nature portfolio

Peer Review File

Connexin43 hemichannel blockade turns microglia neuroprotective and mitigates Alzheimer's disease-related cognitive deficits

Corresponding Author: Professor Chenju Yi

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript describes that connexin 43 hemichannels present in reactive microglia play a relevant role in the progression of AD, suggesting that connexin 43 hemichannels could be a promising therapeutic target. Interesting in vivo approach to study the role of Cx43 in activated microglia in AD. Methodology is sound and could be a relevant contribution to the field.

The data is very interesting but there are some issues that need clarification.

The transgenic model used present reduced memory which could be the consequence of functional recovery or neuronal death or both. Evaluation of neuronal death could help to decide whether reduced Cx43 hemichannels activity with blockers revert the functional state of neurons and to elucidate whether the memory loss was due to reduced functional state. Keeping in mind that AD is associated with aging, a relevant issue to keep in mind is that AD might be worse in aged mice and the potential therapeutic could lose efficacy. Consequently, it could be relevant to perform experiments on transgenic animals of 16 to 24 months old. To that end, it would be needed an inducible transgenic animal to avoid the overexpression of AB plaques at early ages and evaluate with more accuracy the efficacy of Cx43 hemichannels blockade. If this experiment cannot be done, please comment on this possibility.

Line 133. Taken together, these observations suggest that a marked increase in Cx43 is a specific feature characterizing pathological microglia in AD. Why just microglia? Astrocytes also showed a significant increase in Cx43?
Line 157. It has been demonstrated that microglia express Cx36 (doi: 10.1002/jnr.20650). Are levels of Cx36 also affected? Activated microglia also express Cx32 (DOI 10.1074/jbc.M600504200). Is Cx32 also affected?
Line 202. Downregulation of microglial Cx43, however, did not affect the number or the area of GFAP+ astrocytes. Since activated astrocytes release glutamate and ATP (doi: 10.1523/JNEUROSCI.6417-10.2011), What is the role of activated astrocytic Cx43 hemichannels after down-regulation of Cx43 in microglia?
Line 295. This treatment should also reduce the activity of Cx43 in astrocytes. Please describe what happened at this level.
Line 345. Is this conclusion supported by the lack of neuronal death?
Line 366. Please remember that Cx43 hemichannel inhibition also occurred in astrocytes.
Line 406. Vi) improve cognitive function or delay the appearance of cognitive dysfunction?
Line 409. Please include original reference at the end of this remark. Although the role of microglial Cx43 in neuropathology has been suggested by in vitro studies (REFs),
Line 415. Aβ opens Cx43 hemichannels…More accurate would be ...Aβ increases the activity of Cx43 hemichannels.
Line 425. Please discuss why Cx43 hemichannel activity remains high after down-regulation of Cx43 in microglia.
Line 477. Reference 72 does not describe the use of boldine to treat overactive bladder in women. Please include a correct reference.

Reviewer #2

(Remarks to the Author)

This manuscript explores the potential of Cx43 hemichannel inhibition in reducing reactive microglia activity associated with amyloid-beta and its implications for Alzheimer's disease (AD) pathology. While previous studies have examined the role of Cx43 hemichannels in astrocyte activation and peptide-based inhibitors, this study extends these findings to microglia-focused AD treatment using gene knockdown and nanoparticle-delivered hemichannel-blocking peptide drugs. The experiments systematically evaluate the efficacy of these approaches through immunostaining, electrophysiology, and behavioral assessments. The results suggest a promising strategy for combating amyloid-beta-induced AD, although authors need to elaborate on the advantages of this approach over other hemichannel blockers, such as CBX, used as a positive control in this manuscript. Below are specific comments:

1. Some sub-figures in Figure 3 do not align with the descriptions in the text, making readability challenging.

2. Behavioral test data in Figures 3G and 3H require a more detailed explanation. It is unclear how significant differences between conditions, including APP/PS1 and wild-type mice, are determined. Specifically, Figure 3H lacks statistical significance between groups, and the age of the mice for each condition should be clarified in the figure caption.

3. Line 227: The phrase "Cx43 knockout increased morphological complexity of the distal microglia in APP/PS1 mice" needs clarification. What does "morphological complexity" refer to in a physical sense?

4. The term "CBX" should be defined for clarity. According to the manuscript, CBX also blocks the Cx43 hemichannel. It appears that CBX refers to Carbenoxolone, which is also reported to block hemichannels in microglia, as evidenced in some figures. The authors should carefully address the potential advantages of the peptide drugs over CBX in the Discussion section, particularly with respect to their translatability to clinical applications.

5. Figure 5E: The TEM images of liposomes are too low in resolution to distinguish individual particles clearly.

6. For the analysis of the nanolipid gel, what does the "peptide only" condition represent?

7. Additionally, the stability of the liposomes in serum-supplemented media mimicking blood should be assessed. Figure 5H should include this data, as liposomes often dissociate more rapidly in such conditions. If a gel structure is indeed present, it could improve stability.

8. Line 313: The statement, "LNP-packaged peptides were maintained at a relatively high level in the brain lysates (42.9 ± 17.1% compared to day 1, N = 6 mice, p = 0.0068) but were not detectable in serum (Fig 5I)," appears inaccurate. The plot shows detectable levels in serum. This discrepancy needs to be addressed.

9. For Figure S5, details on the number of mice used to generate the physiological and biochemical data in panels S5e to S5i should be included.

Reviewer #3

(Remarks to the Author)
Su et al. present a new manuscript studying the role of microglia Cx43 hemichannels in a mouse model of amyloid deposition. While there are some interesting trends in the mouse models and AD patient tissue, there is reduced enthusiasm for this manuscript as a whole due to several major flaws of the experimental design and analysis. Moreover, considering the large amount of non-microglia Cx43 around plaques, its still unclear how important microglia Cx43 is over astrocytic (or oligodendrocytic) Cx43. Further, there is a lack of data proving a new peptide reagent blocker of Cx43 binds its target and works as stated and so the last portion of the study cannot be confidently assessed due to this severe shortcoming. In this reviewer's opinion, the manuscript is not ready for publication and must address several major issues. Below are specific critiques of the experimental design and data analysis.

Regarding experiments in Fig 1:
Lots of Cx43 outside or Iba1 positive cells. Only considered looking at astrocytes? The GFAP positive cells look underwhelming and wimpy in main figure but better in the Supplement. Why is that? Is this the best antibody for this Gfap, there are much better reagents to label a more complete astrocyte cell morphology.
Only modest difference in peri-plaque microglia compared to distal microglia. Yet, there is quite a robust enrichment of Cx43 signal in the peri-plaque area in general. Similarly, only a modes difference between peri-plaque and distal astrocytes. If not in microglia nor astrocytes, then what cells? This raises the issue of using GFAP as a marker of astrocytes. It is a intermediate filament (cytoskeletal) protein and you do not get the full morphology of the cell as compared to S100Beta or ALDH1L1 staining. These issues raise concerns about the overall approach and potential artifacts driving the current interpretation of this data.
This reviewer is not convinced that Cx43 is selectively upregulated only in microglia. Furthermore, the authors raise the point that Cx43 can be used to form a panglia syncytia between microglia and macroglia. The authors need to do staining for oligodendrocytes/OPCs and performs a similar analysis. The extent of Cx43 upregulation around the plaque and relatively minimal amount of that in microglia does not match their conclusion. The authors should show data of how much Cx43 that is outside Iba1 segmentation.

Regarding AD pathology. The authors first 3 data panels look at Cx43 expression as a relationship between RNA

expression and disease severity (Braak stage). While these are modest trends the relationship is between Cx43 and tau tangle distribution. The authors need to add markers for tau tangles (dye or antibody labeling) and perform the same types of analysis.

Regarding experiments in Figure 2:

Need to show data from other areas of plaque deposition such as cortex. More representative of the cellular composition and anatomy as examined in the human brain slices.

In addition to performing stats by distance to plaque surface, the authors need to control for plaque size within age groups and between age group. Naturally, there will be larger plaques in the 9m animals, and so there will be size bins that do not match the 4m group, which is ok.

I don't understand how the EtBr experiments show any specificity to Cx43 hemichannels. Presumably any random dye would also do this but this one sticks to nucleic acids so you can visualize the cell nuclei? Also, the details of the inhibition are unclear. Are you preloading the inhibitors then adding dye? Presumably any dye bulk loaded at high concentration to an acute brain slice can enter the cell independent of hemichannels, and plaque-associated microglia are highly phagocytic, so it is not surprising you see greater EtBr signal in those microglia compared to distal ones. The importance of Cx43 here is only forced up the reader because of the use of the selective inhibitors. These experiments are not clear and thus leave this reviewer unsure how to interpret them and determine if the conclusions are acceptable. One would need to load individual cells with a capillary pipette to visualize spreading of dye and blockade of hemichannels and gap junctions.

Regarding experiments in Figure 3:

The comparison between plaques in APP/PS1 versus APP/PS1 Cx43 KO mice is flawed. Authors describe the proportion of plaque morphotypes (filamentous vs compact) is shifted towards more compact plaques. However, when looking at RTN3 and LAMP1 areas around plaques between the mouse groups, they do not consider this in their grouping. They should identify filamentous plaques and compact plaques by a strict criterion and then measure RTN3 and LAMP1 signal for the same size plaques in each category. Only doing it by this way can they make any claims that the plaques in the Cx43 KO mice are less damaging if indeed the results hold. As it stands, the images in the representative panels clearly show they did not do this and all they are measuring is RTN3 and LAMP1 as a consequence of total area of Methoxy signal per plaque.

Regarding experiments in Figure 4:

RNAscope and IHC experiments are needed to validate key genes identified from the Bulk cell RNA-seq findings localize which signatures are associated with peri-plaque and distal microglia. One set of data discordance is seen in panel 4D where more abeta inside microglia is observed by imaging in Cx43 KO mice, but the RNA-seq data does not show any differential expression patterns in genes for lysosomal or phagocytosis. How do the authors explain this kind of discrepancy? This is where the spatial validation of DEG would refine the interpretation of findings here and increase the impact of the manuscript.

Regarding all remaining experiments using TAT-Cx43266-283 and TAT-Cx43@LNPs:

I have one major concern. The authors do not show directly labeling of their TAT-Cx43 reagent on cultured microglia, microglia (or astrocytes) in acute brain slices, or after delivered via ip injection. If indeed this reagent is binding to Cx43 then why can't you prove that at the cellular level? You've conjugated a Cy5.5 molecule for the in vivo image experiment but cannot show us by microscopy? Before I can assess the meaning or value of all the work done in the rest of the manuscript, I would need to see unambiguous evidence of the cellular localization of TAT-Cx43@LNPs to Cx43 (co-labeled with antibody). If you can prove this is the case, then I do not know how to judge the rest of the paper.

The in vivo imaging with a low-resolution method showing some weak signal from the head is not convincing. First of all, the animal in those images are contorted in all kinds of ways with the head twisted, how can you get reliable signal from the same area at each time point. This is sloppy. You need to position mouse and hold its head in the same position for each measurement and zoom into the mouse head. These non-invasive systems do have the ability to zoom in. The brain lysate measurements are also not necessarily convincing either. For all we know, all you are showing us is that your TAT-Cx43@LNPs have bound to Cx43 on brain endothelial cells and not actually entered brain parenchyma and bound to the Cx43 on microglia or astrocytes.

Assuming this all worked out and you can prove that TAT-Cx43@LNPs binds to microglia and astrocytic Cx43, you will need to also include the APP/PS1 mice with Cx43 conditional KO in microglia and astrocytes mice as controls to show that you do not get further amelioration of the amyloid phenotypes to bolster the notion that your experimental reagent is indeed working through microglia and/or astrocytes.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Thank you very much for responding satisfactorily all comments and critics. I do not have further suggestions. It is a very good contribution to the field.

Reviewer #2

(Remarks to the Author)
The authors addressed my original comments very well. The revision is qualified for recommendation to "ACCEPT".

Reviewer #3

(Remarks to the Author)
The authors address some minor technical concerns sufficiently, but they fall short of addressing the major flaws of their experimental design and thus many of their primary conclusions are left unsubstantiated. In particular, this manuscript lacks unambiguous evidence that targeting Cx43 with the TAT-Cx43@LPs approach is working as they believe it to be. Therefore, this paper remains unready for publication with the current claims. See below for specific critiques of issues still unaddressed.

1. Of course, oligodendrocytes and OPCs are present near plaques (doi.org/10.1016/j.neurobiolaging.2020.05.016)! These cells are everywhere. They are both affected and contribute to plaque deposition. Recently, they were shown by two independent groups to participate in Aβ plaque generation (DOI: 10.1038/s41593-024-01730-3; doi.org/10.1186/s13024-024-00759-z). Having said that, it is true that OPCs and Oligos do not express Cx43 at baseline conditions, however, neither do microglia at baseline according to the same RNAseq databases used widely by the community to validate gene expression by cell type. As it stands, there is still Cx43 not accounted for in microglia nor astrocytes around plaques. Thus, you have to rule out the OPCs/Oligos to not upregulate Cx43 in plaque proximity. Also, since you still have an appreciable amount of Cx43 in plaque regions even after the conditional knockout in microglia. Even if you argue about some failed recombination leading to inefficient knockdown, taking all this evidence together argues that you are missing something quite obvious about plaque-associated Cx43 expression and it needs to be resolved.

