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Peer Review File

Aberrant Excitatory Neuronal ERBB4 Promotes Alzheimer's Disease Pathology

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Any redactions in this file are there to maintain patient confidentiality, the confidentiality of unpublished data, or to remove third-party material.

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

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)

This is a rather comprehensive study with many intriguing results and some main conclusions that, if true, would represent what the authors call a “paradigm shift” for Alzheimer’s disease research. Remarkably, they show evidence in mouse amyloidosis models (mainly 5xFAD) that Abeta induces, early in disease pathogenesis, a class of “Disease Initiating Excitatory Neurons” (DIENs), which are driven by aberrantly high expression of ErbB4 in those excitatory neurons. Elevated ErbB4 activity leads to neuronal hyperactivity, increased phagocytosis of excitatory synapses by microglia and astrocytes, but reduced phagocytosis of inhibitory synapses, and hence corresponding opposite changes in density of excitatory and inhibitory synapses. More amazingly, AAV-CRISPR mediated deletion of Erbb4 from hippocampal excitatory neurons (CA1) rescued neuronal network imbalance, synapse loss, microgliosis, astrogliosis, and even amyloid plaque load and behavioral abnormalities in 5xFAD mice. Moreover, Erbb4 overexpression in a wild-type mouse can recapitulate many of these disease phenotypes. The whole story, which is too wide in scope to summarize here in a short paragraph, is backed up by multiple layers of evidence, some of which could be described/explained in more detail (for instance, the RNAseq data and interpretation is presented in somewhat superficial and summary manner, presumably due to space limitations).

General comments

The strengths of the paper are: its conceptual novelty (especially that Erbb4 and DIENs are the primary drivers of neuronal network dysregulation and synapse loss, rather than activation of microglia), the broad scope of experiments, and its potential impact on the AD field.

However, there are a number of general weaknesses or gaps; some arise in part because this study is so wide-ranging and far-reaching:

It is difficult to understand, without being told more about the specific details of the RNAseq data and analysis, how the authors homed in on Erbb4 as the gene that drives the formation and dysfunction of DIENs.

Are the findings of this study relevant to human AD? The whole manuscript is based on mouse models of amyloidosis, and yet the authors often tend to overwrite the conclusions as though they apply to human AD. I’d like to know more about whether Erbb4 is elevated in excitatory neurons of human AD brain tissue (there are many databases out there to query, including early AD from live biopsies from humans [for example, Gazestani V…Macosko E, Cell 2023]. Does Erbb4 have any human genetics association with AD?

The entire premise of the study comes out of single nucleus RNAseq analysis, which can be misleading because of bias of nucleus isolation, and because snRNAseq changes often do not reflect what is happening in total mRNA in the cell or bulk. At the least, it would good to measure Erbb4 RNA levels in a more naturalistic preparation (in vivo in intact brain)-- using RNAscope or some similar RNA measurement approach that measures all the RNA in a brain section.

The study is highly focused on hippocampus (in particular, CA1 region), and it is unclear how widely this mechanism can be extrapolated to other parts of the brain.

The study is perhaps over-reliant on inferences from RNAseq and from immunostaining of activity markers and counting of inhibitory and excitatory synapses in brain sections. It would be extremely helpful to get some electrophysiology data to support the central role of ErbB4 in excitatory neurons (DIENs) in the disruption of E/I activities.

In multiple experiments (which I won't list here), whether the result is positive or negative, the n number appeared to be as low as n =3. This seems to be inappropriate for in vivo experiments, especially ones involving AAV injections (which are known to be highly variable).

More specific comments:

UMAP plots are not very quantitative ways to display RNA expression changes, or the degree of transcriptional change. For some experiments and specific genes they could be supplemented with bar graphs, or volcano plots.

I think it is not justified to say that this study "implies that neuroinflammation and phagocyitic hyperactivity of glial cells may not be the primary cause of synapse elimination". Such interpretations ("chicken and egg") should be softened or removed.

