements – the speckle-to-pixel size ratio is maintained at approximately 2 [1], a stabilized laser diode with long coherence length is used [3], and a linear polarizer in cross-polarization configuration is installed [2,4]. The parameters were maintained constant, and no changes to the system were introduced during the study. Furthermore, we have validated the parameters' stability by measuring the contrast of static scattering phantom several times during the study and found it unchanging. These steps ensure that LSCI measurements are compatible longitudinally and between the animal groups. While we agree with the reviewer that the thin-skull preparation might affect blood flow index measurements – we expect it to be independent of the animal group and, therefore, not affect the conclusions of the study. Furthermore, we minimize the possible influence of the skull tissue on the BFI measurements by using temporal contrast analysis, which is less sensitive to static scattering [5].

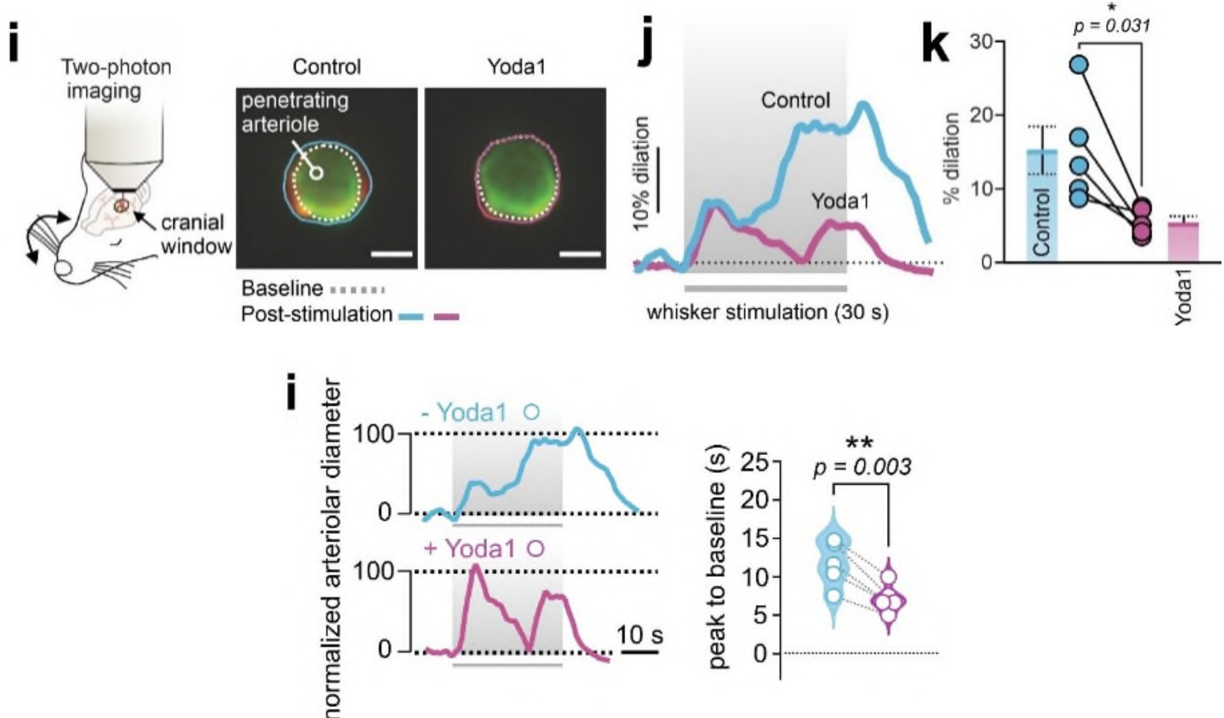
1. *Sunil S, Zilpelwar S, Boas DA, Postnov DD. Guidelines for obtaining an absolute blood flow index with laser speckle contrast imaging. bioRxiv. 2021 Apr 4:2021-04.*
2. *González Olmos A, Zilpelwar S, Sunil S, Boas DA, Postnov DD. Optimizing the precision of laser speckle contrast imaging. Scientific Reports. 2023 Oct 20;13(1):17970.*
3. *Postnov DD, Cheng X, Erdener SE, Boas DA. Choosing a laser for laser speckle contrast imaging. Scientific reports. 2019 Feb 22;9(1):2542.*
4. *Akther S, Mikkelsen MB, Postnov DD. Choosing a polarisation configuration for dynamic light scattering and laser speckle contrast imaging. Biomedical Optics Express. 2024 Jan 1;15(1):336-45.*
5. *Boas DA, Dunn AK. Laser speckle contrast imaging in biomedical optics. Journal of biomedical optics. 2010 Jan 13;15(1):011109.*

In response to this comment, we have made further clarifications and explanations in the methodology section:

"To achieve reliable and repeatable measurements with LSCI, we ensured that the imaging parameters were in optimal range – the speckle-to-pixel size ratio was maintained at approximately 2[91], a stabilized laser diode with long coherence length was used[92], and a linear polarizer in cross-polarization configuration was installed[90,93]. The parameters were maintained constant, and no changes to the system were introduced during the study. Furthermore, we have validated the parameters' stability by measuring the contrast of static scattering phantom several times during the study and found it unchanging. These steps ensure that LSCI measurements are compatible longitudinally and between the animal groups. Furthermore, in the analysis step, we minimized the possible influence of the skull tissue on the BFI measurements by using temporal contrast analysis over 25 consecutive frames, which is known to be less sensitive to static scattering[94]. "

3. A key finding of the paper is that the recovery from FH is faster. Although laser speckle imaging is valid, it does not directly measure dilation. It would be nice to verify that the post stimulus return to baseline of arteriole dilation is indeed faster, as suggested. If the authors have 2P imaging capabilities this would be straightforward to access.

*In response to the reviewer's comment, we performed a new set of 2-photon laser scanning microscopy experiments to measure arteriolar dilation in response to whisker stimulation while Piezo1 activity is manipulated. Whisker stimulation evoked profound dilation of penetrating arterioles. The latter was inhibited after the application of Yoda1 on the cranial window (**Figure. 1i, j, k**). Further, arteriolar diameter return to baseline was faster in the presence of Yoda1 (**Figure. 3i**).*



4. I am slightly concerned about the variability between Cre control mice as can be seen in the CO_2 experiments. Slco1c1-Cre- control mice used in the 2 different groups appear quite different from each other. For example, the tau of the recovery is 504s in Fig.5e and 74.2s in Fig. 5i (which is even faster than the GOF mouse). I do see it is noted on L319 of discussion that Cre-negative controls exhibited different maximal hyperemic responses and kinetics – which is attributed to likely being due to background differences. Although interesting, if true these differences in genetic background appear to exceed the effect of the Piezo manipulations for CO_2 experiments. This would be something that would benefit from being properly characterized and included in the results. I wonder whether Cre+ controls with the same background could be included to help solidify the robustness of this data set.

We understand the reviewer's concern. Our response is broken down below:

i- Regarding the hypercapnia experiments, we performed additional experiments to assess tau values and other kinetic parameters with greater precision. On average, tau

*recovery in control mice **Fig. 5f** is ~190s compared to ~90s in **Fig. 5m**. We believe that the reason for these differences is the genetic background of the mice as explained below (ii). The reason that the values in the revised manuscript are a little shifted from the original manuscript is we originally averaged all traces and then assessed tau values. This approach did not take into consideration the varying lag times in different mice till the onset of recovery (now shown in **Fig. 5g**). Analysis of individual tau values (a single value based on the fitting from a mouse) overcomes this issue and more accurately captures differences.*

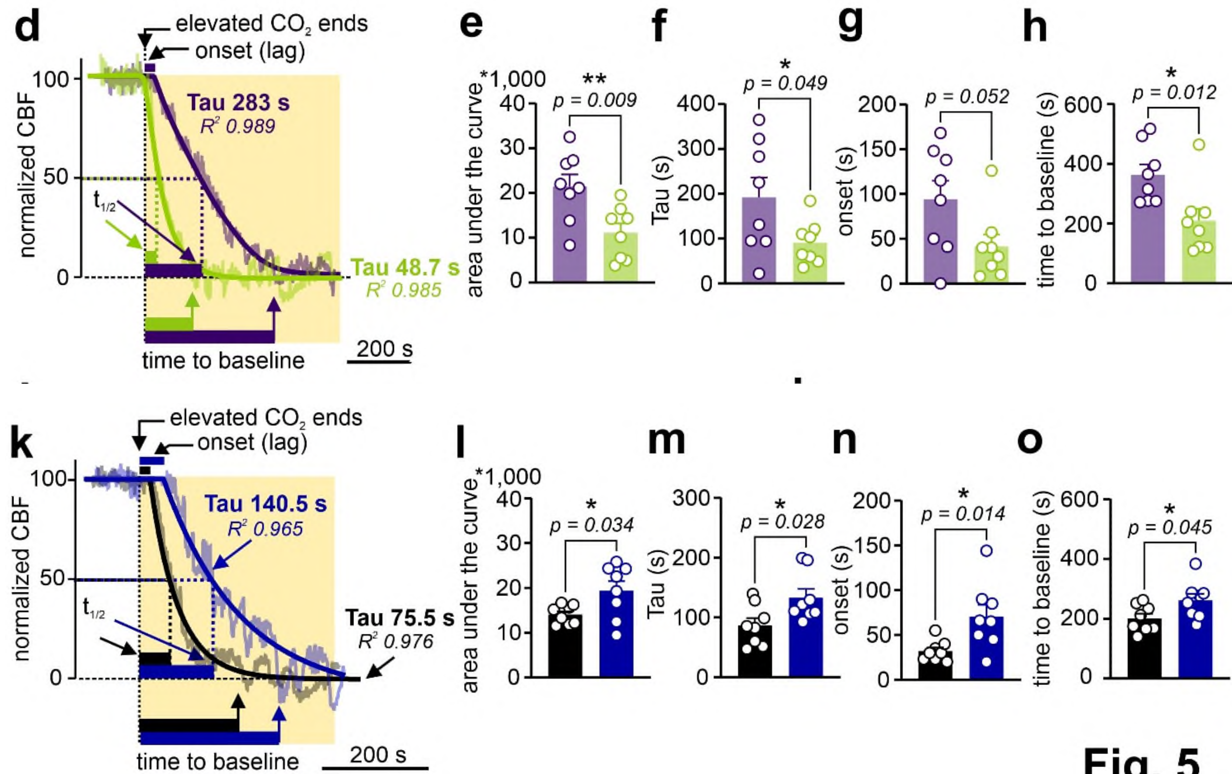
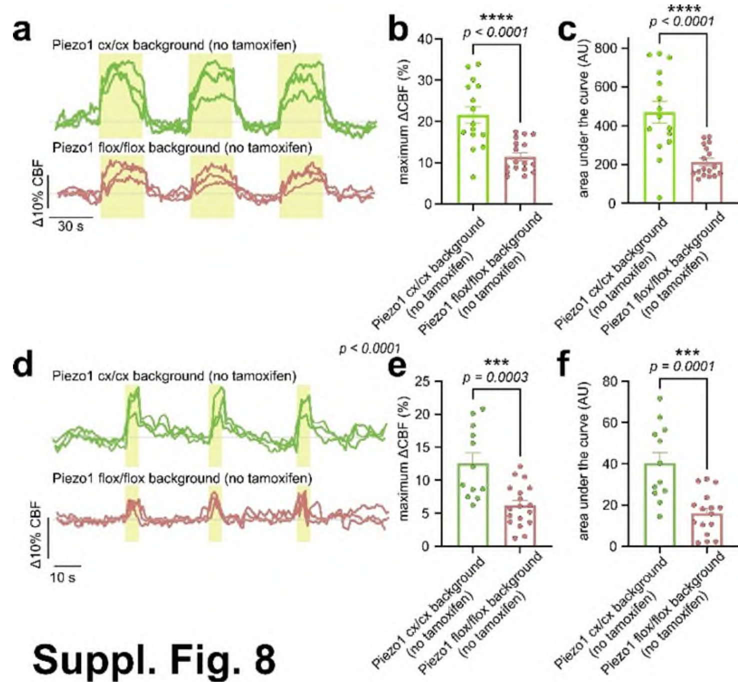