2. You need to stratify your existing data further by breaking down by plaque size and plaque morphotype are well within the scope of your manuscript. You have the data already, so you can analyze it readily. As it stands, your data is only modestly different in most comparisons in human AD and mouse models. There is a likely scenario that your data is only significant for certain plaque types/sizes, which is okay, but you need to define that so anybody who tries to replicate your work and does so more rigorously than you have now can replicate the data.

3. Lack of unambiguous data showing TAT-Cx43-Cy5.5 is targeting Cx43 in brain microglia. The additional data showing antibody stained Cx43 colocalize with a few random Cy5.5 puncta is problematic for the following reasons:

A) You do not provide controls of the TAT-Cy5.5 (with or without the LPs for acute slice versus the in vivo IP injection experiments). Show clearly the controls and do high resolution confocal microscopy with XZ and YZ orthogonal views to calculate true colocalization. What fluorophore did you use for the secondary antibody (no details in legend or methods) to stain Cx43? Need to rule out given the dimness of the Cy5.5 in the images as evident by high background, you may be cross-exciting the fluorophore for anti-Cx43 and getting spectral bleed through. As it stands there is just some random chance you have puncta that happen to appear close enough together to get an artificial colocalization using loose criterion on image processing software.

B) Assuming your signal is still only specific to the condition when you administer TAT-Cx43-Cy5.5 and not TAT- Cy5.5, then show with additional immunostaining for IBA1 and Aldhdl1 that the TAT-Cx43-Cy5.5 is targeting microglia. Its clear it won't be microglia specific, but you must show that TAT-Cx43-Cy5.5 is indeed binding to plaque-associated microglia using high resolution confocal microscopy. Without these data, and especially because the authors protest doing the experiment in the conditional Cx43 knockout mice, the existing claims about the TAT-Cx43-Cy5.5@LPs made by the authors do not stand. Considering the heart and liver get just as much bulk uptake of their TAT-Cx43-Cy5.5, there could just be some systemic effect. In this vein, the authors do not include a key control for experiments shown in Fig5J-I. You show comparison peptide with LNPs to peptide without LNPs, but you do not show experiments where you inject TAT without the Cx43(266-283). You have to rule out the increased brain retention just because of the LNPs.

4. The title of the manuscript is not appropriate. It reads as a title for a future review or perspective piece. Change the title to a more conventional title used for primary research articles.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The authors have addressed most of remaining concerns. For example, they have added key controls missing in many of the

experiments, which were casting doubt about the validity of their results, especially for the LNP delivery of TAT-Cx43. Now, with more attention to detail, the authors have a stronger manuscript.

Nevertheless some claims regarding their LNP based TAT-Cx43 approach are borderline. I suggest adding to their discussion, which already includes some talk of caveats/limitations of the study, some language around the timing of use of this approach. For example, the Cx43 KO only showed a difference for large filamentous plaque, no other size groups or compact plaques. What does that mean, and since the authors did not include this level of analysis in Figure 7, we can't know how effective this would be at different stages of amyloid deposition, or in models that have concomitant tau neuropathology.

Importantly, given the above statement, the new data showing that Cx43 KO ONLY impacts large, fibrillar plaques, this data needs to be in the main figure 3, not in the supplement.

Finally, the title is now acceptable. However, regarding nomenclature, the authors often use early and late stage of AD in their text. This is a model of amyloid-beta deposition, one of two neuropathological protein lesions in AD. The model is not AD. You can say mouse models of amyloid deposition, and early and late stage of amyloid deposition, but not AD (which requires both abeta and tau pathologies). Fix the text accordingly.

Response to Reviewers' comments for manuscript NCOMMS-24-67169-T

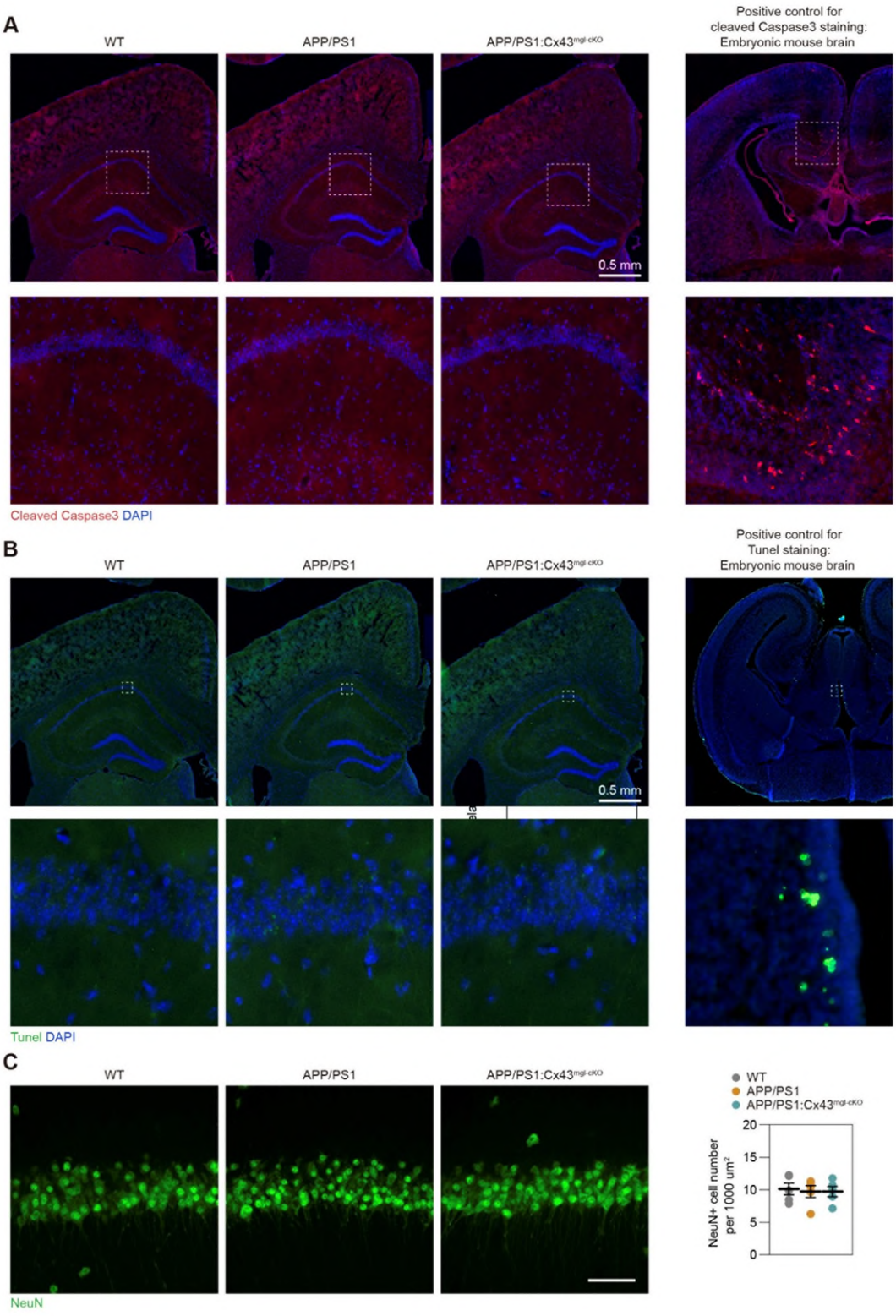
REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript describes that connexin 43 hemichannels present in reactive microglia play a relevant role in the progression of AD, suggesting that connexin 43 hemichannels could be a promising therapeutic target. Interesting in vivo approach to study the role of Cx43 in activated microglia in AD. Methodology is sound and could be a relevant contribution to the field.

The data is very interesting but there are some issues that need clarification.

The transgenic model used present reduced memory which could be the consequence of functional recovery or neuronal death or both. Evaluation of neuronal death could help to decide whether reduced Cx43 hemichannels activity with blockers revert the functional state of neurons and to elucidate whether the memory loss was due to reduced functional state.
Our reply: Thank you for raising this question. To evaluate neuronal death, we performed cleaved-caspase3 (c-Casp3) staining and Tunel assay in the brain sections of WT, APP/PS1, and APP/PS1:Cx43mgl-cKO mice, using the embryonic brain as the positive control for cell death as shown on a Figure below and in **Supplementary Fig. 3b-d**. We did not observe positive staining c-Casp3+ or Tunel+ cells in the brains of APP/PS1 mouse (Panels A and B in the Figure below). We also performed NeuN staining to quantify neuronal number in the hippocampus, which was not significantly changed among three experimental groups (Panel C). Our findings are consistent with the current consensus that there is no neuronal loss in the APP/PS1 mouse model (https://www.alzforum.org/research-models/appswepsen1de9-c57bl6). Therefore, cognitive decline in APP/PS1 mice is most likely due to the aberrant neuronal function, while inhibition of Cx43 hemichannels can restore the abnormal neuronal functions.
However, we acknowledge that the neuronal loss is the leading pathology in humans with AD. Previous studies demonstrated that inhibition of Cx43 hemichannels can reduce neuronal death in Aβ treated primary cultures, or in other neurodegenerative disease models.
We have included this evidence and the related statements in the Result and Discussion section.



Keeping in mind that AD is associated with aging, a relevant issue to keep in mind is that AD might be worse in aged mice and the potential therapeutic could lose efficacy. Consequently, it could be relevant to perform experiments on transgenic animals of 16 to 24 months old. To that end, it would be needed an inducible transgenic animal to avoid the overexpression of AB plaques at early ages and evaluate with more accuracy the efficacy of Cx43 hemichannels

blockade. If this experiment cannot be done, please comment on this possibility.

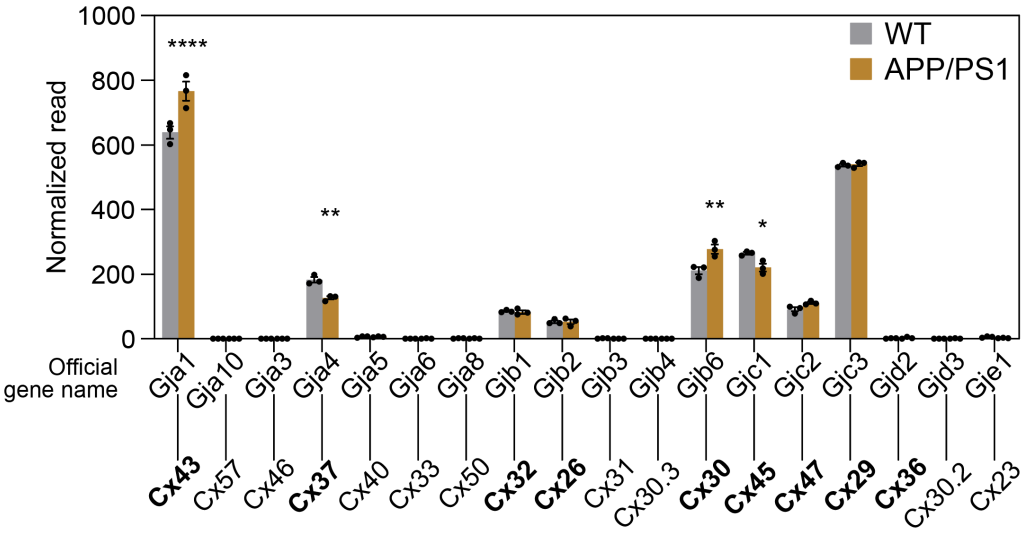
Thank you for the suggestions. The aging factor is indeed a limitation for the majority of AD mouse model studies. However, current AD mouse models developed pathological change at relatively early life stage. APP/PS1 mice, for instance, develop Aβ deposition at 4-month-old, comparable to 20~30-year-old in human, and cognitive decline before 10-month-old (comparable to ~40 years old in human). In this aspect, APP/PS1 mice, similarly to other mouse models, are unsuitable to study the interaction of aging and AD pathogenesis, or the efficacy of Cx43 hemichannel inhibition in aged AD condition. Taken these together, to further confirm the efficacy of Cx43 hemichannel inhibitors in AD, we need to test them in other models, including inducible, aging-dependent, AD mouse models. We have incorporated the related statements in the Discussion section.

Line 133. Taken together, these observations suggest that a marked increase in Cx43 is a specific feature characterizing pathological microglia in AD. Why just microglia? Astrocytes also showed a significant increase in Cx43?

Thank you for this comment. Indeed, as noted in our study, both microglia and astrocytes exhibit increased Cx43 levels in AD. We fully recognize the importance of astrocytic Cx43 in disease pathology. In fact, our previous work (DOI: 10.1038/cdd.2016.63) extensively explored the role of astroglial Cx43 in AD, highlighting its contributions to neuronal oxidative stress. However, our observations indicate that the magnitude of Cx43 upregulation is significantly higher in microglia compared to astrocytes. Present study focuses specifically on microglial Cx43 due to its pronounced association with neuroinflammatory pathways in AD. Therefore, we aimed to emphasize the change of microglial Cx43 in AD pathology in the summary sentence of this section.

Line 157. It has been demonstrated that microglia express Cx36 (doi: 10.1002/jnr.20650). Are levels of Cx36 also affected? Activated microglia also express Cx32 (DOI 10.1074/jbc.M600504200). Is Cx32 also affected?