I find the bioinformatics experiment on patient derived iPSC-neurons quite unconvincing. I would suggest to remove.

I am curious whether any toxic protein aggregate (besides Abeta) would induce Erbb4 expression in excitatory neurons.

What is the "control guide RNA" for the AAV-CRISPR experiments?

In the AAV "rescue" experiments (Fig 3 and related supplemental figures): is it practical to do RNAseq analysis of microglia and astrocytes in response to Erbb4 deletion in excitatory neurons? IHC of a couple of markers (e.g AXL) is not as satisfying as transcriptomic analysis.

The ideal validation experiment would be to show that whole brain or whole body Erbb4 knockout (or Erbb4 het +/-) would protect mice in some way from the full 5xFAD phenotype (whether it be amyloid load, gliosis, synapse loss etc). I acknowledge this might take a long time, and be beyond the scope of this already large study.

Referee #2

(Remarks to the Author)
The paper by Lee and colleagues has identified a population of pyramidal neurons that express ERBB4 in mouse models of amyloidogenesis that may reflect Alzheimer's disease (AD). The data suggest this population is responsible for the emergence of defining features of AD such as neuroinflammation, synapse loss and behavioral changes. The work also suggests that synapse loss due to glia is probably not directly causing such effects but happens downstream of ERBB4 expression in the pyramidal neuron subgroup. The authors arrived at these conclusions using molecular methods and both knockout and overexpression of ERBB4 in AD model and control mice respectively. Overall, this is an interesting study but several points need clarification and additional work.

Among the major comments below are listed points that need to be addressed with experiments. Among the less major points are comments that require additional clarifications or data to make some of the points clearer. If all such comments could be addressed, this could be a valuable contribution to the literature.

Major points
1. Large parts of the study are reliant on snRNAseq data. However, there are no details for the quality control metrics for any of the data. These should be provided. How many cells we assessed in each group? How was contamination accounted for? How many animals were used? How many batches of mice were used? How were the data analyzed? Some of these details are needed in the results. As written, the snRNAseq sections are superficial and appear to lack rigor. This aspect needs to be improved.

2. ERBB4 KO improves the AD-related readouts. However, the study lacks mechanism for how this happens. It is understood that the authors tried to perform snRNAseq in the last figure to address this, but this did not show how ERBB4 causes the changes in control mice and how KO of ERBB4 in AD mice rescues the phenotype. Since the authors are claiming this one gene controls a broad spectrum of AD phenotypes, it is critical that they provide some mechanism for how all these things happen. Such data are needed to support the bold claims that are made and to support the primary importance of ERBB4 that they claim.

3. The relevance to AD readouts is supported by behavioral analysis in the last figure. However, the behavioral experiments

are simplistic and a greater range of AD-related phenotypes need to be assessed. Such evaluations could also include some in vivo electrophysiology to assess sleep wake cycles and LTP. The authors are making an amazing claim that ERBB4 alone in some pyramidal neurons causes all the major behavioral disruptions of AD in mice. This could potentially be important but they must do better by performing far better and more detailed behavior experiments that are relevant to AD.

4. Key experiments exploring ERBB4 in AD model mice such as behavior should be repeated in at least one additional mouse model. APP/PS1 seems obvious because they used it in Ext Fig 1. I

5. The authors have not studied Alzheimer's disease. They have studied a mouse model of amyloidogenesis which may be related to AD. They must change the title and the text to reflect this.

6. The authors must explore how ERBB4 and the snRNAseq data changes in the last figure are related to the vast amounts of data for human AD. This is critical to do. Are the mechanisms they find seen in humans? Much deeper analysis is needed.

7. The statistics section needs to be expanded and additional details provided such as how and why certain statistical tests were chosen. Why is a student's t test always the best test? Was there never any none normally distributed data in the project? Or did they assume normal distributions in all the data? Such details are needed for the behavior experiments.