Fig. 5

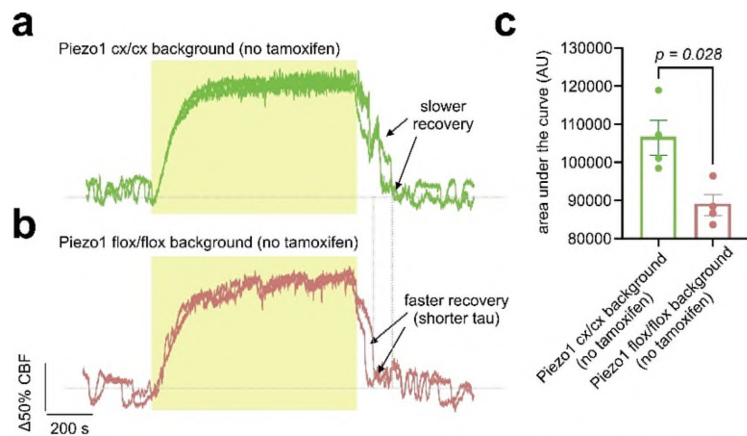
*ii- The differences observed here between different controls are largely due to differences in genetic backgrounds of the floxed Piezo1 (Piezo1$^{flox/flox}$) and the knock-in Piezo1 GOF (Piezo1$^{cx/cx}$) mice. Piezo1$^{flox/flox}$ mice (from Jackson Labs) were generated in C57BL/6 background. On the other hand, Piezo1$^{cx/cx}$ mice (kindly provided by Dr. Ardem Patapoutian) were initially generated in BALB/c background. Since Cdh5-Cre and Slco1c1-Cre mice were in the C57BL/6 background, we don't anticipate that these Cre mouse lines are the major source of genetic background differences. Upon crossing Cre and floxed mice, KO mice maintained a C57BL/6 background, but the GOF mice had a mixed BALB/c-C57BL/6 background. Two lines of evidence support that the genetic background could explain the observed differences. **First**, previous work has documented differences in Piezo1 mediated Ca^{2+} signaling in keratinocytes from Piezo1 KO controls (generated using Piezo1$^{flox/flox}$ mice) and Piezo1 GOF controls (generated using Piezo1$^{cx/cx}$ mice). Such differences were attributed to different genetic backgrounds of the two strains (PMID: 34569935), as explained above. Here, we followed a similar approach where in all experiments, mutant mice (KO or GOF) were compared to littermate control mice of the same genetic background. **Second**, from a CBF standpoint, there is strong*

*evidence of key differences in CBF between BALB/c and C57BL/6 mice (e.g., Kang et al., 2015 PMID: 25833343; Kanoke et al., 2020 PMID: 32669022). These differences range from a tendency for higher CBF in BALB/c mice compared with C57BL/6 mice, to lower mean transit time in C57BL/6 mice. Furthermore, the numbers of cerebrovascular branches and collateral vessels are different between the two strains. These changes are consistent with the differences we observed between controls of Piezo1 KO versus GOF controls (**Fig. 3; S6; 5**).*

*iii- As suggested by the reviewer and to help solidify the robustness of our datasets, we performed a new experimental series in which we assessed FH (whisker stimulation) and hypercapnia-induced hyperemia in mice from different genetic backgrounds (i.e., homozygous Piezo1 flox/flox mice versus homozygous Piezo1cx/cx mice) that were not treated with tamoxifen. This experiment serves to answer the question whether pre-existing differences that are independent of Cre-recombinase induction are strong enough to be observed. As depicted in new supplementary figures (**Fig. S8 and S12**), non-tamoxifen treated Piezo1$^{cx/cx}$ mice (Cre+ and Cre-) showed hyperemic responses that were distinct in amplitude and kinetics from Piezo1$^{flox/flox}$ mice (Cre+ and Cre-; no tamoxifen).*



Suppl. Fig. 8



Suppl. Fig. 12

iv- As mentioned above, there is evidence that differences exist between Piezo1$^{cx/cx}$ and Piezo1$^{flox/flox}$ mice, rather than between different Cre lines. While new Cre+ controls with a similar background can be back-engineered, these mice are not currently available. We estimate that backcrossing to obtain controls of similar backgrounds would minimally require 4-6 months, exceeding the time allowed for this revision.

To summarize, we performed new experiments and added the following clarification in the Results section of the revised manuscript in response to the reviewer's comment:

*"Notably, Piezo1$^{cx/cx}$ and Piezo1$^{flox/flox}$ mice have different genetic backgrounds that are known to alter Piezo1-mediated Ca^{2+} signaling in Cre- controls[30]. Further, these backgrounds demonstrate key differences in CBF dynamics[31,32]. Given the differences observed in CBF across control (Cre-) mice here (**Fig. 3d, e**) and the differences seen in Piezo1$^{cx/cx}$ and Piezo1$^{flox/flox}$ Cre- and Cre+ without tamoxifen treatment (i.e., no induction; **Fig. S8**), hyperemic responses were only compared to controls of the same genetic background in all analyses."*

5. What is the rationale for why there is no effect on the rise of CO_2 hyperemia?

Our hypothesis was that like functional hyperemia, hypercapnia-induced hyperemia will be different in magnitude, rise and fall. We consistently observed differences in the recovery phase, but not in the rise. This could be attributed to the dramatic temporal differences between whisker stimulation (30 s) versus the 30-fold longer CO_2 stimulation (900 s). It is important to highlight that the mechanisms underlying different hyperemic responses are distinct. In response to this comment/question, we have now included the following in the results section:

"We expected that—like FH—hypercapnia-induced hyperemia will be different in GOF mice in both magnitude and kinetics, but we observed consistent differences only during recovery. This could be attributed to the huge temporal variability between stimulations (5-30 s versus 900 s), and the distinct mechanisms underlying different hyperemic responses[36,37]."

6. Although interesting that there are some behavioral deficits in the Piezo1 GOF mouse, the link with functional hyperemia is purely correlative and whether it is the result of diminished FH or something else is unknown, and I think this should be made clear.

Our study reports an intriguing observation that altering Piezo1 activity in (brain) endothelial cells is associated with behavioral deficits. We agree with the reviewer that there is no definitive and direct evidence that the NVC deficits observed in Piezo1 GOF mice are responsible for cognitive deficits. As suggested by the reviewer, we have made this clear in the revised manuscript. Below are a few examples:

> *"Whether the neurovascular impairment is responsible for cognitive deficits in GOF mice remains to be confirmed."*
> *"...suggesting a potential link between Piezo1 impact on neurovascular coupling and cognition."*

7. I'm not a behavior expert, but the data in the figure seems odd. For example, in fig 6.c and d it appears the average DI is at 50%, suggesting that the controls don't discriminate to begin with, and the mice failed to learn the task. This questions whether making a comparison between groups is valid to begin with. The pie charts are misleading, as more than half the control mice in fig. c prefer the familiar object location to start with. The cumulative scatter plots, however, do look a bit more convincing.

We agree with the reviewer. The novel object location (NOL) tests revealed discrimination indices that were below 50%. As suggested by the reviewer, this is indicative that the mice failed to learn the task and therefore making comparisons between genotypes would be invalid. Therefore, we removed these results from the revised manuscript. The concern related to pie charts being misleading is legit, and therefore they were removed.

*To address the question whether Piezo1 manipulation affects memory and cognition, we performed new experiments (spontaneous alternation T-maze) which revealed profound deficits in Piezo1 brain-EC GOF mice compared with their respective controls (**Fig. 6g-h**).*

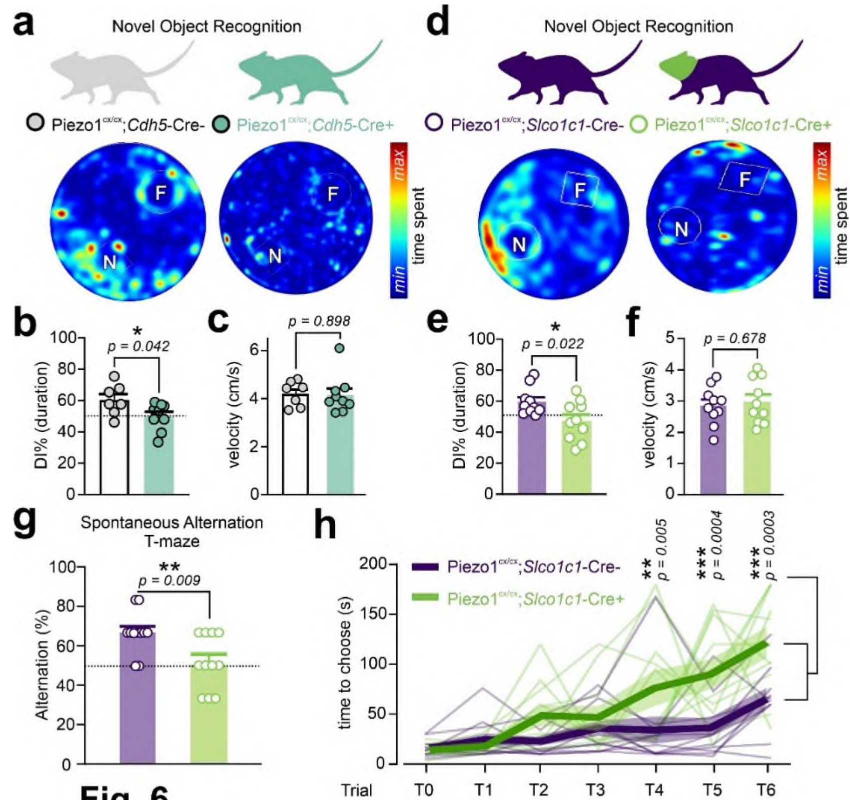


Fig. 6

8. Given the behavioral effects, I wonder if neuronal activity is impacted in the GOF mouse? If so, how does this affect the interpretation of the FH results?