Thank you for this comment. We measured expression level of all connexin genes using RNA-sequencing. As shown in the figure below, Cx43, Cx30, and Cx29 are highly expressed in microglia, and Cx43 expression increased prominently in microglia from APP/PS1 mice. However, expression levels of Cx36 and Cx32 are relatively low in microglia, and not significantly altered in APP/PS1 mice. The discrepancy between our findings and the literature may be due to (i) primary cell culture was used in which microglia transcriptome profile is significantly altered. (ii) different expression detecting methods (PCR in 10.1002/jnr.20650 vs. RNA-seq in our study).



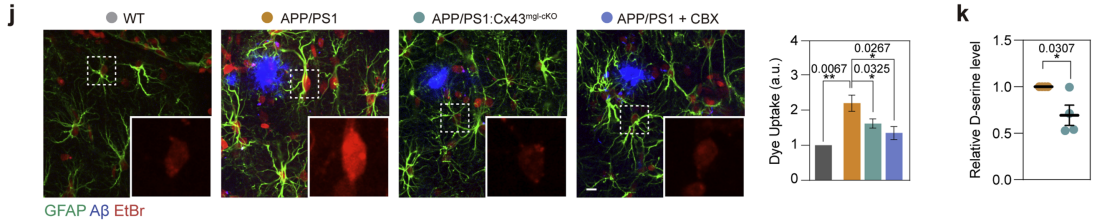
Line 202. Downregulation of microglial Cx43, however, did not affect the number or the area of GFAP+ astrocytes. Since activated astrocytes release glutamate and ATP (doi: 10.1523/JNEUROSCI.6417-10.2011), What is the role of activated astrocytic Cx43 hemichannels after down-regulation of Cx43 in microglia?

Thank you for this insightful comment.

Proinflammatory cytokines from reactive microglia can activate astrocytic connexin hemichannels (doi: 10.1002/glia.22785). Given that the knockout of microglial Cx43 can suppress the proinflammatory profile, we wondered if the astrocytic connexin hemichannel activation was thus affected. To confirm this, we counter-stained the acute brain slices subjected to the dye uptake with astrocyte marker GFAP. We found that the dye uptake of GFAP+ astrocytes was also reduced in APP/PS1:Cx43$^{mgl\text{-}cKO}$ mice compared to APP/PS1 control, indicating a reduced astrocyte connexin hemichannel activity (reported in **Supplementary Fig. 4j** and below for your convenience). Activation of astrocytic connexin hemichannels allows the release of gliotransmitters such as D-serine (10.1523/JNEUROSCI.2204-16.2017). Indeed, we found that the D-serine level in the APP/PS1:Cx43$^{mgl\text{-}cKO}$ brain slice conditional medium was also significantly reduced compared to that of APP/PS1, suggesting reduced astrocytic connexin hemichannel activity (reported in **Supplementary Fig. 4k**). Since our previous study showed that astrocytic connexin hemichannel activation contribute to neuronal damage (10.1038/cdd.2016.63), the reduced astrocytic hemichannel activation in APP/PS1:Cx43$^{mgl\text{-}cKO}$ may ameliorate neuronal stress.

The new data are presented in **Supplementary Fig. 4j, k** (as below) and in the **Result - Turning microglia neuroprotective by microglial-specific Cx43 knockout in AD mice** section**.**

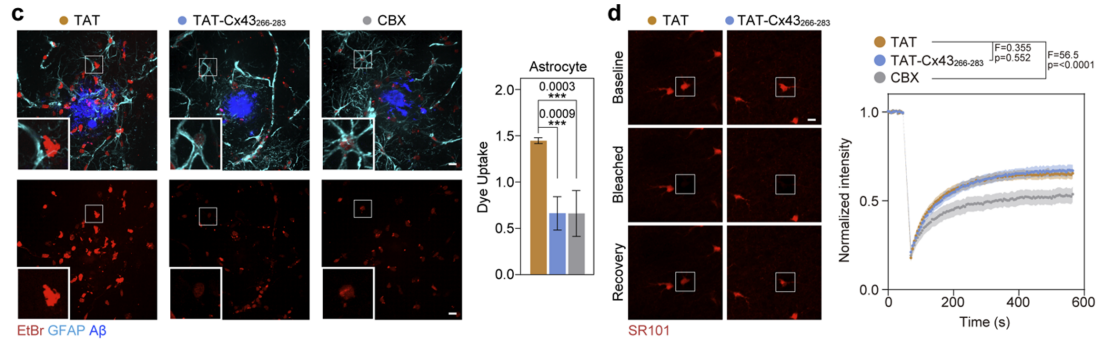
Supplementary Fig. 4



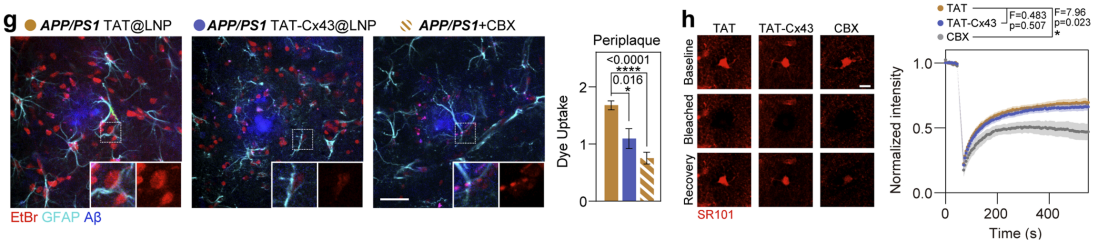
Line 295. This treatment should also reduce the activity of Cx43 in astrocytes. Please describe what happened at this level.

We examined the function of astrocytic Cx43 after the acute TAT-Cx43$_{266-283}$ treatment *ex vivo* and the chronic TAT-Cx43@LNP treatment *in vivo*. In both experiments, the hemichannel activation of astrocyte was significantly reduced reflected by dye uptake experimental results. However, the gap junction function was not affected as shown gap-FRAP experiment (shown in **Supplementary Fig. 5c-d, 6g-h**). Considering our previous study that astrocytic connexin hemichannel activation contribute to neuronal damage in APP/PS1 mice (doi: 10.1038/cdd.2016.63), and the critical contribution of astrocytic gap junction towards neuronal function maintenance [63], these observations are further in favor of the use of TAT-CX43$_{266-283}$ in the treatment of AD. We have revised the manuscript accordingly.

Supplementary Fig. 5



Supplementary Fig. 6



Line 345. Is this conclusion supported by the lack of neuronal death?

As mentioned above, neuronal death was not apparent in the APP/PS1 mouse brains.

Line 366. Please remember that Cx43 hemichannel inhibition also occurred in astrocytes.

Thank you for the suggestion. We revised the statement to include the astrocytic Cx43

hemichannel in the description.

Line 406. Vi) improve cognitive function or delay the appearance of cognitive dysfunction?
We revised the statement to read"alleviated the cognitive decline".

Line 409. Please include original reference at the end of this remark. Although the role of microglial Cx43 in neuropathology has been suggested by in vitro studies (REFs),
Apologize for the oversight. We included a reference.

Line 415. Aβ opens Cx43 hemichannels…More accurate would be ...Aβ increases the activity of Cx43 hemichannels.
Thank you for the suggestion. We revised it accordingly.

Line 425. Please discuss why Cx43 hemichannel activity remains high after down-regulation of Cx43 in microglia.
Thank you for this comment. We assume that you refer to the microglial dye uptake experiment in Fig. 4e. in which Cx43 conditional knockout did not reduce the microglial dye uptake level to that of the negative control, wildtype (WT) group. We believe that this is due to an insufficient Cre-mediated recombination, leading to incomplete Cx43 knockout in microglia in the APP/PS1:$Cx43^{mgl\text{-}cKO}$ model. Indeed, our immunostaining analysis show that APP/PS1: $Cx43^{mgl\text{-}cKO}$ mice reduced microglial Cx43 expression to 42.3 ± 5.0% compared to APP/PS1 (Supplementary Fig. 3a). Regarding the dye uptake experiment, our data in Fig. 2e suggest Cx43 hemichannel activation accounted for the elevated microglial dye uptake in APP/PS1 mice compared to WT. When we perform zero-mean normalization to WT control, Cx43 conditional knockout reduced microglial dye uptake level to 63.1 ± 13.3 % compared to APP/PS1, comparable to the change of Cx43 expression level in the knockout mice.
The above discussion was added to the paper.

Line 477. Reference 72 does not describe the use of boldine to treat overactive bladder in women. Please include a correct reference.
Thank you for these suggestions, we revised manuscript accordingly.

Reviewer #2 (Remarks to the Author):

This manuscript explores the potential of Cx43 hemichannel inhibition in reducing reactive microglia activity associated with amyloid-beta and its implications for Alzheimer's disease (AD) pathology. While previous studies have examined the role of Cx43 hemichannels in astrocyte activation and peptide-based inhibitors, this study extends these findings to microglia-focused AD treatment using gene knockdown and nanoparticle-delivered hemichannel-blocking peptide drugs. The experiments systematically evaluate the efficacy of these approaches through immunostaining, electrophysiology, and behavioral assessments. The results suggest a promising strategy for combating amyloid-beta-induced AD, although

authors need to elaborate on the advantages of this approach over other hemichannel blockers, such as CBX, used as a positive control in this manuscript. Below are specific comments:

1. Some sub-figures in Figure 3 do not align with the descriptions in the text, making readability challenging.

Apologize for the misalignment. We revised the text to reflect the figure content.

2. Behavioral test data in Figures 3G and 3H require a more detailed explanation. It is unclear how significant differences between conditions, including APP/PS1 and wild-type mice, are determined. Specifically, Figure 3H lacks statistical significance between groups, and the age of the mice for each condition should be clarified in the figure caption.

Thank you for your comment. We revised the statement for the behavioral test results, which now reads:

"Conditional knockout of microglial Cx43 in APP/PS1 mice significantly improved cognitive performance as evidenced by the discrimination index in the novel object recognition test (Fig. 3g). Microglial Cx43 ablation in the wildtype background, however, did not additionally improve the cognitive function compared to the wildtype control (Fig. 3g). Several studies identified anxiety or hyperactivity symptom in APP/PS1, similarly to psychiatric manifestations in some AD patients [43, 44]; however, evidence regarding these behavioral deficits in APP/PS1 mice is inconsistent [44]. In our hands, open field test showed no anxiety-like (distance in center) or hyperactivity (total distance) behaviors in APP/PS1 mice compared to WT (Fig. 3h). Microglial Cx43 knockout in APP/PS1 mice or WT mice did not cause abnormality in these behavioral traits, either (Fig. 3h)."

The age of mice in the behavioral test is 9-month-old; statistical differences between groups were determined by unpaired t-test. We have specified these in the figure legend.

3. Line 227: The phrase "Cx43 knockout increased morphological complexity of the distal microglia in APP/PS1 mice" needs clarification. What does "morphological complexity" refer to in a physical sense?

Thank you for your comment. We apologize for the vague expression. Here we meant that the distal microglia in APP/PS1:Cx43$^{mgl\text{-}cKO}$ extend more numerous, complex processes compare to that in APP/PS1 mice. We revised the text accordingly.

4. The term "CBX" should be defined for clarity. According to the manuscript, CBX also blocks the Cx43 hemichannel. It appears that CBX refers to Carbenoxolone, which is also reported to block hemichannels in microglia, as evidenced in some figures. The authors should carefully address the potential advantages of the peptide drugs over CBX in the Discussion section, particularly with respect to their translatability to clinical applications.

Yes. CBX is Carbenoxolone. We defined CBX when it was first mentioned.

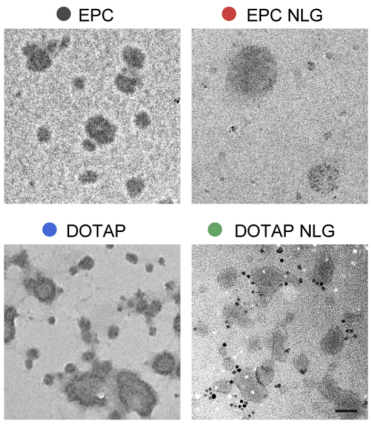
CBX is commonly used to inhibit connexin hemichannels in cell cultures or *ex vivo* brain slice studies. It is, however, unspecific (also target other protein channels such as Panx1 channel), potently inhibits gap junction channels, and could not cross blood-brain barrier (DOI: 10.1016/j.molmed.2024.03.009). We incorporated these statements in the Discussion

section.

5. Figure 5E: The TEM images of liposomes are too low in resolution to distinguish individual particles clearly.

Thank you for your comment. We re-measured the liposomes using higher resolution settings, presented in **Fig. 5e** as shown below. We hope the new images meet standards.

Fig. 5e



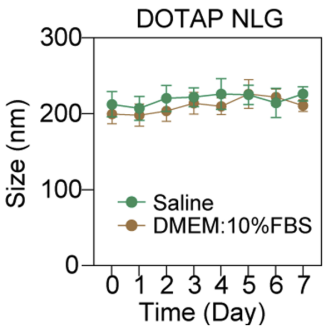
6. For the analysis of the nanolipid gel, what does the "peptide only" condition represent?

Thank you for the comment. In the "peptide only" group, we directly injected the mice with unpackaged peptide. We have changed "peptide only" to "peptide without LNP packaging" to improve clarity.

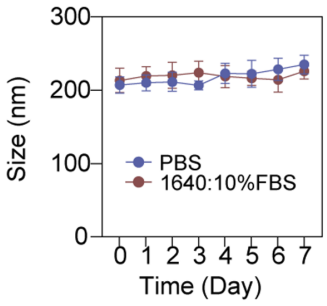
7. Additionally, the stability of the liposomes in serum-supplemented media mimicking blood should be assessed. Figure 5H should include this data, as liposomes often dissociate more rapidly in such conditions. If a gel structure is indeed present, it could improve stability.

Thank you for your suggestion. We repeated this experiment with 4 conditions: Saline control (0.9% NaCl), PBS, DMEM:10%FBS, and 1640:10% FBS. The addition of FBS did not significantly alter the stability of DOTAP NLG liposomes. The **new Fig. 5h** and **Supplementary Fig. 5i** are presented below.

Fig. 5f



Supplementary Fig. 5i



8. Line 313: The statement, "LNP-packaged peptides were maintained at a relatively high

level in the brain lysates (42.9 ± 17.1% compared to day 1, N = 6 mice, p = 0.0068) but were not detectable in serum (Fig 5I)," appears inaccurate. The plot shows detectable levels in serum. This discrepancy needs to be addressed.

Apologies for the inaccuracy of the statement. We revised and the statement now reads, "On 14th day post-injection, LNP-packaged peptides were maintained at a relatively high level in the brain lysates (42.9 ± 17.1% compared to day 1) but dropped to a low level (9.7 ± 13.4% compared to day 1) in serum (Fig 5I)".

9. For Figure S5, details on the number of mice used to generate the physiological and biochemical data in panels S5e to S5i should be included.

Thank you for your comment. We included the sample size information for each experiment accordingly in the figure legend of **Supplementary Fig. 5j-m**.

Reviewer #3 (Remarks to the Author):

Su et al. present a new manuscript studying the role of microglia Cx43 hemichannels in a mouse model of amyloid deposition. While there are some interesting trends in the mouse models and AD patient tissue, there is reduced enthusiasm for this manuscript as a whole due to several major flaws of the experimental design and analysis. Moreover, considering the large amount of non-microglia Cx43 around plaques, its still unclear how important microglia Cx43 is over astrocytic (or oligodendrocytic) Cx43. Further, there is a lack of data proving a new peptide reagent blocker of Cx43 binds its target and works as stated and so the last portion of the study cannot be confidently assessed due to this severe shortcoming. In this reviewer's opinion, the manuscript is not ready for publication and must address several major issues. Below are specific critiques of the experimental design and data analysis.

Regarding experiments in Fig 1:

Lots of Cx43 outside or Iba1 positive cells. Only considered looking at astrocytes? The GFAP positive cells look underwhelming and wimpy in main figure but better in the Supplement. Why is that? Is this the best antibody for this Gfap, there are much better reagents to label a more complete astrocyte cell morphology.

Only modest difference in peri-plaque microglia compared to distal microglia. Yet, there is quite a robust enrichment of Cx43 signal in the peri-plaque area in general. Similarly, only a modes difference between peri-plaque and distal astrocytes. If not in microglia nor astrocytes, then what cells? This raises the issue of using GFAP as a marker of astrocytes. It is a intermediate filament (cytoskeletal) protein and you do not get the full morphology of the cell as compared to S100Beta or ALDH1L1 staining. These issues raise concerns about the overall approach and potential artifacts driving the current interpretation of this data.

This reviewer is not convinced that Cx43 is selectively upregulated only in microglia. Furthermore, the authors raise the point that Cx43 can be used to form a panglia syncytia between microglia and macroglia. The authors need to do staining for oligodendrocytes/OPCs and performs a similar analysis. The extent of Cx43 upregulation

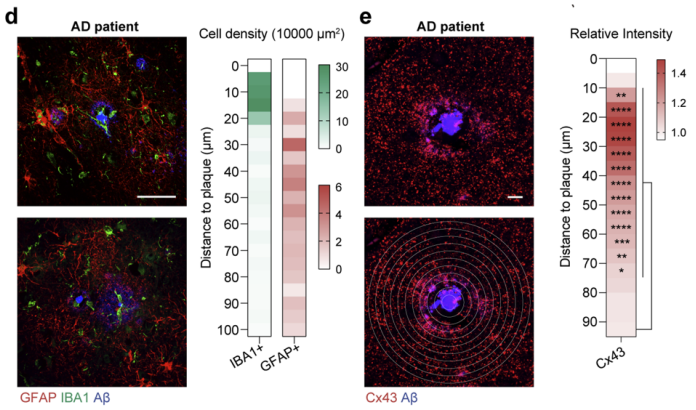
around the plaque and relatively minimal amount of that in microglia does not match their conclusion. The authors should show data of how much Cx43 that is outside Iba1 segmentation.

Thank you for your suggestion. In Fig 1d, the fine processes of GFAP+ would seems dimmer because images were captured using 20x objective, and also the high fluorescence intensity at the cell body. We replaced the images in **Fig 1d** with ones captured using a 60x objective, which clearly presented the astrocytes. However, we acknowledge that GFAP is not an ideal marker for accurately labelling astrocyte morphology when quantifying Cx43 expression. To address this, we additionally stained for ALDH1L1, a more suitable astrocytic marker for defining the astrocytic domain, and quantified Cx43 expression within the ALDH1L1+ astrocytic domain (presented in the **new Supplementary Fig. 1f**). Result showed that astrocytic Cx43 level was also significantly increased in AD patient brains, but the degree of the change (AD vs control: 52.4 ± 8.1% increase, N = 321 and 129 cells, $p < 0.0001$; periplaque vs distal: 51.6 ± 6.6% increase, N = 124 and 197 cells, $p < 0.0001$) was lower compared to that of microglia.

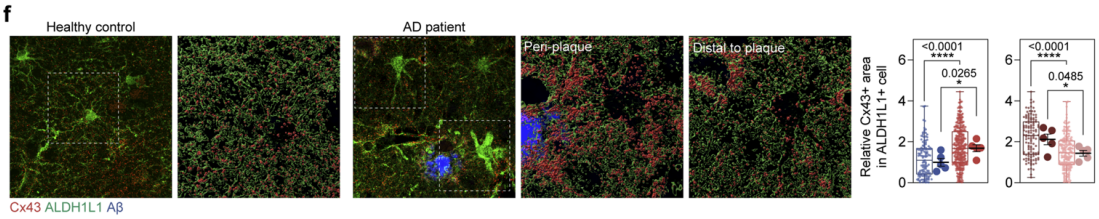
Regarding the cell type-dependent contribution to the elevated periplaque Cx43 expression, we would like to clarify that we did not state Cx43 is increased in microglia only. Rather, we observed that periplaque microglia exhibited a greater increase in Cx43 level compared to periplaque astrocytes. Specifically, the periplaque microglia showed a 61.9 ± 12.4% (N = 85 and 121 cells, $p < 0.0001$) increase in Cx43 expression compared to the distal microglia, while periplaque astrocytes displayed 51.6 ± 6.6% (N = 124 and 197 cells, $p < 0.0001$) increase compared to the distal astrocytes. Regarding elevated Cx43 expression at peri-plaque area, we used heatmap to present the Cx43 expression level at different distance to the plaque. In the **new Fig 1e** (shown below) the highest Cx43 expression is observed at 20~25 μm from the plaque, with a 47.1 ± 5.7% increase compared to the distal region (90 ~ 95 μm), a level comparable to that in astrocyte. These findings indicate that both periplaque microglia and astrocytes contribute to elevated Cx43 levels in the periplaque area.

We did not include data of Cx43 expression in Oligodendrocytes (OL) or oligodendrocyte precursor cells (OPC) for two reasons: (ii) OL or OPC are not present at periplaque area like microglia, and (ii) OL/OPC mainly express different set of connexins (Cx32, Cx47; but do not express Cx43), and thus form heterotypic (Cx32/Cx30 and Cx47/Cx43) gap junction channels with astrocytes (ref: 10.1152/physrev.00043.2018).

Fig. 1



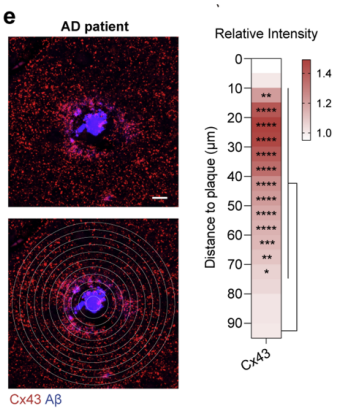
Supplementary Fig. 1



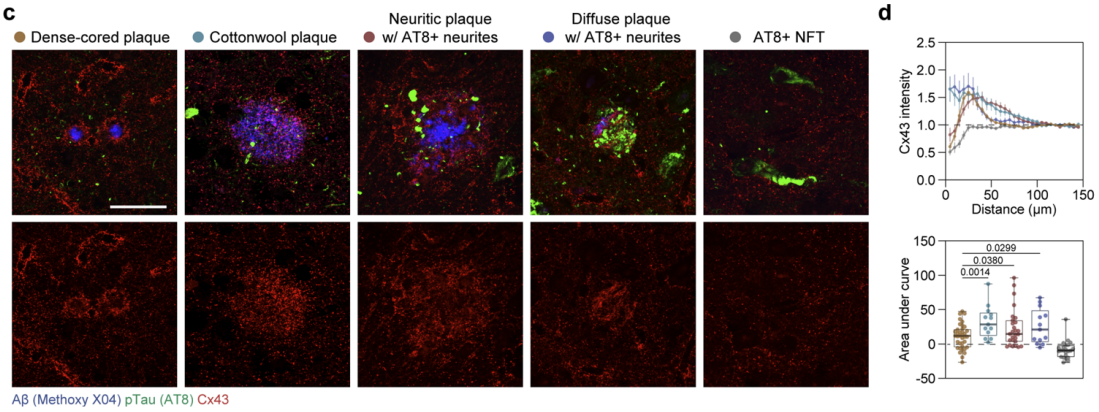
Regarding AD pathology. The authors first 3 data panels look at Cx43 expression as a relationship between RNA expression and disease severity (Braak stage). While these are modest trends the relationship is between Cx43 and tau tangle distribution. The authors need to add markers for tau tangles (dye or antibody labeling) and perform the same types of analysis.

Thank you for your suggestion. We labelled tau tangles using the AT8 antibody and performed similar analysis. We observed that Tau tangles were mainly found in neuronal cell body as well as at the diffuse or neuritic Aβ plaque. Notably, Cx43 expression was not elevated around AT8+ Tau NFT residing in neuron cell body. However, Cx43 expression is significantly increased at plaques surrounded with AT8+ neurites. Moreover, Cx43 enrichment was greater at plaques with AT8+ neurites compared to the dense-cored plaques lacking AT8+ neurites. We have added these data in **Supplementary Fig. 1c-d** and append new quantification to **Fig 1e**.

Fig. 1



Supplementary Fig. 1

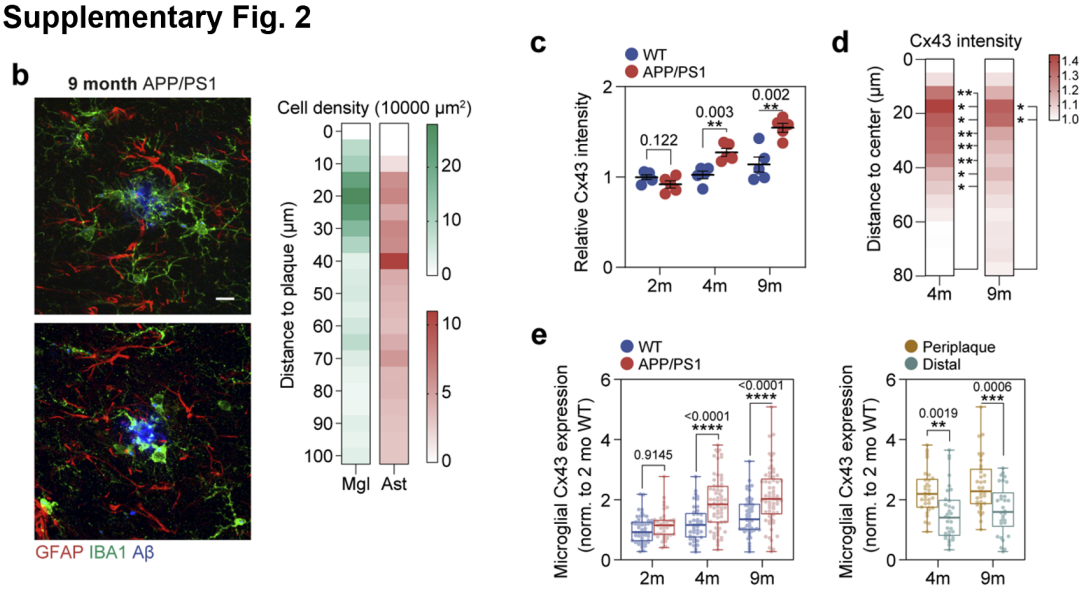


Regarding experiments in Figure 2:

Need to show data from other areas of plaque deposition such as cortex. More representative of the cellular composition and anatomy as examined in the human brain slices.

Thank you for your suggestion. We included the cellular composition and anatomy in **Supplementary Fig. 2b.** The quantification of Cx43 expression change in the cortex is presented in **Supplementary Fig. 2c-e** as shown below.

Supplementary Fig. 2



In addition to performing stats by distance to plaque surface, the authors need to control for plaque size within age groups and between age group. Naturally, there will be larger plaques in the 9m animals, and so there will be size bins that do not match the 4m group, which is ok.