The impact of Piezo1 GOF on the interplay between neuronal activity and functional hyperemia is an essential component of our work. We have found interesting behavioral effects in these mice. However, testing whether neuronal activity is altered in the GOF was beyond the scope of our methods as we focused on characterizing the endothelial component of the neurovascular unit. Ongoing studies are directly aimed at the question whether neural activity is impacted in GOF mice. We respectfully note that the proper assessment of neuronal activity in the different genotypes used here (i.e., EC-Piezo1-GOF, brain-EC-Piezo1-GOF, EC-Piezo1-KO, brain-EC-Piezo1-KO and the respective controls) is a massive undertaking that goes beyond the scope of this study and would require a much longer duration than available for this revision. Further, the question itself is complex because if we find altered neuronal activity in the GOF, it will be important to dissect whether this activity is altered only when the neurovascular unit is intact or whether activity is altered in neurons isolated from GOF mice. Based on our findings

demonstrating dynamic stimulus-dependent alterations, we hypothesize that the former will be true - the neurovascular unit including the GOF endothelial cells is required for acute alterations in neuronal activity.

9. Do the authors have any data indicating the levels of tamoxifen induced recombination in the cdh5 and slco1c1 creERT2 mice using their tamoxifen feeding protocol?

We have used functional assessment of Piezo1 activity after the tamoxifen feeding protocol as we have done previously for Cdh5 KO (Harraz et al., 2022 Circulation Research PMID: 35382561). Since our focus has been the impact of altered Piezo1 function of blood flow control, we systematically used patch clamp electrophysiology to assess Piezo1 channel function. This is included in the current manuscript in ***Fig. 2b, c*** *for Cdh5 GOF as well as our previous study for the KO mice (PMID: 35382561). Please, note that we used a tamoxifen feeding protocol with Slco1c1-CreER^{T2} mice in a previous study (PMID: 32151223) which led to a reduction of the respective gene expression by 72.5%, measured in brain endothelial cells derived from treated mice.*

Minor: L75 "It is unknown, however, whether hyperemia-associated forces themselves affect CBF"– Unknown is a bit too strong wording – e.g. what about the myogenic response?

As suggested, we tuned down the sentence in question as suggested:

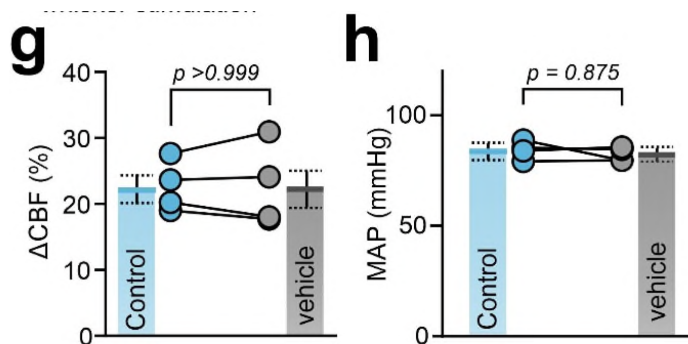
<u>"It is not fully understood, however, how hyperemia-associated forces within the cerebrovasculature could themselves affect CBF."</u>

Minor: I couldn't find any info on the CO_2 experiments in the methods.

The details of the CO_2 inhalation experiment have been added to the Methods section.

Minor: Vehicle controls would be nice for Yoda experiments fig1

We thank the reviewer for the important suggestion. In the revised manuscript, we have performed a new experiment (shown in ***Fig. 1g, h****) to assess the impact of cortical vehicle (aCSF supplemented with DMSO) on functional hyperemia and mean arterial blood pressure. In contrast to Yoda1, DMSO failed to affect functional hyperemic responses.*

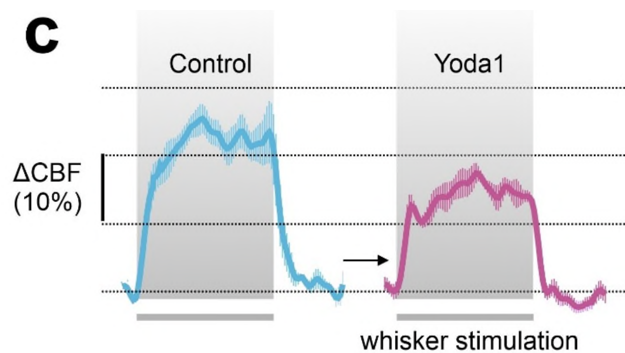


REVIEWER #2

In this study, Lim et al. propose a mechanism by which endothelial cell Piezo 1 ion channels contribute to the recovery of cerebral blood flow following a hyperemic response. The authors used gain of function and knockout Piezo 1 transgenic mice to demonstrate that the enhanced/loss of channel activity, changes the waveform of the functional hyperemia response. Specifically, increased activity blunts FH and accelerates recovery, whereas loss of function increases the FH response. The use of brain endothelial cells-specific mice in this study is impressive, and overall, the data supports their hypothesis, making the Piezo 1 ion channel an integral player in the process of CBF regulation. The authors also demonstrate a role for Piezo1 in reversing CBF following hypercapnia. Interestingly, the recovery rate does not affect the response magnitude here, suggesting different mechanisms involved. Finally, the authors provide evidence that GOF mice also show deficits in memory, suggesting that the impact of channel function can alter cognition. A few comments are listed below.

We thank the reviewer for their comments. Please, find our point-by-point responses below.

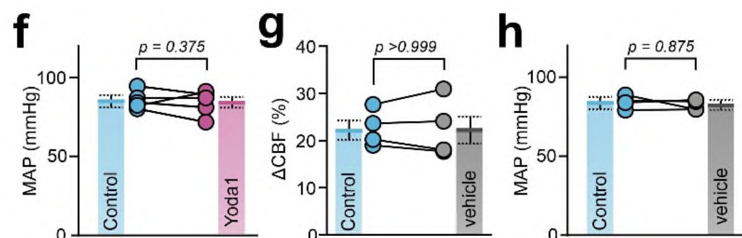
Fig.1 was created from an acute window and a 60-second stimulation. Many protocols average a series of consecutive WS runs to reduce variations. Based on the information provided, this does not seem to be the case, with each run shown corresponding to a single stimulus. How do the average waveforms compare? This comment is relevant to all the WS runs in the manuscript if not averaged.



*We thank the reviewer for the question. We previously used representative traces for whisker stimulation-evoked functional hyperemia (e.g., PMID: 34351870; 33875602; 28319610; 33763649). As noted by the reviewer, some protocols average consecutive whisker stimulation runs and display overall averages. In the original manuscript, all WS displays were representative traces reflecting the median of the data, rather than averaged traces (in **Fig. 1, 2, 3, 4**). To address the reviewer's comment, we included the average waveforms of whisker stimulation induced FH in C57BL/6 mice before and after Yoda1 application (**Fig. 1c**) in the revised manuscript. These averaged responses were similar to the representative traces used originally. For consistency throughout the manuscript, we kept single run examples that reflect the median response in other figures.*

What were the actual MAP values (as opposed to deltas) during the recordings with Yoda1?

*The actual MAP values before and after Yoda1 as well as before and after vehicle are now included in **Fig. 1**.*



Before experiments were started, a minimum 7-day "washout period" was established. How many days after tamoxifen treatment were the studies conducted? Was there consistency in the timing for all experiments?

The washout duration was 3-5 weeks for most experiments (imaging). The behavioral tests were performed after a ~7-10-week washout period. We have added this information in the methods section.

"Tamoxifen treatment was followed by a washout period before experimental intervention (3-5 weeks for CBF experiments, 7-10 weeks for behavioral studies)."

The rationale for looking at the structural changes is not clear unless there is an expectation that the GOF mice experience a stimulus (e.g., ischemia) that would lead to changes in vascular density. Is this the case? It is a bit unclear how much information is gained from these images. In addition, more details are needed in the methods sections regarding the acquisition and analysis of the data shown in the images in Fig 2j-k. Which cortical layer do images/analysis correspond to? Was this consistent across the acquisition? Same for the hippocampus, was there a specific region acquired/analyzed?

We thank the reviewer for the opportunity to clarify. Mechanical stimuli and the sensitivity to shear stress play a critical role in angiogenesis and vessel survival in the periphery. We observed functional changes in functional hyperemia in GOF mice. Therefore, we aimed to assess morphological changes, to confirm or exclude the possibility that the observed functional changes were secondary to a structural impairment. We added the following clarification in the Results section:

"Structural changes in the cerebral vasculature could lead to a defect in FH. To test whether cerebrovascular structural changes in GOF mice underlie the impaired CBF responses, we stained brain slices from the cortex and hippocampus for ECs and basement membrane, as we have done before[27]."

In the Methods section, we describe how vessel density and string vessels are measured and analyzed:

"Images were taken using confocal microscopes (Leica, SP5 and Stellaris 5). Image stacks were taken (50 µm thickness, 5 µm steps) and analyses were performed on z-projections. Empty basement membrane tubes (string vessels) were defined as endothelial-negative and basement membrane-positive structures, thinner than 4 µm and measured manually using ImageJ."

The cortical and hippocampal regions we chose were similar and consistent across the acquisition and between the samples. We included this information in the Methods section:

"The cortical and hippocampal regions were similar and consistent across the acquisition and between the samples. Cortical images contained layers 2-4 and hippocampal images were taken in the regions of the dentate gyrus and CA1."

Fig.3. The data is compelling; however, to better assess the differences observed in d,e,h, the entire waveform needs to be shown (as in Fig 5, for example). Also, at times, it is unclear if the WS was averaged or not. Fig 3 d/e refers to "amplitudes of hyperemic responses to three consecutive WS"… were the waveforms of three runs averaged and

compared? The protocol for the WS needs to be clarify as it seems different between figures.

As suggested by the reviewer, we have included entire waveforms of FH from the different genotypes studied. To avoid any confusion to the reader, we decided to replace figures 3d and 3e with representations of FH responses and scatter plots showing that FH amplitude differs when Piezo1 activity changes. Additional details have been included in all figure legends to clarify the protocols for WS that were used.

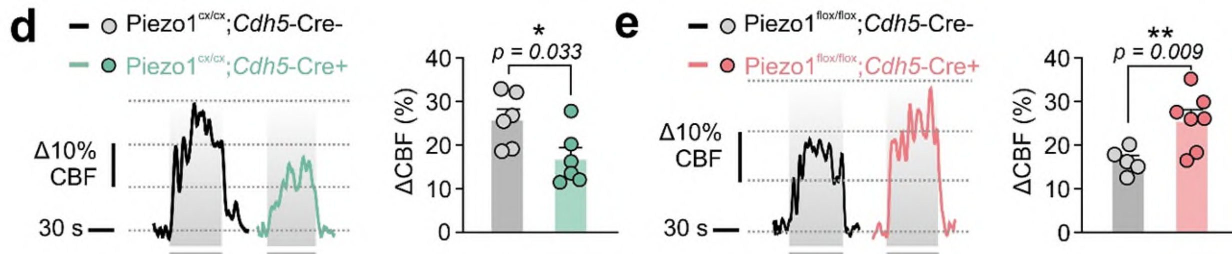


Fig.4: Was the phenotype of the brain EC-specific mouse different from the global GOF mouse?