We appreciate the reviewer's insightful comment on the importance of controlling for plaque size across age groups. We acknowledge that plaque size increases with age in this model (9m vs. 4m), which inherently limits direct size-matching between groups. While a stratified analysis based on plaque size or type could provide additional nuance, our primary objective in this analysis is to highlight the rapid response of Cx43 expression to Aβ deposition. Notably, Cx43 is upregulated at periplaque regions as early as 4 months of age, when Aβ plaque pathology first arises. We agree that investigating the plaque size dependent regulation of Cx43 expression is an important direction for future studies, and we appreciate for this constructive suggestion.

I don't understand how the EtBr experiments show any specificity to Cx43 hemichannels. Presumably any random dye would also do this but this one sticks to nucleic acids so you can visualize the cell nuclei? Also, the details of the inhibition are unclear. Are you preloading the inhibitors then adding dye? Presumably any dye bulk loaded at high concentration to an acute brain slice can enter the cell independent of hemichannels, and plaque-associated microglia are highly phagocytic, so it is not surprising you see greater EtBr signal in those microglia compared to distal ones. The importance of Cx43 here is only forced up the reader because of the use of the selective inhibitors. These experiments are not clear and thus leave this reviewer unsure how to interpret them and determine if the conclusions are acceptable. One would need to load individual cells with a capillary pipette to visualize spreading of dye and blockade of hemichannels and gap junctions.

We apologize for lack of details in our initial description of this experiment. Dye uptake experiment is a widely used assay to monitor opening of hemichannels (10.1152/physrev.00043.2018, section VIII-B); this assay utilizes poor selectivity of connexin hemichannels, which allow the passage of small molecules with a molecular weight of < 1.5

kDa (10.1152/physrev.00043.2018). Several dyes were used in dye uptake experiment, including EtBr, DAPI, and carboxyfluorescein, all of them cannot cross the lipid bilayer but permeate through connexin hemichannels. The reliability of this method in assessing connexin hemichannel activity has been confirmed studies using connexin knockout models (10.1073/pnas.012589799; 10.1523/JNEUROSCI.0861-15.2015).

For our study, we selected EtBr for the dye uptake experiment due to: (i) carboxyfluorescein diffuses across the cell body after entering through hemichannel, making it implausible to perform cell type specific dye uptake analysis; (ii) both EtBr and DAPI bind to DNA upon entering the cell, facilitating cell-type identification. However, the autofluorescence of Aβ plaques interferes with the DAPI detection, making ErBr better choice.

In our protocol, we pre-incubated the acute brain slices with hemichannel inhibitors for 15 min before adding EtBr. EtBr dye was added to the recording buffer at a concentration of 5 μM, and the slices were incubated with EtBr for 10 min, then rinsed 3 times with the recording ACSF (5 min each), fixed with 4% PFA for 1 hour, and subsequently proceeded for immunostaining (See **Method – Dye uptake**).

Regarding the concern that increased EtBr signal observed may result from phagocytosis rather than hemichannel activity, this is unlikely for several reasons. Firstly, the time frame is insufficient for phagocytosis to account for the observed signal. The process of phagosome formation, maturation and fusion with lysosome typically takes ~10 min (10.1038/s42003-022-03988-4), which is the same duration as our EtBr treatment. Even if EtBr was phagocytosed, it would remain trapped within the phagosome due to its limited permeability across the lipid bilayer. Secondly, Cx43 inhibition and conditional knockout reduce EtBr uptake. Both pharmacological blockade of Cx43 hemichannels as well as conditional knockout of Cx43 in microglia significantly reduced the EtBr dye uptake in periplaque microglia. These results both indicated that the observed EtBr uptake is mediated by Cx43 hemichannel activation.

We incorporated the experimental details into the **Method - dye uptake experiment** section.

Regarding experiments in Figure 3:

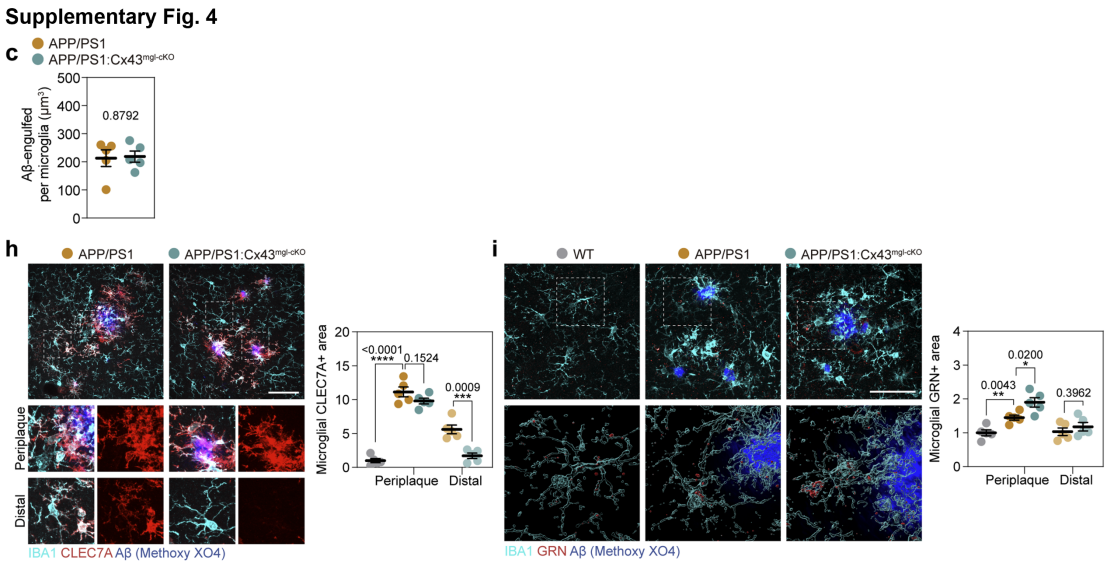
The comparison between plaques in APP/PS1 versus APP/PS1 Cx43 KO mice is flawed. Authors describe the proportion of plaque morphotypes (filamentous vs compact) is shifted towards more compact plaques. However, when looking at RTN3 and LAMP1 areas around plaques between the mouse groups, they do not consider this in their grouping. They should identify filamentous plaques and compact plaques by a strict criterion and then measure RTN3 and LAMP1 signal for the same size plaques in each category. Only doing it by this way can they make any claims that the plaques in the Cx43 KO mice are less damaging if indeed the results hold. As it stands, the images in the representative panels clearly show they did not do this and all they are measuring is RTN3 and LAMP1 as a consequence of total area of Methoxy signal per plaque.

In our study, we observed that microglial Cx43 ablation increased periplaque microglial clustering, consistent with previous findings linking microglia-mediated plaque compaction to reduced neurite dystrophy (10.1016/j.neuron.2016.05.003; 10.1038/s41467-019-11674-z).

Our analysis of total RTN3 and LAMP1 signals relative to total Methoxy (plaque area) also supports our hypothesis. Therefore, we have revised the Results section to clarify that the observed reduction in RTN3/LAMP1 signals reflects an overall decrease in periplaque neurite dystrophy, in line with increased plaque compaction in Cx43 KO mice.
We agree that rigorously distinguishing between filamentous and compact plaques, followed by morphotype-specific analysis of RTN3 and LAMP1 signals, would enhance our understanding of plaque-shape associated neurotoxicity. However, this is beyond the scope of this study. In future relevant studies, we will implement strict morphotype classification and size-matched plaque analyses in Alzheimer's disease.

Regarding experiments in Figure 4:

RNAscope and IHC experiments are needed to validate key genes identified from the Bulk cell RNA-seq findings localize which signatures are associated with peri-plaque and distal microglia. One set of data discordance is seen in panel 4D where more abeta inside microglia is observed by imaging in Cx43 KO mice, but the RNA-seq data does not show any differential expression patterns in genes for lysosomal or phagocytosis. How do the authors explain this kind of discrepancy? This is where the spatial validation of DEG would refine the interpretation of findings here and increase the impact of the manuscript.
Thank you for the suggestion. In Fig 4d, we showed a higher percentage of Methoxy X04+ Aβ plaque within the microglia domain in Cx43 cKO mice. We attribute this to an increased number of periplaque microglia, rather than enhanced Aβ phagocytosis by individual microglia. Indeed, when we normalized the microglia-engulfed Aβ to the number of periplaque microglia, no significant difference was found between the control and Cx43 cKO mice (**Supplementary Fig. 4c**). That said, we agree that the spatial validation of DEG would further strengthen our manuscript. To address this, we performed immunostaining to examine the expression of two selected DEGs, Clec7a (downregulated in Cx43 cKO mice) and Grn (upregulated in Cx43 cKO mice). Interestingly, Clec7a showed only a trend toward reduction in periplaque microglia of Cx43 cKO mice, but was significantly reduced in distal microglia. In contrast, Grn was significantly increased in peri-plaque microglia of Cx43 cKO mice, but not the distal microglia (**Supplementary Fig. 4h-i**). Overall, RNA sequencing data on Clec7a and Grn from the total cortex and hippocampus have been verified by immunostaining analysis.

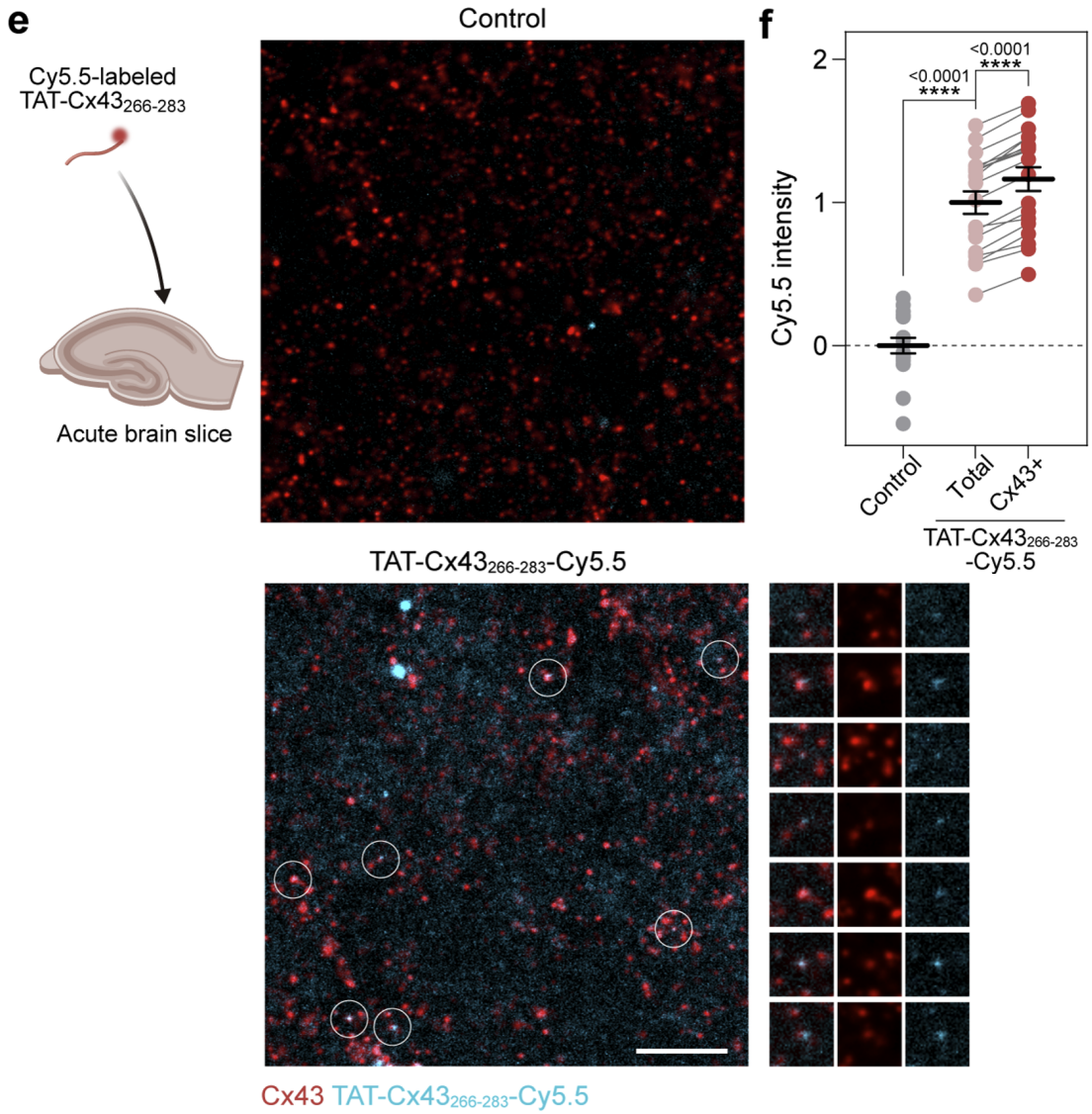


Regarding all remaining experiments using TAT-Cx43266-283 and TAT-Cx43@LNPs:

I have one major concern. The authors do not show directly labeling of their TAT-Cx43 reagent on cultured microglia, microglia (or astrocytes) in acute brain slices, or after delivered via ip injection. If indeed this reagent is binding to Cx43 then why can't you prove that at the cellular level? You've conjugated a Cy5.5 molecule for the in vivo image experiment but cannot show us by microscopy? Before I can assess the meaning or value of all the work done in the rest of the manuscript, I would need to see unambiguous evidence of the cellular localization of TAT-Cx43@LNPs to Cx43 (co-labeled with antibody). If you can prove this is the case, then I do not know how to judge the rest of the paper.

Thank you for the suggestion. We performed an additional experiment in which acute brain slices were treated with TAT-Cx43$_{266-283}$-Cy5.5, followed by fixation and immunostaining of Cx43. We observed that an enrichment of TAT-Cx43$_{266-283}$-Cy5.5 at the Cx43+ puncta (**Supplementary Fig. 5e**). Furthermore, quantification of Cy5.5 intensity within the total volume and the Cx43+ regions revealed a significant increase in Cy5.5 intensity specifically within the Cx43+ regions (**Supplementary Fig. 5f**). These findings support the notion that TAT-Cx43$_{266-283}$ interact with Cx43 to suppress hemichannel activation.

Supplementary Fig. 5

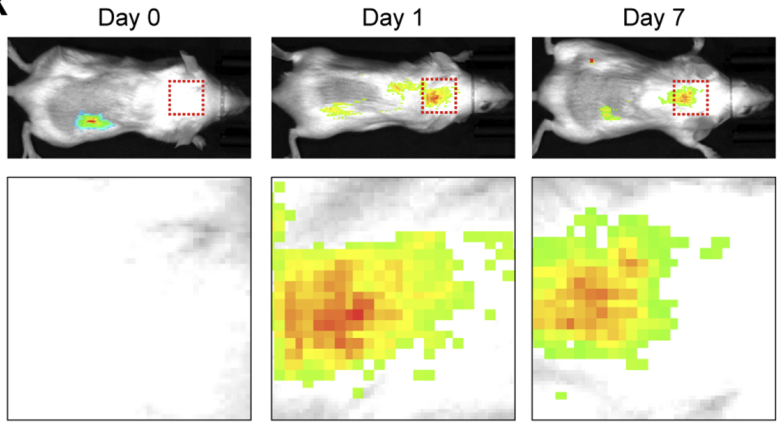


The in vivo imaging with a low-resolution method showing some weak signal from the head is not convincing. First of all, the animal in those images are contorted in all kinds of ways with the head twisted, how can you get reliable signal from the same area at each time point. This is sloppy. You need to position mouse and hold its head in the same position for each measurement and zoom into the mouse head. These non-invasive systems do have the ability to zoom in. The brain lysate measurements are also not necessarily convincing either. For all we know, all you are showing us is that your TAT-Cx43@LNPs have bound to Cx43 on brain endothelial cells and not actually entered brain parenchyma and bound to the Cx43 on microglia or astrocytes.

We apologize for the low-quality images. We repeated the biofluorescence imaging experiment, with the mouse head positioned at the same position **(See new Fig 5k).** Result shows that Cy5.5 signal persists in the brain for up to 7 days post injection.

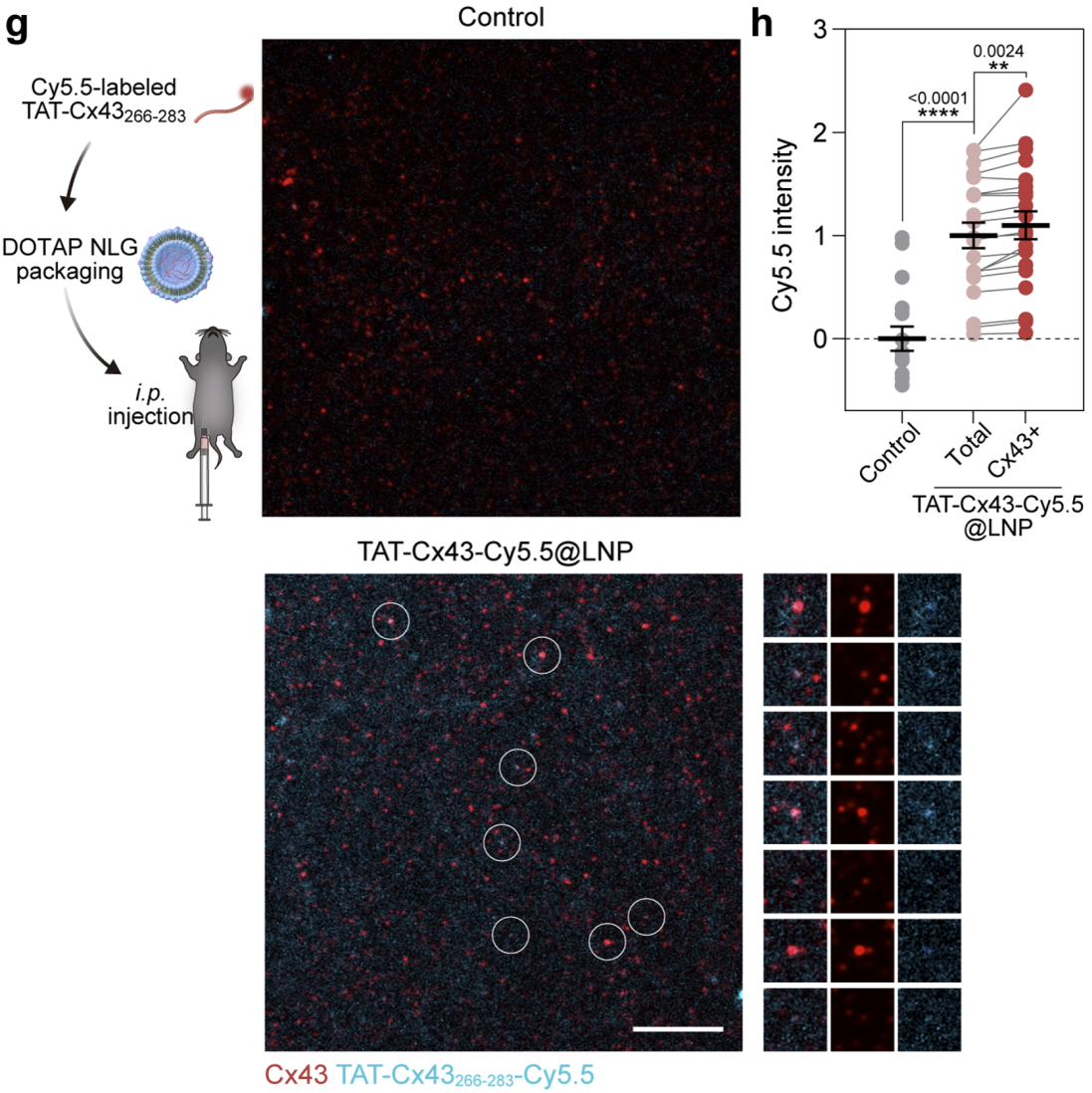
Fig. 5

k



To further confirm the entry of LNP-packaged peptide into the brain parenchyma, we performed imaging of brain slices on day 1 post injection. Mice injected with TAT-Cx43-Cy5.5@LNP injected mice exhibited higher Cy5.5 signal intensity in the brain parenchyma compared to the controls. Consistent with the *ex vivo* brain slice treatment, Cy5.5 signals were also enriched at Cx43+ puncta (**Supplementary Fig. 5g-h**), further supporting the targeted delivery of the peptide.

Supplementary Fig. 5



Assuming this all worked out and you can prove that TAT-Cx43@LNPs binds to microglia and astrocytic Cx43, you will need to also include the APP/PS1 mice with Cx43 conditional KO in microglia and astrocytes mice as controls to show that you do not get further amelioration of the amyloid phenotypes to bolster the notion that your experimental reagent is indeed working through microglia and/or astrocytes.

We appreciate the reviewer's insightful suggestion that including APP/PS1 mice with conditional Cx43 KO in microglia/astrocytes as controls would further strengthen the mechanistic link between TAT-Cx43@LNPs and its cellular targets. However, after careful consideration, we regret that implementing this experiment is currently unfeasible due to the following constraints:

(i) Generating and validating conditional Cx43 KO mice crossed with the APP/PS1 model would require significant time (12~18 months for breeding/characterization) and cost, delaying the timely dissemination of our findings.

(ii) Our institution strictly adheres to the 3R principles (Reduction, Refinement, Replacement) in animal research. Initiating a new large-scale breeding colony would not only have limited

value beyond this study, but it would also add minimal benefit to our existing robust dataset. (iii) As noted by the reviewer, our current evidence including colocalization assays, hemichannel functional assays, and amyloid phenotype rescue in APP/PS1 mice, already provides strong mechanistic support for the specificity and benefit of Cx43 hemichannel targeting.

While we acknowledge the value of this additional control, we believe our revised manuscript, along with the additional analyses now included, sufficiently address this concern.

Response to Reviewers' comments:

Reviewer #3 (Remarks to the Author):

The authors address some minor technical concerns sufficiently, but they fall short of addressing the major flaws of their experimental design and thus many of their primary conclusions are left unsubstantiated. In particular, this manuscript lacks unambiguous evidence that targeting Cx43 with the TAT-Cx43@LPs approach is working as they believe it to be. Therefore, this paper remains unready for publication with the current claims. See below for specific critiques of issues still unaddressed.

1. Of course, oligodendrocytes and OPCs are present near plaques (doi.org/10.1016/j.neurobiolaging.2020.05.016)! These cells are everywhere. They are both affected and contribute to plaque deposition. Recently, they were shown by two independent groups to participate in Aβ plaque generation (DOI: 10.1038/s41593-024-01730-3; doi.org/10.1186/s13024-024-00759-z). Having said that, it is true that OPCs and Oligos do not express Cx43 at baseline conditions, however, neither do microglia at baseline according to the same RNAseq databases used widely by the community to validate gene expression by cell type. As it stands, there is still Cx43 not accounted for in microglia nor astrocytes around plaques. Thus, you have to rule out the OPCs/Oligos to not upregulate Cx43 in plaque proximity. Also, since you still have an appreciable amount of Cx43 in plaque regions even after the conditional knockout in microglia. Even if you argue about some failed recombination leading to inefficient knockdown, taking all this evidence together argues that you are missing something quite obvious about plaque-associated Cx43 expression and it needs to be resolved.

Thank you for your comment. While OPCs and oligodendrocytes (OLs) are present near plaques, their spatial distribution is less enriched compared to microglia. We quantified the distribution of OPCs (PDGFRA+) and OLs (CC1+) in relationship to plaque using the same approach we used with microglia and astrocytes, which demonstrated the marked increase in microglia at the periplaque area, but not OPCs and OLs (See new **Supplementary Fig 2b**).

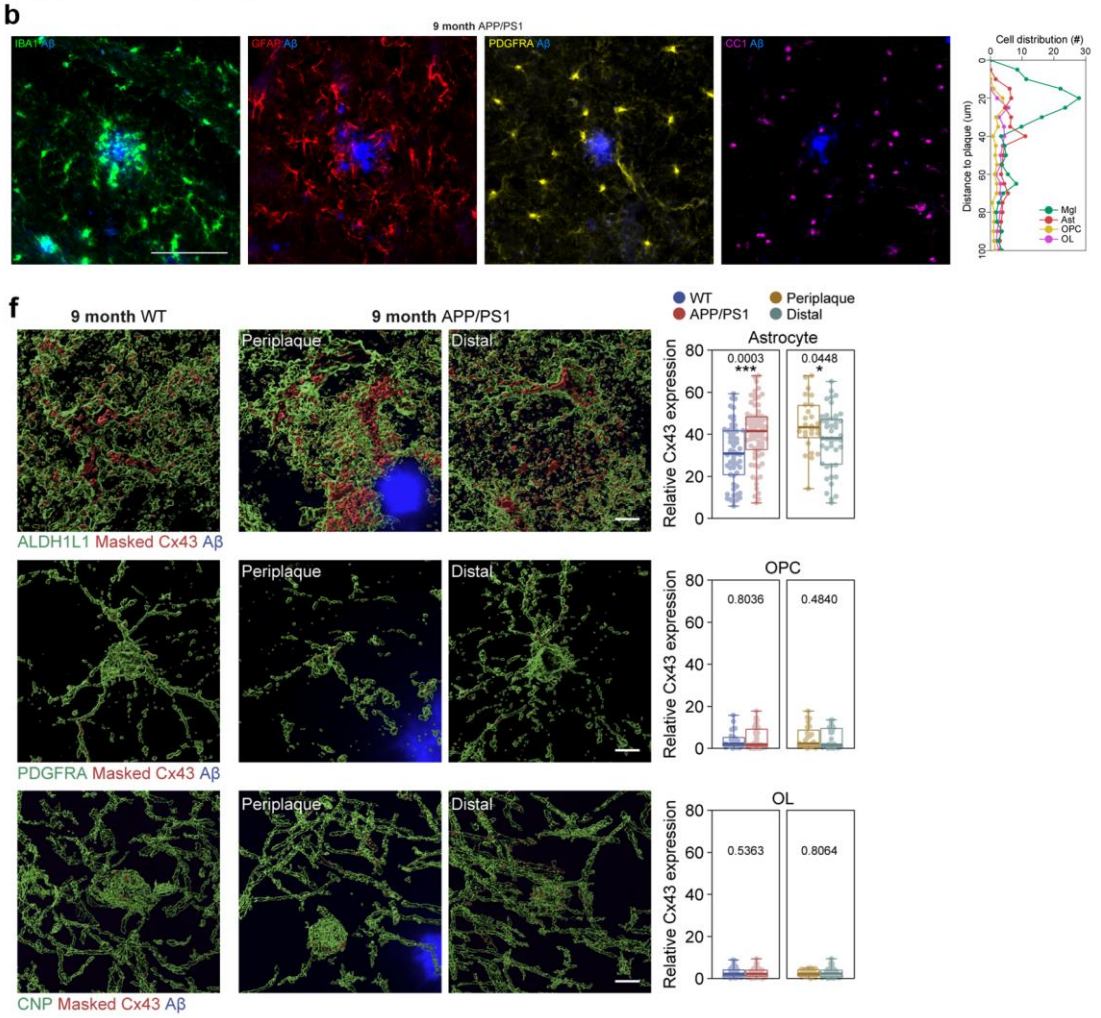
To examine whether Cx43 expression in oligodendroglial cells is associated with the presence of plaques, we performed immunostaining and quantified expression of Cx43 in OPC (PDGFRA-labeled) and OL (CNP-labeled) domain in 9-month-old WT and APP/PS1 mice . Results showed that Cx43 level in OPC or OL was low in both WT and APP/PS1 mice; neither it was significantly increased in periplaque cells (See new **Supplementary Fig 2f**). In contrast, Cx43 staining in ALDH1L1+ astrocytes (See new **Supplementary Fig 2f**) and IBA1+ microglia (Fig 2d-e) was significantly upregulated in APP/PS1 mice brains compared to WT, and Cx43 levels are higher in periplaque astrocytes and microglia compared to distal ones. These data support the hypothesis that the elevated Cx43 level was associated with the presence of plaques, and they are mainly located in periplaque astrocytes and microglia, but not in the oligodendroglia.