We observed similar suppression of FH in both genotypes (i.e., pan-EC and brain-EC), as shown in the manuscript. In behavioral tests where both genotypes were used (e.g., novel object recognition), we observed similar deficits. Some other experiments employed only the brain-EC-specific mouse model, since we aimed to minimize possible peripheral effects of Piezo1 manipulation, given the literature supporting a vascular role for Piezo1 in the peripheral vascular networks (Wang et al., 2016 JCI; Rode et al., 2017 Nat Comm).

Was the anesthesia for the hypercapnia experiments the same as with the WS? a-chloralose and urethane?

Yes, the same anesthesia was used. We have clarified this in the revised manuscript.

"Hypercapnia (elevated CO_2) was evoked in anesthetized mice (urethane and α-chloralose) by elevating CO_2 in the inhaled air to 10% while monitoring CBF."

Discussion: Line 286. What is meant by "most ECs (~85-95%) reside in the capillary bed"

We apologize for the lack of clarity. We have edited the sentence in question, and it now reads:

"However, most brain ECs (~80%) are capillary ECs[53], where functional expression of K_{Ca} channels is lacking[8]."

The notion that Piezo1 in EC acts as a sensor of hyperemia and provides feedback to return CBF to baseline is interesting; if this were the case for an efficient system, at some point the channel function must override vasodilatory mechanisms**.** At what point of the FH response is the short-circuit of the EC hyperpolarization taking place?

This is a great question. At this point, we are uncertain about the critical short-circuit point. From an electrophysiological standpoint, our direct measurements of the major contributor to hyperpolarization (i.e., Kir2.1 current, Harraz et al., 2018 PNAS; Longden et al., 2017 Nat Neurosci) and the known channel properties (Longden and Nelson, 2015) suggest that the outward hyperpolarizing current at physiological conditions is ~6 fA.

Elevation of external K^+ to 10 mM would increase Kir2 current at this voltage to ~260 fA, that is sufficient to ensure conduction fidelity (Longden et al., 2017), and presumably exceeds the overall inward (depolarizing) current. For Piezo1, we measured an open probability (NPo) of 0.002 (Harraz et al., 2022), and the unitary current (i) at physiological Vm is ~ 0.7 pA. Based on the assumptions that: i) each patch had 0.8 channels, ii) Piezo1 channel is uniformly distributed across the plasma membrane of capillary EC (surface area ~800 μm^2), and iii) surface area of a patch is 4 μm^2:

$$I_{Piezo1} = NPo * i * 0.8 * 800/4 = 0.002 * 0.7 * 0.7 * 200 = 0.196\ pA$$

Therefore, our guesstimate is that the inward Piezo1 current under baseline condition is ~200 fA, a value lower than the estimated hyperpolarizing Kir2.1 current during hyperemia. We speculate that mechanical forces increase Piezo1 open probability, producing a current in the pico-ampere range—sufficient to effectively short circuit K^+-induced hyperpolarization and cripple this key Kir2.1-based NVC mechanism. Taken together, these calculations imply that the increase in Piezo1 activity during hyperemia would be able to induce a depolarization that would overcome hyperpolarizing signals.

Further, Piezo1 activation in response to forces has been observed by our group and others. An important consideration is that responses can be virtually immediate (Harraz et al., Circ Res 2022), but we also observed immediate and delayed openings in our electrophysiological recordings. Others have shown that endothelial Piezo1 signaling demonstrates slow inactivation kinetics (Shi et al., Cell Reports 2020), suggesting that Piezo1 engagement could lead to sustained responses. It is therefore unclear at what point of the FH response Piezo1 can be engaged to affect CBF. This is an area of active research in our laboratory.

In response to the reviewer's comment, we have enhanced our discussion of the proposed short-circuit role of Piezo1 in the Discussion section:

"The evidence presented here demonstrates that Piezo1 activation depolarizes ECs, consistent with a role for Piezo1 as a feedback mechanism to reset endothelial V_m. We suggest that increases in Piezo1 inward depolarizing current during FH may surpass hyperpolarization and act as a short-circuit to reset V_m."

The section discussing NO-Ca^{2+} and how hemodynamic forces engaged Piezo1-induced Ca^{2+} changes leading to EC depolarization is interesting but unclear. If shear-induced forces lead to the generation of NO via Ca^{2+}, etc… what determines the end of the vasodilatory signaling? Differences in the ion channel expression composition in ECs from different vascular segments, in the cell-cell interaction, and in the levels of Ca^{2+} in the ECs?

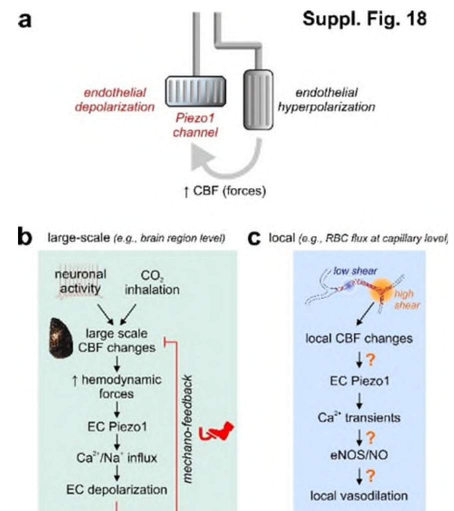
Line 314. The authors state that Piezo1 is crucial for CBF recovery "after" hyperemia. However, enhancement (GOF)/absence (KO) of channel function did seem to alter the magnitude of the response (Fig 3d,e). Are the channels involved throughout the hyperemic response? Under physiological conditions, would these channels have a rapid closing? A schematic illustrating the potential sequence of events driving EC Ca^{2+}-mediated vasodilations vs vasoconstrictions would clarify these concepts/pathways.

We combine our response to both comments by the reviewer for better clarity.

As we mention in the discussion, different scenarios could follow brain EC Piezo1 activation and Ca^{2+}/Na^{+} influx. Ca^{2+} transients could activate endothelial NO synthase (eNOS) leading to the generation of the short-lived vasodilator NO that could dilate only proximal capillaries and arterioles, leading ultimately to a local increase in blood flow (Longden et al., 2021 Sci Adv). However, we have shown earlier that in deep capillaries there was no correlation between Ca^{2+} signals and blood flow (Longden et al., 2021 Sci Adv), suggesting that differences in cell-cell interactions (EC-mural cell) play an important role in this signaling cascade. Additionally, differences in the repertoire of ion channel expression composition in ECs from different vascular segments can determine the effect of Ca^{2+} on Ca^{2+}-activated targets. Furthermore, we have preliminary evidence for a different study that endothelial Piezo1 regulates CBF at a local level (Ca^{2+}/NO-dependent) and a large-scale level (this study). The rationale for this is that not all hemodynamic triggers are the same. A local change in red blood cell (RBC) flux promotes a mechanical stimulus that differs spatiotemporally and in magnitude from hemodynamic changes in extended active brain regions (e.g., somatosensory cortex). We have observed that spatially restricted changes in forces (e.g., change in shear stress in one capillary but not in neighboring segments) evoke Piezo1-mediated Ca^{2+} signals leading to nitric oxide (NO) generation and local vasodilation. On the other hand, large-scale changes in forces (e.g., in thousands of vascular segments in the somatosensory cortex during FH) lead to a cation influx through Piezo1 channels that, in turn, acts as a repolarizing signal to restrict hyperpolarization-mediated FH, much like a built-in brake system. The findings in our study that FH and hypercapnia-induced hyperemia are affected by Piezo1 manipulations are consistent with a feedback inhibitory role for Piezo1. Such a role cannot be explained by NO signaling, but rather by a change in V_m. Considering the critical role for hyperpolarizing signals in driving hyperemia, we proposed that Piezo1 could act as circuit breakers.

<u>Are the channels involved throughout the hyperemic response?</u> Based on our findings and the inhibition of short FH responses (5s), it is likely that Piezo1 channels are engaged throughout FH. We showed before (Harraz et al., 2022 Circ Res) that changes in flow trigger Piezo1 activation throughout the duration of stimulus.

<u>Under physiological conditions, would these channels have a rapid closing?</u> As mentioned earlier, others have shown that endothelial Piezo1 exhibits slow inactivation kinetics (Shi et al., Cell Reports 2020), contrary to the dogma that Piezo1 inactivates and closes very rapidly (within milliseconds). Our previous analysis corroborates these findings. We observed long lasting Ca^{2+} transients and inward currents that were Piezo1 mediated (Harraz et al., 2022 Circ Res).



<u>A schematic illustrating the potential sequence of events driving EC Ca^{2+}-mediated vasodilation vs vasoconstrictions would clarify these concepts/pathways</u>.

As suggested by the reviewer, we have included a new figure (Fig. S18) in the revised manuscript depicting

different paradigms for Piezo1 signaling in the brain endothelium.

Intriguingly, the CO_2 hyperemic response did not show a significant change in the magnitude of the response, albeit changes in the recovery. What explains this? Or why are there such pronounced differences with different stimuli (WS, CO_2), considering the putative hemodynamic forces driving activation of the channels may be the same?

Similar to our response to a similar comment from Reviewer #1, our hypothesis was that like FH, hypercapnia-induced hyperemia will be different in magnitude, rise and fall when Piezo1 activity is altered. We consistently observed differences in the recovery phase, but not in the rise. This could be attributed to the dramatic temporal differences between whisker stimulation versus the 30-fold longer CO_2 stimulation (900 s). It is also important to highlight that the mechanisms underlying both hyperemic responses could be distinct. In response to this comment/question, which has been legitimately raised by other reviewers, we have included the following in the results section:

"We expected that—like FH—hypercapnia-induced hyperemia will be different in GOF mice in both magnitude and kinetics, but we observed consistent differences only during recovery. This could be attributed to the huge temporal variability between stimulations (5-30 s versus 900 s), and the distinct mechanisms underlying different hyperemic responses[36,37]."

The methods section needs further details to help other investigators consider the study's limitations. When appropriate, specify acute cranial window vs thin skull. Given the impact of intracranial pressure, acute inflammation, and other factors on the preparation and potential data, this information needs to be clearly defined in the methods section. A limitation section is encouraged.

Thanks for bringing this up. We have added additional details to the Methods section as well as the Results, Figures, and Figure Legends. Further, limitations were included in the revised manuscript as necessary.

Sometimes Vm or VM is used.

We used a uniform abbreviation throughout the revised manuscript.

REVIEWER #3

In their manuscript "Mechano-feedback control of brain blood flow", Lim et al. introduce endothelial PIEZO1 as a built-in brake on functional hyperemia. They show convincingly that endothelial PIEZO1 gain-of-function alters the magnitude and dynamics of blood-flow response. They also show behavioral differences in endothelial PIEZO1 GOF mice. Their discovery of a functional hyperemia "brake" is an important advance for the field. However, there are some concerns that need to be addressed.

We thank the reviewer for the positive remarks and for considering this work "an important advance for the field". Please, find below our responses to the concerns raised.

Major concerns:

1. The biggest concerns surround behavioral testing. The first concern is the claim that neurovascular coupling deficits in endothelial PIEZO1 GOF mice are directly responsible for cognitive deficits. While the authors show differences in NOR, there is no direct evidence that this is due to the NVC changes shown in the rest of the manuscript. Additionally:

- The NOL test seems not to have worked as expected, as the control groups have DI% of ~50 (the exact numbers are not presented). Given this, perhaps the NOL assay should be removed from the study.
- The pie charts for both NOR and NOL (and the associated very low p values) are misleading as they bin data into "discriminative" (DI% >50) and "non-discriminative" (DI% <50)… but only data points =50 are actually "non-discriminative"; data points below 50 show a preference for the familiar object/location.
- Given that the authors found a significant motor coordination deficit in GOF mice, how can we be sure that this does not affect the exploration of the different objects in the NOR assay?
- Given the racial implications of suggesting a link between cognitive deficits and an allele found in high frequency in African Americans, it is crucial that these cognition experiments be exceptionally rigorous. Because the NOL test did not seem to work in control mice, it would be nice to use a second test of memory/learning in its place (regardless of what the results are). It is also important to be sure that the NOR test is properly powered.

We understand the reviewer's concern surrounding the behavioral experiments. Please, find below our specific responses surrounding behavioral tests.

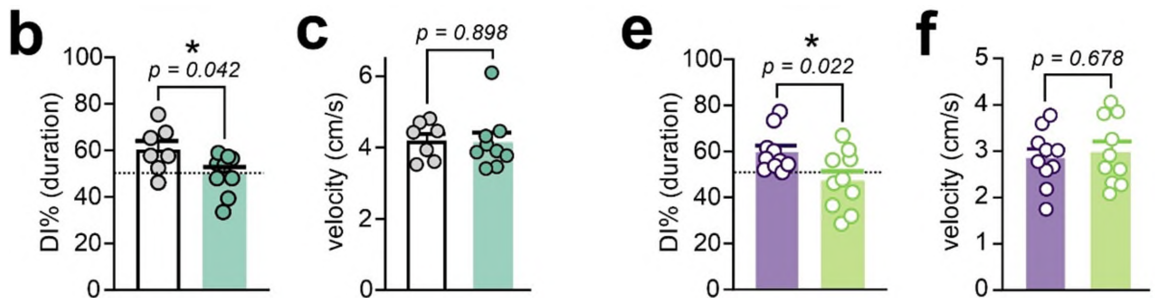
- *Claim that NVC deficits in PIEZO1 GOF mice are responsible for cognitive deficits: We agree with the reviewer that our study reports an intriguing observation that Piezo1 activity alteration changes cognition. We agree that our data do not provide direct or definitive evidence that neurovascular coupling deficits are responsible for cognitive deficits. In the revised manuscript, we have made this clear.*
  "EC Piezo1 function modification translates to altered performance in behavioral tests suggesting a potential link between Piezo1 impact on neurovascular coupling and cognition."

"Whether the neurovascular impairment is responsible for cognitive deficits in GOF mice remains to be confirmed."

- *Novel Object Location (NOL) test: As we mentioned earlier in our response to Reviewer #1, we agree that the NOL tests revealed discrimination indices that were largely below 50%. As suggested by the reviewer, this is indicative that the mice failed to learn the task and therefore making comparisons between genotypes would be invalid. We removed these results from the revised manuscript on the basis that even control mice have DI% around 50%.*

- *Pie charts: The concern related to pie charts being misleading is legit, and therefore they were removed in the revised manuscript.*

- *Motor coordination deficits: We thank the reviewer for this important question. We are confident that the exploration of objects in the NOR tests wasn't driven by motor deficits. Despite a mild deficit in motor coordination that was only noted in the first trial on day 1 of the rotarod tests (out of 10 trials over two days), motor learning was similar between GOF mice and controls. To directly address the question, we have included different parameters of motor function that were measured during the NOR tests. **Fig. 6** and **Fig. S13** now show the distances travelled and velocities of all mice in the NOR tests. These analyses revealed no difference in motor activity.*



- *Second test of memory/learning: We agree with the reviewer that a second test would be helpful. In the revised manuscript, a new behavioral test (Spontaneous alternation T-maze test) is included. This test was performed to assess working memory. The percentage of alternation, an index of working memory, was significantly lower in brain-EC-GOF mice compared with controls (**Fig. 6g**), consistent with impaired memory.*

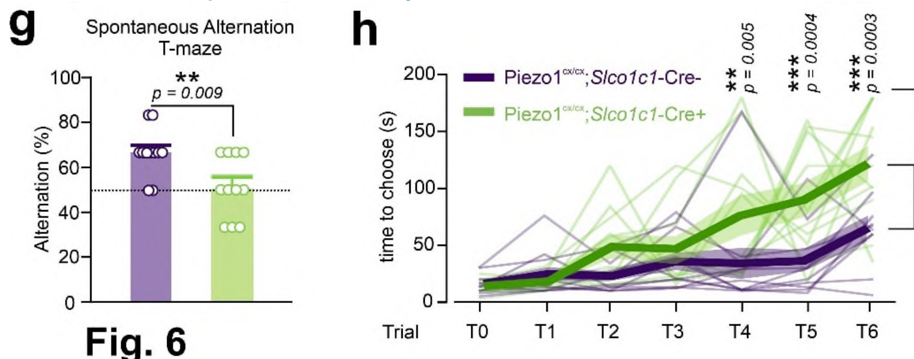


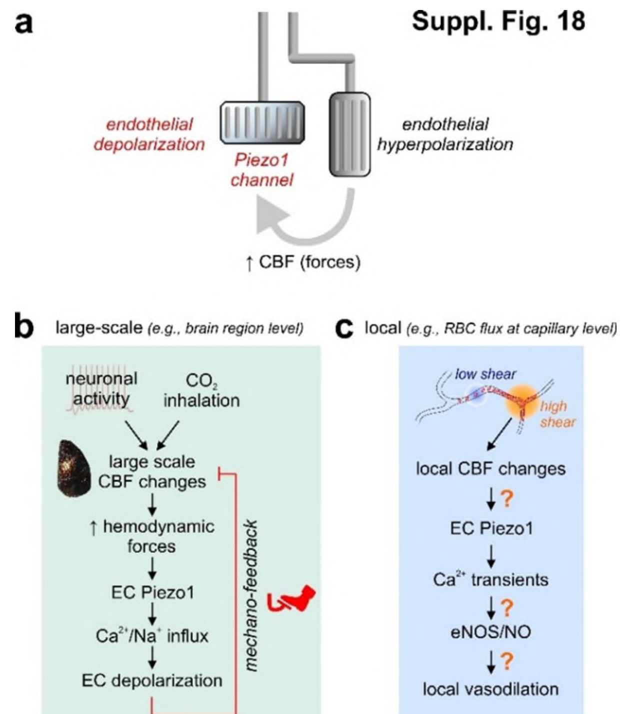
Fig. 6

2. The other central issue is a lack of discussion or experimentation regarding which part of the vascular tree this PIEZO1-medated NVC brake is occurring. While the hypothesis is explained clearly, this discussion is lacking. What is the rough contribution of arterioles and capillaries to the CBF dynamics picked up by the Doppler imaging? Would you expect that ex vivo PIEZO1 GOF arterioles would have decreased dilation response to both capillary and arteriole K^+ stimulation?

*Thank you for raising this point. Piezo1 is functionally expressed in almost all central nervous system ECs, including arteriolar, capillary and venular ECs (Vanlandewijck et al., 2018 Nature). We have previously shown that changing flow throughout the retinal vascular network elicited Piezo1 mediated Ca^{2+} signals in arterioles, capillaries and venules (Harraz et al., Circ Res 2022). The hyperemic responses described here (i.e., FH or CO_2 induced hyperemia) are presumably big enough to engage all parts of the vascular tree, a scenario that is quite distinct from scenarios of more localized differences (please, see new **Fig. S18**). We speculate that all these parts of the vascular tree contribute to the NVC braking mechanisms. It remains to be tested whether arterioles or capillaries are the predominant contributors to this feedback mechanism. To that end, we speculate a significant contribution for capillaries to the mechano-feedback mechanism because: **i**) Piezo1-mediated Ca^{2+} signals cannot activate Ca^{2+}-activated K^+ channels; and **ii**) the majority of Ca^{2+} signals and transients in deep capillaries do not translate into vasodilation and blood flow increases. We would like to note that contribution of capillaries versus arterioles in Piezo1-mediated mechano-feedback mechanism is an area of active research in our laboratory. A logical step, as suggested by the reviewer, is testing whether capillaries from Piezo1-GOF mice exhibit reduced sensitivity to K^+ vasodilation. These experiments and others are underway.*

In response to the reviewer's comment, we have discussed these aspects in the revised manuscript in the Discussion section:

*"FH occurs throughout the vascular tree in the somatosensory cortex (**Fig. S18**)—where Piezo1 is expressed in arteriolar, capillary and venular ECs[14,22]—and we speculate that entire vascular networks are implicated in the feedback mechanism. It remains unclear where within these networks the feedback mechanism predominates. Given that the majority of Ca^{2+} transients in deep capillaries do not translate into vasodilation[50] and that capillary Ca^{2+} signals do not trigger K_{Ca}-mediated hyperpolarization[8], we expect that capillaries are a major contributor to mechano-feedback mechanisms. Experimental investigations are required to determine the contribution of capillaries and arterioles to Piezo1-mediated braking."*



*We further include a new supplementary figure (**Fig. S18**) that highlights different possibilities downstream of Piezo1 activation.*

There are several other minor issues:

1. Please report actual p values throughout the manuscript, rather than just p<0.05, etc.

We have included p values throughout the manuscript in all figures.

2. It would be helpful to discuss the logistics of the cortical Yoda1 application, including what vessels are exposed to the aCSF. Why was cortical application chosen over iv or ip (which would presumably have less Yoda1 action on other brain cells)?