In Cx43 mgl-cKO mice, we showed a significant reduction of Cx43 expression in periplaque microglia (evidenced by the IBA1-masked signal and the quantification,

supplementary Fig 3a). We think the residual Cx43 expression in the periplaque area in Cx43 mgl-cKO would be located in the periplaque astrocytes given their increased Cx43 levels.

Together, these data support our conclusion that periplaque Cx43 elevation is driven by microglia and astrocytes, with minimal involvement of oligodendroglial cells. We have included these new data in the **Result, Section 2, paragraphs 1 and 2.**

Supplementary Fig 2



Supplementary Fig 2: b. Distribution of glial cells in relation to plaques. Images show IBA1 (microglia), GFAP (astrocyte), PDGFRA (oligodendrocyte precursor cell, OPC), CC1 (oligodendrocyte, OL) and Aβ staining in the hippocampus from 9-month-old APP/PS1 mice. Scale bar: 100 μm. Right, quantification of microglia, astrocyte, OPC, and OL distribution at different distances from Aβ plaques. Cells around >15 plaques were quantified. **f** Representative images and quantification of Cx43 expression in ALDH1L1+ astrocyte, PDGFRA+ OPC, or CNP+ OL domain, in the hippocampus from WT and APP/PS1 mice at 9-month-old. Cx43+ volume was normalized to the cell domain volume, displayed in percentage. Scale bar: 5 μm. For astrocytes, N = 57 (WT), 71 (APP/PS1, 26 periplaque, 45 distal) cells. For OPC, N = 27 (WT), 56 (APP/PS1, 25 periplaque, 31 distal) cells. For OL, N = 33 (WT), 52 (APP/PS1, 20 periplaque, 32 distal) cells.

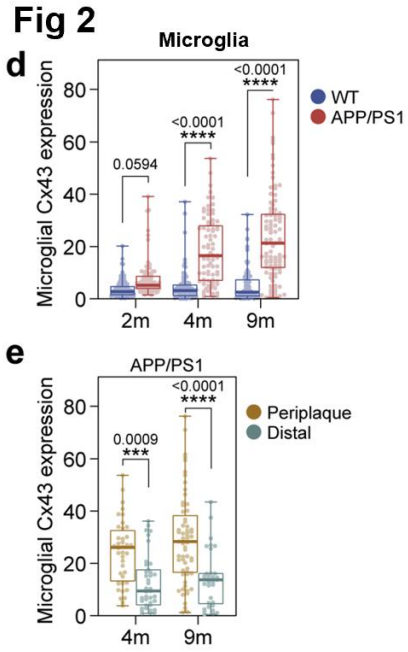
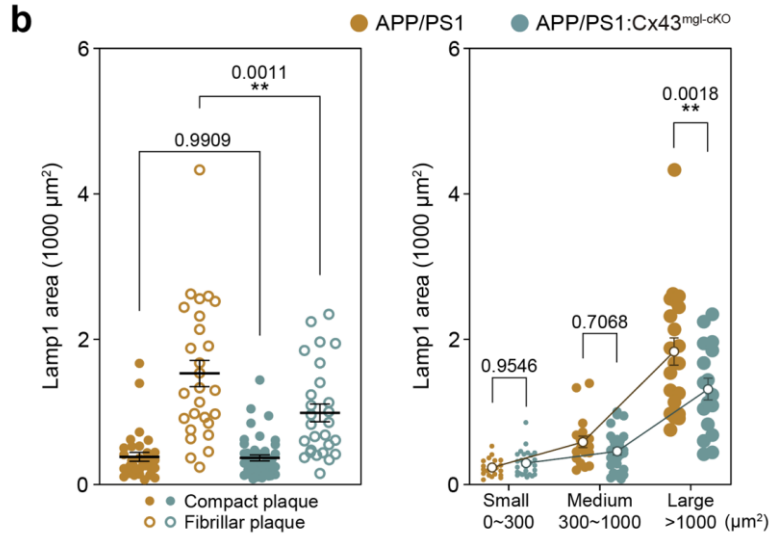


Fig 2: d Quantification of Cx43+ area within IBA1+ microglial domain in WT and APP/PS1 mice. Cx43+ volume was normalized to the cell domain volume, displayed in percentage. N = 97, 92, 79, 79, 90, 95 cells, respectively, from 5 mice. **e** Comparison of Cx43+ area within IBA1+ domain between periplaque and distal microglia. N = 40, 39, 61, 34 cells from 5 mice.

2. You need to stratify your existing data further by breaking down by plaque size and plaque morphotype are well within the scope of your manuscript. You have the data already, so you can analyze it readily. As it stands, your data is only modestly different in most comparisons in human AD and mouse models. There is a likely scenario that your data is only significant for certain plaque types/sizes, which is okay, but you need to define that so anybody who tries to replicate your work and does so more rigorously than you have now can replicate the data.

Thank you for the suggestion. We re-analyzed the Lamp1+ neurite dystrophy area and the matching plaque size in APP/PS1 controls and APP/PS1:Cx43$^{mgl\text{-}cKO}$ mice. We then compared dystrophic neurites around compact and fibrillar plaque, and found that Cx43 cKO did not affect the Lamp1+ dystrophic neurites around compact plaques, but significantly reduced Lamp1+ dystrophic neurites around fibrillar plaques. We further classified plaques according to size, separating them into small (0~300 μm^2), medium (300~1000 μm^2) and large (>1000 μm^2). It appeared that Cx43 cKO significantly alleviated neurite dystrophy around large plaques, but not that of the small or medium plaques (See new **Supplementary Fig 3b)**. These findings highlight the role of microglial Cx43 in Aβ-associated pathology depends on plaque type and size. We have added this to the **Result, Section 3, paragraph 2**.

Supplementary Fig 3



Supplementary Fig 3: b Left panel: Lamp1+ neurite dystrophy area around compact and fibrillar plaque in APP/PS1 and APP/PS1:Cx43$^{mgl\text{-}cKO}$ mice. N = 60 (APP/PS1, 33 compact, 27 fibrillar), 73 (APP/PS1:Cx43$^{mgl\text{-}cKO}$, 46 compact, 27 fibrillar) plaques. Right panel: Lamp1+ neurite dystrophy area around small (0~300 µm^2), medium (300~1000 µm^2) and large (>1000 µm^2) plaque. For APP/PS1 mice, N = 21 (small), 18 (medium), 21 (large) plaques. For APP/PS1:Cx43$^{mgl\text{-}cKO}$, N = 27 (small), 29 (medium), 17 plaques (large). Data were analysed by Two-way ANOVA.

3. Lack of unambiguous data showing TAT-Cx43-Cy5.5 is targeting Cx43 in brain microglia. The additional data showing antibody stained Cx43 colocalize with a few random Cy5.5 puncta is problematic for the following reasons:

A) You do not provide controls of the TAT-Cy5.5 (with or without the LPs for acute slice versus the in vivo IP injection experiments). Show clearly the controls and do high resolution confocal microscopy with XZ and YZ orthogonal views to calculate true colocalization. What fluorophore did you use for the secondary antibody (no details in legend or methods) to stain Cx43? Need to rule out given the dimness of the Cy5.5 in the images as evident by high background, you may be cross-exciting the fluorophore for anti-Cx43 and getting spectral bleed through. As it stands there is just some random chance you have puncta that happen to appear close enough together to get an artificial colocalization using loose criterion on image processing software.

Thank you for your comment. We added the TAT-Cy5.5 group in the acute brain slice treatment. TAT-Cy5.5 treatment elevated the Cy5.5 signal in the acute brain slice, however, they did not colocalize with Cx43+ puncta as shown in the representative image and the quantification of Cy5.5 intensity in Cx43+ domain (compared to the total mean intensity). This supports the specificity of only TAT-Cx43$_{266\text{-}283}$-Cy5.5 targeting microglial Cx43.

We also added the orthogonal view to show the true localization of TAT-Cx43$_{266\text{-}283}$-Cy5.5 and the Cx43 signals. We used Alexa Fluor 555-conjugated antibody to label Cx43 to reduce chromatic aberration (which would significantly affect colocalization analysis, see

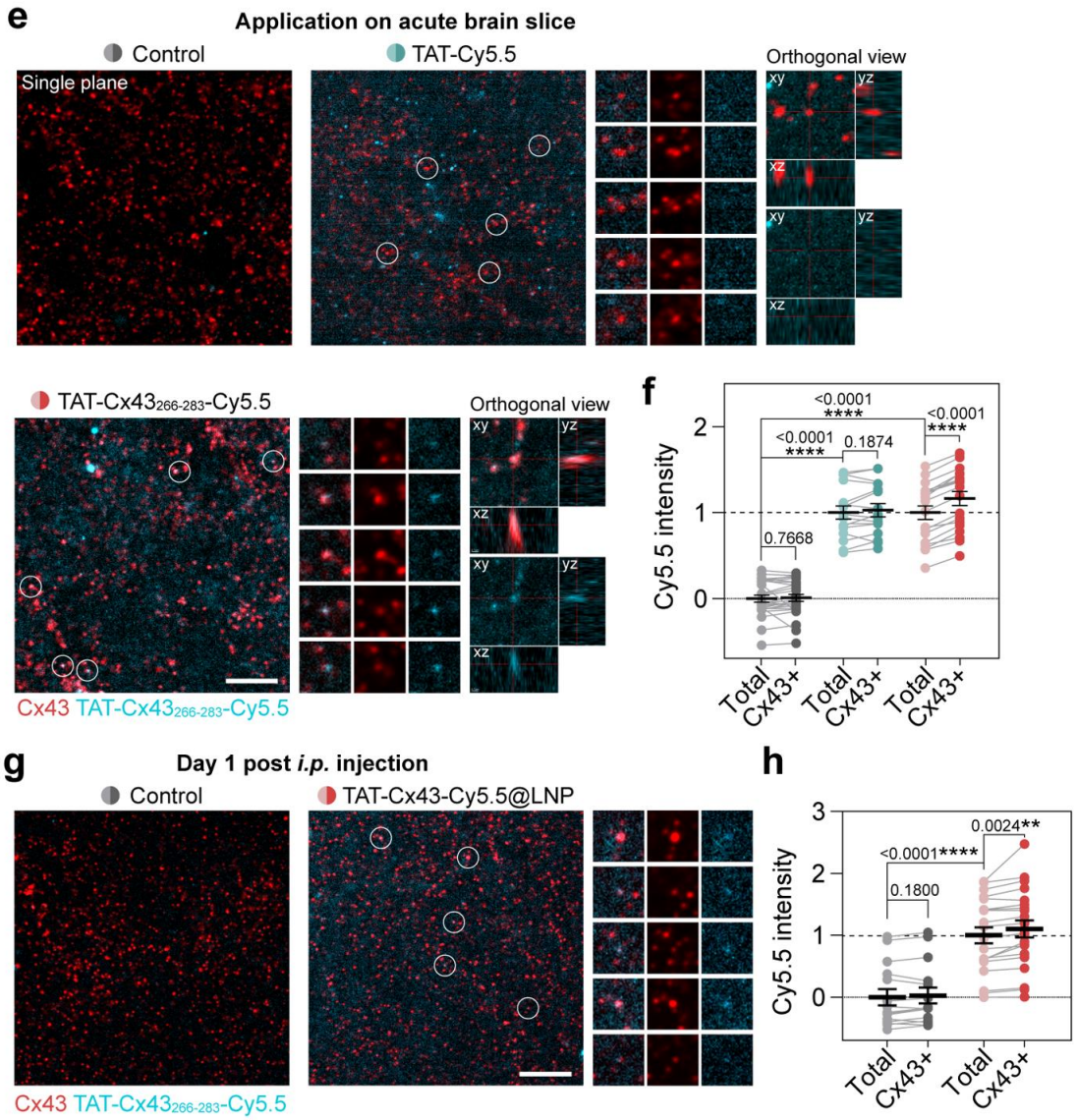
Jonkman et al., 2020). However, it may introduce spectral bleed-through. To examine this possibility, we also quantified the Cy5.5 signal within the Cx43+ domain in the empty control and the TAT-Cy5.5 control groups, which did not show significant changes compared to the Cy5.5 signal of the total image (See **new supplementary Fig. 5e-f**). Similarly, in the TAT-Cx43@LNP treatment experiment, Cy5.5 signal in the Cx43+ domain of the control group was not significantly different from that of the total image (See **new supplementary Fig. 5h**), suggesting that the spectral bleed-through did not interfere with our colocalization analysis.