*The reviewer raises an important point. While designing this experiment, we had several considerations in mind: **i**) while intravenous (iv) administration was a logical route to target endothelial cells, we decided to avoid it since RBCs express functional Piezo1 channels and they have been shown to change shape in response to Yoda1 (e.g., PMID: 26001274; 37071200); **ii**) Intraperitoneal injection of Yoda1 has been used previously, but there is no evidence that it can reach the cerebrovasculature; and **iii**) we and others have previously shown that cortical application of drugs and pharmacological modulators can alter cerebral endothelial signaling (PMID: 28319610; 35349300; 34351870). We included a discussion of these logistics in the revised manuscript methods.*

The following was included in the Methods section:

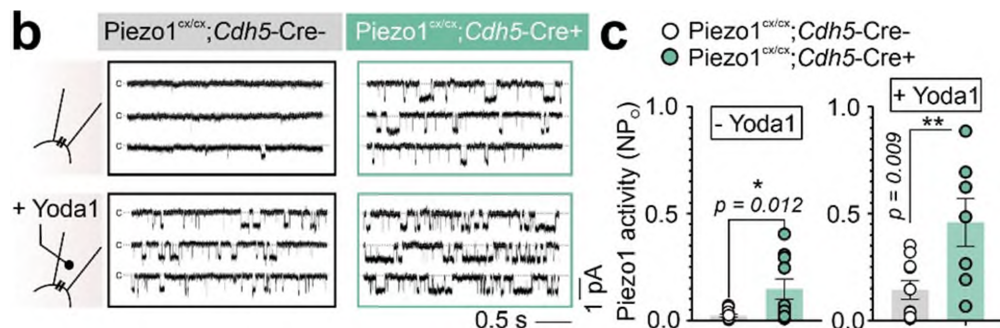
"We have previously shown that cortical application of drugs and pharmacological modulators can alter cerebral endothelial signaling[8,9]. Intravenous administration of Yoda1 was avoided because RBCs express functional Piezo1 channels and can therefore change shape in response to Yoda1[25]. Intraperitoneal injection was not employed, since there is no evidence that Yoda1 can reach the cerebrovasculature through this route."

3. It is a bit confusing to report that PIEZO1 GOF FH was "reduced by X%" when the units of measurement are % themselves. It would be clearer to just state the max ΔCBF (%) for each genotype group.

*Thanks for this comment and suggestion. We have modified all instances where we described FH suppression so that we state the FH values in different genotypes (e.g., Compared with controls, the maximum hyperemic responses were reduced in GOF mice from 14.7±1.6% to 9.7±0.7% (30 s stimulation) or from 10.3±1% to 7.4±0.6% (5 s stimulation) (**Fig. 2f-i**).).*

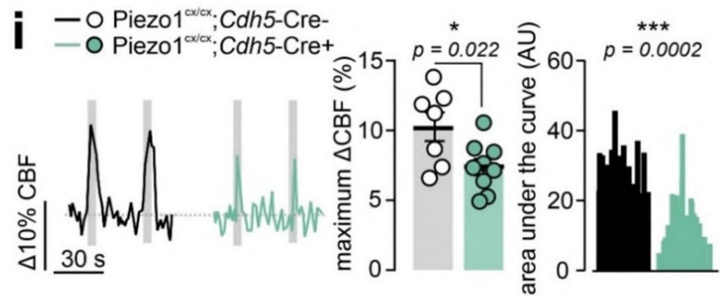
4. Figure 2b labeling is confusing—it seems like it is the key to 2c (which does not have a key).

*We apologize for the confusion. In the revised manuscript, **Fig. 2b, 2c** have been modified to enhance clarity.*



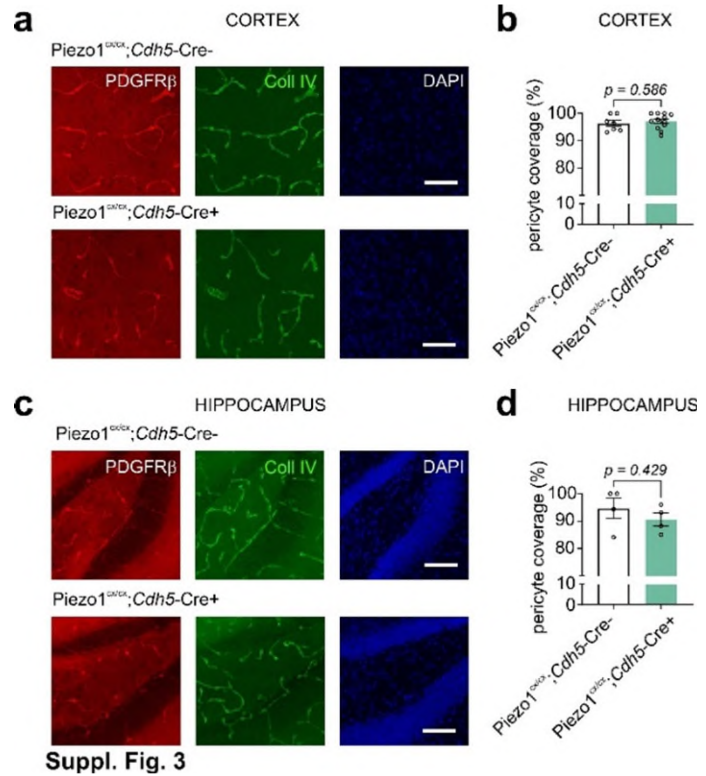
5. The n in the data in Figure 2i is very uneven, and it seems as if some of the data is missing from the right graph, as the GOF dataset should presumably have 2.5x more data points than the control (n=17 vs 43)?

Thank you for catching this missight. We have performed additional experiments, and the revised figure now includes balanced number of replicates (n= 7 control and 9 GOF mice; AUC analyses: n=41 stimulations in control mice and 43 in GOF mice).



6. The authors looked for changes in neurons, astrocytes, and microglia numbers and SMA+ vessel length. It seems strange to neglect pericytes coverage given the subject matter.

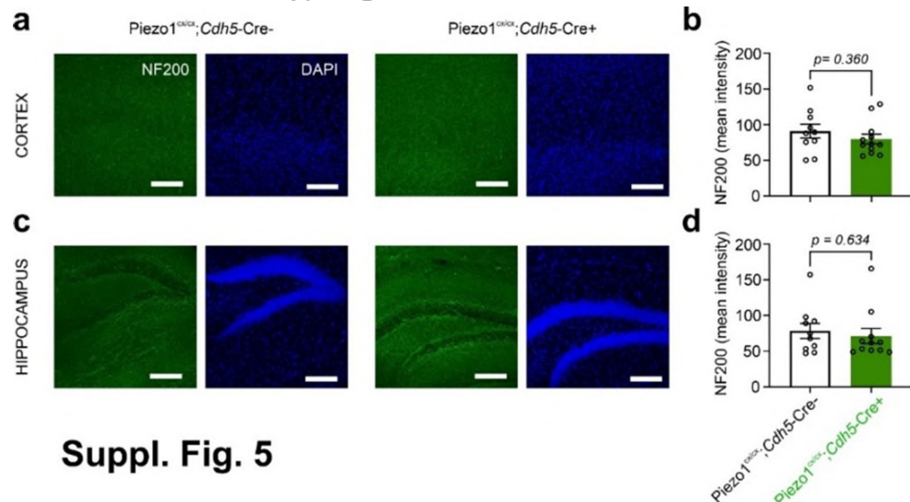
We thank the reviewer for this important comment. We agree that pericyte coverage is an important parameter that we considered. In response to the reviewer's comment, we performed additional pericyte staining using PDGFRβ as a marker and could show that there was no difference between control and GOF mice (new ***Fig. S3****).*



Suppl. Fig. 3

7. In Figure S2, the NeuN staining in hippocampus appears to be vascular

The reviewer is right, the NeuN antibody used was raised in mice and the tissues that were stained were not perfused. Therefore, the secondary antibody detects the immunoglobulins in the plasma as well. That's why the staining also shows the vasculature. However, the vessels only represent a small part of the tissue

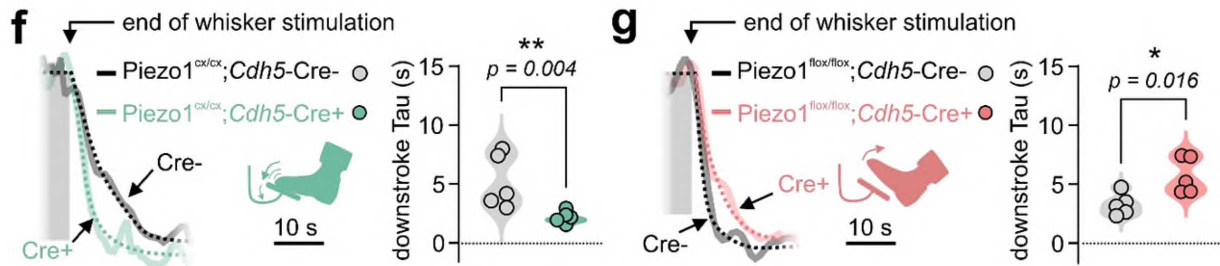


Suppl. Fig. 5

compared to neurons and since there is no difference between control and GOF mice in vascular density, the contribution of the vasculature to the values is likely negligible. With that said, to verify the NeuN staining, we performed measurements of an additional new marker, neurofilament 200 (NF200), which again shows no difference between control and GOF mice in the hippocampus and cortex (new ***Fig. S5****).*

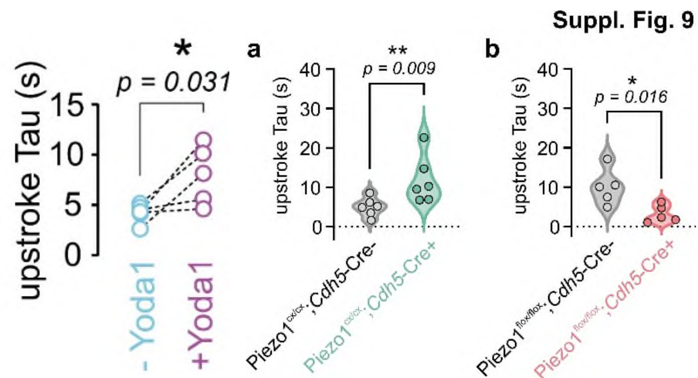
8. In Figure 3, it would be helpful to see the trace of the downstroke (as shown in 3h for Yoda1) for the GOF and KO mice.

We agree with the reviewer's suggestion. The revised manuscript includes representative traces of the downstroke in GOF and KO mice as well as their respective controls.



9. In supplemental figures 6-7, please report n of recordings. Also, for consistency, each data point should be one animal. It does not seem right to use each recording as a data point for upstroke and each mouse as a point for downstroke analyses.

*The authors apologize for this missight. In the revised manuscript, we included n of recordings, and each data point represents a single mouse in upstroke as well as downstroke analyses (****Fig. 3, S9, S10****).*



10. In Figures 3 and 4, it is confusing that the data presentation of Slco1c1-CreERT2 GOF line as well as of the KO line are quite different from that presented in Figure 2 for the Cdh5-CreERT2 GOF line. It would be helpful to have the same graph types for each line of mice to allow the reader to make comparisons across different metrics. For instance, the area under the curve data for the Slco1c1 line (4c and 4e, right) seem to be from one representative mouse rather than showing all the data. Relatedly, what is the purpose of showing of the 3 stimulations separately across time (eg in Fig 3d)? Do you expect any differences between these values across time?

Thank you for raising this point. We understand that the original manuscript included different graph types, which made comparisons harder and presumably confusing. In order to address this concern, we have now revised figures 3 and 4 so that:

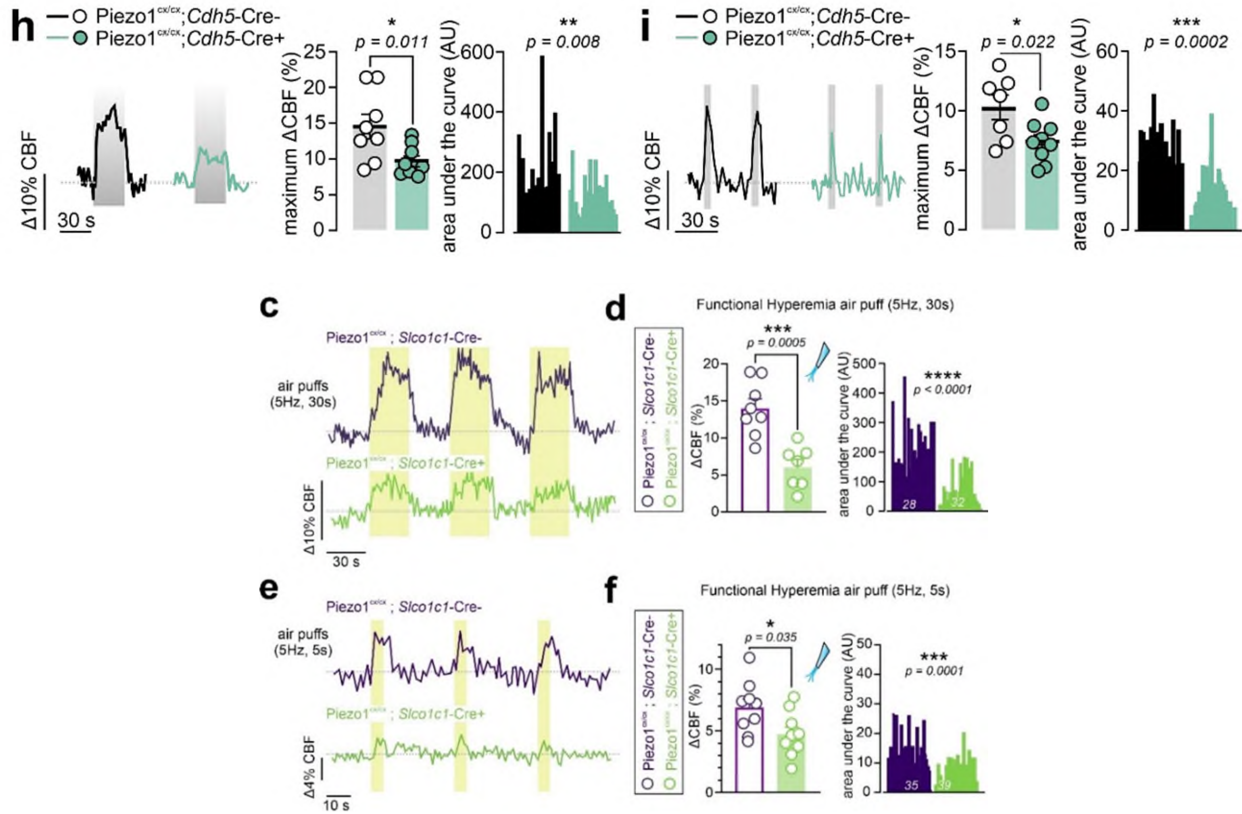
i*) area under the curve analyses are presented in the same way (Fig. 2h-i and 4d, 4f)*

ii) *we no longer show area under the curve for 3 consecutive stimulations in Fig. 4*

***iii**) we no longer show the magnitude of 3 consecutive stimulations over time (**Fig. 3d** in the first submission)*

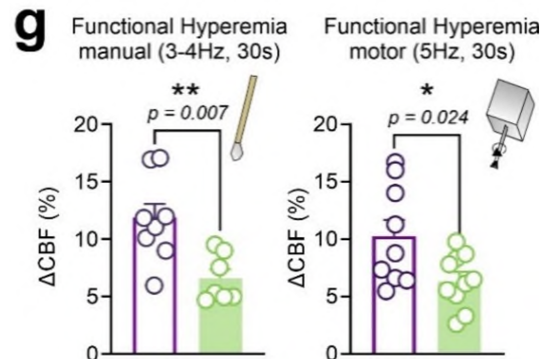
***iv**) we now show representative traces of FH responses from Cdh5-CreERT2 GOF and KO lines along with their respective controls (in new **Fig. 3d** and **3e**).*

We did not systematically test whether there will be differences in FH magnitudes over time.



11. Is the n in Fig 4g sufficient?

*The number of replicates wasn't sufficient in **Fig. 4g** in the original manuscript. We performed new experiments to address this issue.*



12. It would be helpful to offer a hypothesis as to why PIEZO1 KO mice would exhibit increased FH in response to whisker stimulation (Fig 3d) but not CO_2 (Fig 5g).

In the revised manuscript, we provided the following:

<u>"We expected that—like FH—hypercapnia-induced hyperemia will be different in GOF mice in both magnitude and kinetics, but we observed consistent differences only during recovery. This could be attributed to the huge temporal variability between stimulations (5-30 s versus 900 s), and the distinct mechanisms underlying different hyperemic responses[36,37]."</u>

13. For the data presented in Figure 6, The methods' equation for DI% says that NO and FO is "the time spent or the frequency of exploring". Was duration or frequency used to calculate DI%?

We calculated the discrimination index using both methods (time or frequency). They were shown in the original manuscript, and they are still shown in ***Fig. 6*** *as well as* ***Fig. S13****.*

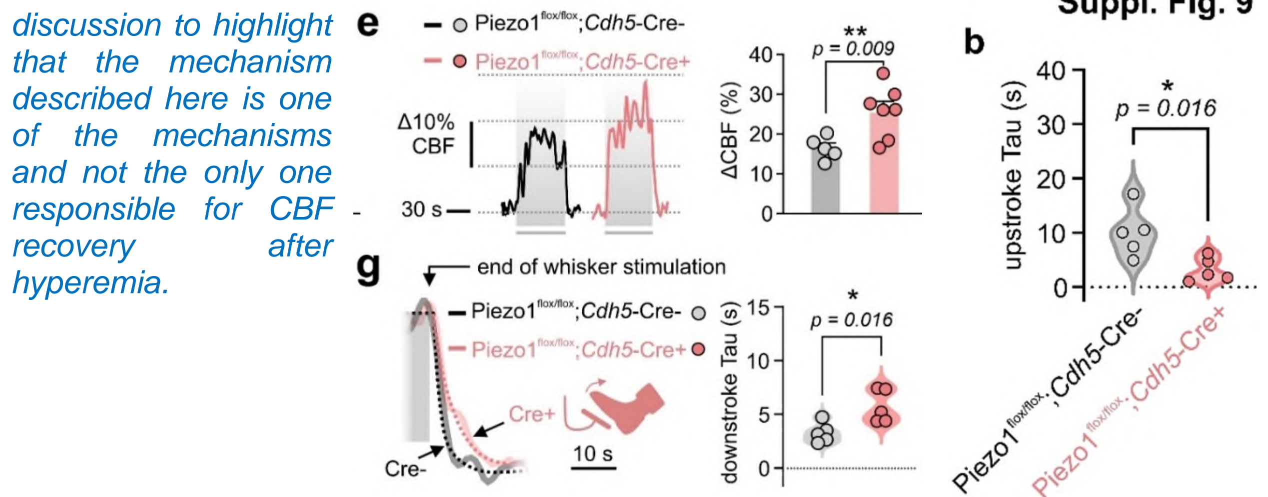
14. In the discussion, the authors acknowledge that the control cohorts of the GOF and KO mice are very different, perhaps due to strain differences. It would be helpful for this to also be earlier in the text to avoid confusion while looking at the figures.

Thank you for the suggestion. We have now mentioned the genetic differences in background earlier in the manuscript. The following was included earlier in the Results section:

*"Notably, Piezo1$^{cx/cx}$ and Piezo1$^{flox/flox}$ mice have different genetic backgrounds that are known to alter Piezo1-mediated Ca^{2+} signaling in Cre- controls[30]. Further, these backgrounds demonstrate key differences in CBF dynamics[31,32]. Given the differences observed in CBF across control (Cre-) mice here (****Fig. 3d, e****) and the differences seen in Piezo1$^{cx/cx}$ and Piezo1$^{flox/flox}$ Cre- and Cre+ without tamoxifen treatment (i.e., no induction;* ***Fig. S8****), hyperemic responses were only compared to controls of the same genetic background in all analyses."*

15. The discussion calls PIEZO1 "the key, but not only" brake for hyperemia, but the only trace data from KO mice shows them recovering completely, just slightly more slowly (after CO2-induced hyperemia). No trace at all is shown for KO mice in somatosensory experiment. Further, there are no statistics on the ∆CBF for KO mice (Fig 3d), only for the downstroke of tau. Thus, it seems as if data as presented do not support PIEZO1 being the key brake on hyperemia. That said, because there are not really any known brakes for hyperemia, any effect in this regard is interesting.

We thank the reviewer for bringing this up. In the revision, we have included traces from KO mice and their controls in ***Fig. 3e****. We have also included statistics of KO functional hyperemia magnitude, downstroke and upstroke (****Fig. S9****). We also changed the statement in the discussion to highlight that the mechanism described here is one of the mechanisms and not the only one responsible for CBF recovery after hyperemia.*



16. The discussion (lines 340-342) makes it sound as if there are no studies on mechanical force sensing and CSF dynamics, however Piezo1 has been shown to be involved in CSF flow regulation (PMID: 37917195).

Thank you for bringing up this important point. We have edited the sentence to reflect the active research in these areas.

"The influence of mechanical forces on these functions is an active research area, with recent evidence suggesting that Piezo1 is involved in cerebrospinal fluid flow regulation[70]."

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The revised manuscript has greatly improved. I just have one additional question and comment based on the new data.

Can the authors quantify the baseline diameter of the arterioles that were measured before and after Yoda1 treatment and include this in the results. It appears from the images in Fig. 1i, that the baseline diameter of the example arteriole is larger after Yoda1 application (similar in size to the stim. evoked increase without yoda1). If the arteriole is already dilated at baseline in Yoda1, this would have important implications on the interpretation of the results, and the manuscript would need to be revised accordingly.

The methodology for 2-photon imaging is insufficient. “Dilations were quantified using custom MATLAB scripts” – please specify the measurements and calculations. Please specify if a single trial or multiple trials were performed and averaged in each mouse? Does n=5 (fig. legend) indicate 5 mice or 5 arterioles?

Reviewer #2 (Remarks to the Author):

This is a revised study by Lim et al. Here, the authors use genetic/pharmacologic approaches to manipulate endothelial Piezo1 channel function and evaluate its contribution to cerebral blood flow, functional hyperemia, and cognition. The authors have addressed my prior concerns and clearly provided compelling evidence that Piezo 1 acts as a brake during the FH response, facilitating CBF recovery from baseline. These observations advance our understanding of the cellular processes implicated in the fine-tuned regulation of CBF during FH.