In summary, in response to the reviewer's concerns, we confirmed specific colocalization of TAT-Cx43$_{266\text{-}283}$-Cy5.5 with Cx43+ puncta using orthogonal imaging and Alexa Fluor 555-conjugated antibodies, with controls (TAT-Cy5.5 and empty control), which demonstrate no significant spectral bleed-through or artificial colocalization. These analyses validate the specificity of our findings and rule out technical artifacts in colocalization measurements. This content was added to **Result, section 5, paragraph 3**.

Reference:

1. Jonkman J, Brown CM, Wright GD, Anderson KI, North AJ. Tutorial: guidance for quantitative confocal microscopy. Nature protocols 15, 1585-1611 (2020).

Supplementary Fig 5



Supplementary Fig 5: (e) TAT or TAT-Cx43$_{266-283}$ peptide was conjugated with Cy5.5 fluorophore before applied to acute brain slices, followed by counterstaining of Cx43 (secondary antibody, Alexa Fluor 555 conjugated). Representative images show control, TAT-Cy5.5, and TAT-Cx43$_{266-283}$-Cy5.5 treated brain slice. Scale bar: 10 µm. Enrichment of Cy5.5 signal at Cx43+ puncta is highlighted in white circles. Orthogonal view of TAT-Cx43$_{266-283}$-Cy5.5 group demonstrates the true colocalization of Cy5.5 and Cx43 signal. **(f)** Quantification of Cy5.5 signal intensity in control, TAT-Cy5.5, and TAT-Cx43$_{266-283}$-Cy5.5 treated brain slices. The Cy5.5 intensity in Cx43+ volume was further quantified for each group. Pairwise comparison was performed to compare Cy5.5 intensity in total and Cx43+ volume. N = 18, 16 and 19 images from 3 brain slices. **(g)** Acute brain slices on day 1 post TAT-Cx43-Cy5.5@LNP intraperitoneal injection subjected to Cy5.5 and Cx43 counterstaining. Scale bar: 10 µm. White circles highlight examples of Cy5.5 enrichment at Cx43+ puncta. **h** Quantification of Cy5.5 intensity in control and TAT-Cx43-Cy5.5@LNP treated mouse brain slices. N = 16 and 21 images from 3 mice.

B) Assuming your signal is still only specific to the condition when you administer TAT-Cx43-Cy5.5 and not TAT- Cy5.5, then show with additional immunostaining for IBA1

and Aldhdl1 that the TAT-Cx43-Cy5.5 is targeting microglia. Its clear it won't be microglia specific, but you must show that TAT-Cx43-Cy5.5 is indeed binding to plaque-associated microglia using high resolution confocal microscopy. Without these data, and especially because the authors protest doing the experiment in the conditional Cx43 knockout mice, the existing claims about the TAT-Cx43-Cy5.5@LPs made by the authors do not stand. Considering the heart and liver get just as much bulk uptake of their TAT-Cx43-Cy5.5, there could just be some systemic effect. In this vein, the authors do not include a key control for experiments shown in Fig5J-I. You show comparison peptide with LNPs to peptide without LNPs, but you do not show experiments where you inject TAT without the Cx43(266-283). You have to rule out the increased brain retention just because of the LNPs.

Thank you for your comment.

To demonstrate that TAT-$Cx43_{266-283}$ interact with periplaque microglia, we performed immunostaining of IBA1 as per request. The results confirm the colocalization of TAT-$Cx43_{266-283}$ and Cx43 in the periplaque microglia (See **new supplementary Fig. 5i**), supporting the conclusion that TAT-Cx43 delivered by LNP indeed directly interact with periplaque microglial Cx43 to suppress its hemichannel function.

Our data showed that TAT-Cx43@LNP could enter brain parenchyma, interact with Cx43 to suppress hemichannel activation (including that of periplaque microglia), and thus promote microglia-plaque interaction to limit neuropil damage. Results also show that TAT-Cx43@LNP does not interfere with the normal function of vital organs expressing Cx43, including the heart, liver, and kidney (supplementary Fig 5l-n). Together these data suggest a central mechanism of TAT-Cx43@LNP in alleviating the cognitive deficit and neural pathology in APP/PS1 mice. It is possible that the drug may affect the peripheral organs to alleviate the central pathology, which is however beyond the scope of this study. We have added this to the **Discussion section, paragraph 5**.

TAT-$Cx43_{266-283}$ peptide is able to enter the brain parenchyma due to the TAT sequence that facilitates the BBB transport (Stalmans et al., 2015; Abudara et al., 2014). To verify if TAT sequence promotes brain retention, we synthesized the $Cx43_{266-283}$ peptide, labelled it with Cy5.5, and measured its levels in the brain and serum on day 1, 3, and 7 post i.p. injection. The result showed that $Cx43_{266-283}$ peptide concentration in serum was similar to that of the TAT-$Cx43_{266-283}$ peptide (without LNP packing). However, the $Cx43_{266-283}$ peptide failed to enter the brain evidenced by the lack of Cy5.5 signal from days 1-7 post injection (See **new Fig. 5l**). We appreciate the reviewer's suggestion to include a TAT-only control. However, we believe this experiment is not necessary for the current study, as the role of the TAT sequence in facilitating BBB transportation is already well-established (Stalmans et al., 2015; Abudara et al., 2014), and our above data confirm its functional contribution for BBB transport and brain retention of $Cx43_{266-283}$. Therefore, injecting TAT alone (without a cargo peptide) would not provide additional mechanistic insight.

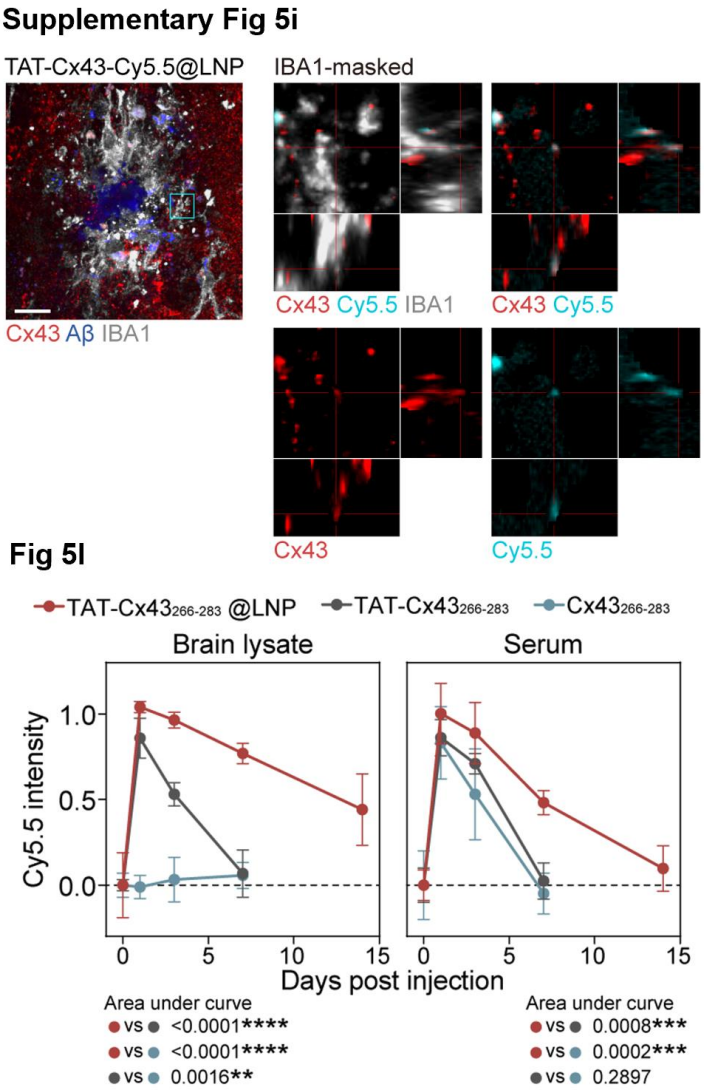
In summary, we demonstrated specific targeting of TAT-$Cx43_{266-283}$-Cy5.5 to periplaque microglia via IBA1 colocalization and confirmed that the TAT sequence is essential for brain entry, as non-TAT peptide failed to accumulate in the brain despite comparable serum levels. These data, alongside organ-specific safety assessments, validate the central mechanism of TAT-Cx43@LNP. The related results were described in the **Result,**

section 5, paragraph 4.

Reference

1. Stalmans S, et al. Cell-Penetrating Peptides Selectively Cross the Blood-Brain Barrier In Vivo. PloS one 10, e0139652 (2015).

2. Abudara V, et al. The connexin43 mimetic peptide Gap19 inhibits hemichannels without altering gap junctional communication in astrocytes. Frontiers in cellular neuroscience 8, 306 (2014).



Supplementary Fig 5: i Representative image of Cx43 and Cy5.5 colocalization in periplaque IBA1+ microglia. Scale bar: 10 µm. Orthogonal view shows IBA1, Cx43, and Cy5.5 signal, as well as IBA1-masked Cx43 and Cy5.5 signal.

Fig 5: l Microplate reader analysis of Cy5.5 intensity of brain lysate and serum collected at day 0, 1, 3, 7, 14 post-injection of TAT-Cx43$_{266-283}$ peptide with or without DOTAP-NLG packaging, as well as Cx43$_{266-283}$ peptide without DOTAP-NLG packaging. N = 5 (TAT-Cx43$_{266-283}$@LNP), 3 (TAT-Cx43$_{266-283}$), and 3 (Cx43$_{266-283}$) experiments. The area

under curve were calculated for statistical analysis (unpaired t-test).

4. The title of the manuscript is not appropriate. It reads as a title for a future review or perspective piece. Change the title to a more conventional title used for primary research articles.

Thank you for your suggestion. We have changed the title to “Connexin43 hemichannel blockade turns microglia neuroprotective and mitigates Alzheimer's disease-related cognitive deficits”.

Response to Reviewers' comments:

Reviewer #3 (Remarks to the Author):

The authors have addressed most of remaining concerns. For example, they have added key controls missing in many of the experiments, which were casting doubt about the validity of their results, especially for the LNP delivery of TAT-Cx43. Now, with more attention to detail, the authors have a stronger manuscript.

Nevertheless some claims regarding their LNP based TAT-Cx43 approach are borderline. I suggest adding to their discussion, which already includes some talk of caveats/limitations of the study, some language around the timing of use of this approach. For example, the Cx43 KO only showed a difference for large filamentous plaque, no other size groups or compact plaques. What does that mean, and since the authors did not include this level of analysis in Figure 7, we can't know how effective this would be at different stages of amyloid deposition, or in models that have concomitant tau neuropathology.

We thank the reviewer for this comment. While our intervention window (inducible knockout: 8.5 months–9.5 months; TAT-Cx43@LNP: 8 months–9.5 months) is shorter than constitutive knockout studies, it still elicited robust rescue of cognitive deficits and neuropathology. Notably, early TAT-Cx43@LNP administration (3.5–5.5 months) reduced Aβ plaque burden by 41.2 ± 13.2% (Fig. 7), demonstrating its potential for pathology prevention. These results highlight the therapeutic potential for Cx43-targeted interventions even in advanced stages. We added this statement to the text.

Regarding the plaque size- and type-dependent effects of microglial Cx43 ablation on periplaque neurite dystrophy, we propose two key considerations: First, compact or smaller plaques are inherently associated with reduced neurite dystrophy. Consequently, the microglial phenotypic changes induced by Cx43 KO may lack sufficient additional impact to further mitigate dystrophy in these regions. Second, and critically, when contextualizing these findings holistically, microglial Cx43 KO enhances plaque compaction, a process that diminishes the overall burden of dystrophic neurites by reducing the proportion of diffuse, neurite-injurious plaques. Thus, while local microglial effects near preexisting compact plaques may appear limited, the systemic shift toward plaque compaction represents a neuroprotective mechanism at a broader scale.

We added parts of these discussion to the result section (section 3, paragraph 2), and in discussion section (paragraph 3).

Importantly, given the above statement, the new data showing that Cx43 KO ONLY impacts large, fibrillar plaques, this data needs to be in the main figure 3, not in the supplement.

We thank the reviewer for this suggestion. We moved this piece of data to the new Fig 3e.

Finally, the title is now acceptable. However, regarding nomenclature, the authors often use early and late stage of AD in their text. This is a model of amyloid-beta deposition, one of two neuropathological protein lesions in AD. The model is not AD. You can say mouse models of amyloid deposition, and early and late stage of amyloid deposition, but not AD (which requires both abeta and tau pathologies). Fix the text accordingly.

We thank the reviewer for this comment. We revised the text accordingly.