Reviewer #3 (Remarks to the Author):

The authors have done an excellent job responding to reviews. There are a few additional issues with the revised version:

I am not sure that the images of the feet on brakes in Figure 3 are helpful; they are a bit confusing as is. If they are kept, it might be helpful to make them smaller and include a gray one next to each signifying the baseline brake. Also, is there a reason that the GOF foot is different (motion and an extra arrow) than the Yoda1 foot? The data seem fairly similar.

It seems strange that downstroke is featured in the main figures but upstroke is relegated to the supplement. It might make more sense to put the mouse lines upstroke and downstroke in Figure 3, and put the Yoda1 upstroke and downstroke in supplement (combine 3h-i with S10 in supplement).

With the current arrangement, you risk readers not realizing there was any difference in upstroke, and this effect seems like an important piece of data.

The rebuttal states that the paper no longer shows the three simulations over time, but this is still the case in 4c and e. Since the reader is comparing 2h-i (Cdh5-Cre GOF) to 4 c and e (Slco1c1-Cre GOF) it would be helpful to have the data presented in the same way—perhaps one long stimulation and two consecutive short stimulations (as in 2 h-I), or just one stimulation for all experiments. The fact that the data for the two strains are presented differently makes the reader think it might be a different experimental paradigm in the Slco1c1 mice.

The NOR behavioral data for the KO seem strange. It appears as if the KO does not have any ability to discriminate the novel object, and that the reason for the non-significance is that the test is not working as expected in WT mice. I would suggest removing any behavior experiments in which the WT does not show the expected phenotype.

There is random bit of red font in the methods section for sharp electrode recordings.

REVIEWER COMMENTS

REVIEWER #1

The revised manuscript has greatly improved.

We thank the reviewer for their positive remarks and laudatory comments. The feedback was quite helpful in improving the manuscript.

I just have one additional question and comment based on the new data. Can the authors quantify the baseline diameter of the arterioles that were measured before and after Yoda1 treatment and include this in the results. It appears from the images in Fig. 1i, that the baseline diameter of the example arteriole is larger after Yoda1 application (similar in size to the stim. evoked increase withoutyoda1). If the arteriole is already dilated at baseline in Yoda1, this would have important implications on the interpretation of the results, and the manuscript would need to be revised accordingly.

In response to the reviewer's comment, we have included arteriolar baseline diameters before and after Yoda1 application. Arteriolar diameters were not different. The following has been added in the Results section in the revised manuscript:

*"Penetrating arterioles in the barrel cortex were visualized in anesthetized C57BL/6J mice, and whisker stimulation evoked vasodilation was significantly reduced after Yoda1 application (from 15.2±3.3% to 5.5±0.7%, **Fig. 1j, k**), despite no change in baseline arteriolar diameter before (7.2±0.7 µm) and after (7.6±0.5 µm) Yoda1 application."*

The methodology for 2-photon imaging is insufficient. "Dilations were quantified using custom MATLAB scripts" – please specify the measurements and calculations. Please specify if a single trial or multiple trials were performed and averaged in each mouse? Does n=5 (fig. legend) indicate 5 mice or 5 arterioles?

We thank the reviewers for bringing this up. The revised manuscript includes detailed information for the 2-photon imaging experiment:

*"Diameter measurements across time were measured using a custom MATLAB script written by D. Isaacs that calculated the full width at half maximum (FWHM) of a line profile placed over a PA for each frame of the xyt recording. To avoid contamination by shot noise and other sources, once the FWHM of the line profile for the PA under study had been calculated for each frame, any individual value greater than 3*SD (standard deviation) from the baseline mean was replaced with "NaN" in the diameter vs. time trace. Where possible, multiple trials were performed and averaged in each mouse. The baseline was defined as the average FWHM in µm in the 25 s prior to stimulation and this served as the reference point in the percent change in diameter measurements. The change in diameter extracted from the data describes the maximal change observed relative to the baseline established for the PA being imaged."*

The number of replicates displayed in Fig. 1 refers to the number of mice (i.e., n=5 indicates 5 mice), similar to the laser Doppler flowmetry experiment in the same figure. We have clarified this in the figure legend in the revised manuscript.

REVIEWER #2

This is a revised study by Lim et al. Here, the authors use genetic/pharmacologic approaches to manipulate endothelial Piezo1 channel function and evaluate its contribution to cerebral blood flow, functional hyperemia, and cognition. The authors have addressed my prior concerns and clearly provided compelling evidence that Piezo 1 acts as a brake during the FH response, facilitating CBF recovery from baseline. These observations advance our understanding of the cellular processes implicated in the fine-tuned regulation of CBF during FH.

The authors thank the reviewer for the positive remarks. We believe that addressing the reviewer's suggestions has made the evidence presented in the manuscript stronger.

REVIEWER #3

The authors have done an excellent job responding to reviews.

Thank you very much for the laudatory remarks. The reviewers' feedback was crucial for improving the manuscript.

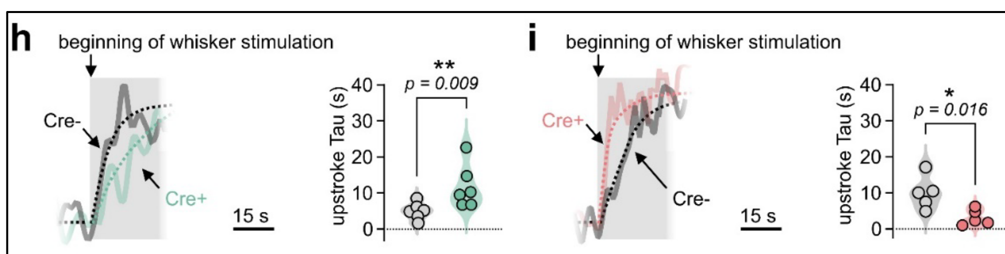
There are a few additional issues with the revised version:

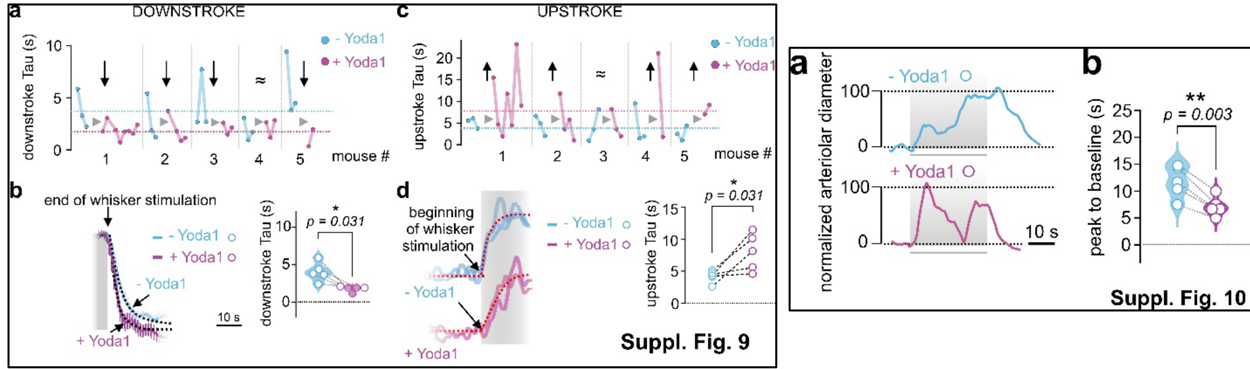
I am not sure that the images of the feet on brakes in Figure 3 are helpful; they are a bit confusing as is. If they are kept, it might be helpful to make them smaller and include a gray one next to each signifying the baseline brake. Also, is there a reason that the GOF foot is different (motion and an extra arrow) than the Yoda1 foot? The data seem fairly similar.

In response to the reviewer's comment and to avoid confusion, we decided to remove these brakes schematics.

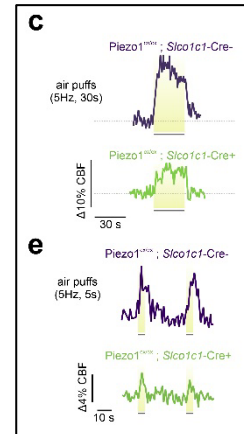
It seems strange that downstroke is featured in the main figures, but upstroke is relegated to the supplement. It might make more sense to put the mouse lines upstroke and downstroke in Figure 3 and put the Yoda1 upstroke and downstroke in supplement (combine 3h-i with S10 in supplement). With the current arrangement, you risk readers not realizing there was any difference in upstroke, and this effect seems like an important piece of data.

*The reviewer raises an important point and makes a good suggestion. We concur. In the revised manuscript, FH upstroke kinetics (traces and averaged time constants) are now presented in main **Fig. 3h** and **3i**. Yoda1-induced alterations in FH kinetics are now presented in **Suppl. Fig. S9** (blood flow) and **Suppl. Fig. S10** (arteriolar diameter).*

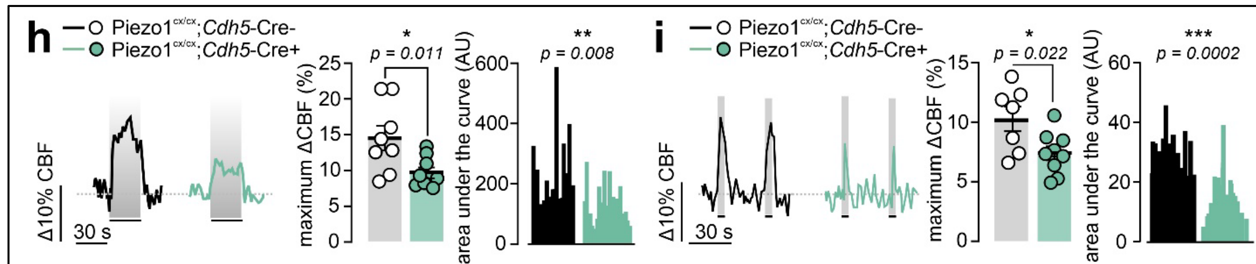




The rebuttal states that the paper no longer shows the three simulations over time, but this is still the case in4c and e. Since the reader is comparing 2h-i (Cdh5-Cre GOF) to 4 c and e (Slco1c1-Cre GOF) it would be helpful to have the data presented in the same way—perhaps one long stimulation and two consecutive short stimulations (as in 2 h-I), or just one stimulation for all experiments. The fact that the data for the two strains are presented differently makes the reader think it might be a different experimental paradigm in the Slco1c1 mice.



*We agree with the reviewer that consistent data presentation will enhance clarity and reduce confusion. As suggested by the reviewer, we have revised **Fig. 4c**, **e**, so that the responses are presented similar to those in **Fig. 2h-i**.*



The NOR behavioral data for the KO seem strange. It appears as if the KO does not have any ability to discriminate the novel object, and that the reason for the non-significance is that the test is not working as expected in WT mice. I would suggest removing any behavior experiments in which the WT does not show the expected phenotype.

*As suggested by the reviewer, the NOR data for KO mice (previously **Fig. S14**) has been removed on the basis that WT mice likely failed to learn the task, making comparisons between genotypes invalid.*

There is random bit of red font in the methods section for sharp electrode recordings.

This has been fixed.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

I have no further questions, the paper can be accepted in my opinion.