Less major points

8. Line 24. The word <contribute> is too strong. It is more accurate to say neuroinflammation and synapses loss <accompany> cognitive decline. Is there any evidence that neuroinflammation causes the effects directly or synergistically? What is that evidence?

9. Line 79. Is it the relative instability of EGFP or the relative intensity of EGFP. This needs to be clarified.

10. Near the section at line 94. It should be described what the phagocytic index is in the graphs. This seems to be some unitless number. This needs to be defined in the results or the graphs need to be changed to have real values that represent phagocytosis per cell, for example. This is needed so that people can interpret and repeat the experiments.

11. The section between lines 105 and 114 is too strong and should be toned down. Opposing changes in excitation and inhibition could be important. The brain does not simply reflect the aggregate balance between excitation and inhibition. This section needs to be rewritten and comes across as simple minded.

12. The results section for snRNAseq must report numbers of cells and mice

13. Near line 154, they should name at least some of the genes

14. Line 157 it seems very premature to define the neurons as disease initiating excitatory neurons. This must be stated only when the field has had time to repeat the work. Disease-related excitatory neurons seem better.

15. Near line 168-174. Some MERSCOPE or in situ hybridization may be needed to show ERBB4 mRNA is found in the somata of pyramidal neurons.

16. Near lines 181-185. The data mentioned for humans could not be found in Fig 5j. This analysis should be in Fig 2. As stated earlier the human analysis needs to be expanded.

17. Lines 188-198. It is not clear how it was possible to reduce expression of ERBB4 only in those neurons that showed an increase. How was this done? This must be stated.

18. Lines 215-229. The assessment of neuroinflammation using three markers is superficial. Neuroinflammation must be evaluated broadly.

19. Near lines 259-266. The data for the late reduction of ERBB4 should be included in the main manuscript

20. Line 265. The authors should stop using poor language such as "preventative care". They have assessed mice. They need to tone things down.

21. Lines 269-296. For the ectopic expression experiments how did they ensure the number of neurons expressing ERBB4 and its expression levels was like in AD mice? This must be clarified. Obviously simply overexpressing ERBB4 in all neurons is not a very good experiment.

22. Lines 307-310. That section needs to be expanded more and as written is superficial.

23. Lines 311-313. I do not understand what they mean when they state overexpression of ERBB4 resulted in the emergence of a population of novel neurons expressing ERBB4. Of course, this happened because they overexpressed it. Do they mean something more? Or is this another example of exaggeration?

24. Line 357. I don't think the work is a paradigm shift. It is a valuable study. They should tone down their language.

25. Are there differences in the phagocytic index between Fig 1 and 3? It would be very useful to have real values on the y-axis rather than some arbitrary index that is hard to compare between figures and other studies.

26. Throughout the figures, the images showing green synaptic blobs inside astrocytes based on reconstructions of astrocytes (eg Fig 1a and others) could be replaced with detailed colocalization analysis. This would be more valuable to the field.

27. Check the naming of the figures in the text because this was confusing.

Referee #3

(Remarks to the Author)
This study by Lee et al. shows that during Alzheimer's disease (AD) progression, astrocytes and microglia increase phagocytosis of excitatory synapses but reduce phagocytosis of inhibitory synapses. Also, astrocyte-mediated synapse elimination was altered earlier than microglial one, in both APP/PS1 and 5xFAD mice. Using single-nucleus RNA sequencing, the researchers identified "Disease-Initiating Excitatory Neurons" (DIENs) marked by increased Erbb4 expression as an early event in AD mouse models. Deleting Erbb4 in excitatory neurons substantially prevented multiple AD symptoms, such as excitatory neuronal hyperactivity, inhibitory neuronal hypoactivity, abnormal synapse elimination, reactive gliosis, and even amyloid beta (Ab) accumulation, leading to significant recovery of cognitive functions. On the other hand, overexpressing Erbb4 in wild-type neurons mimicked AD phenotypes without plaques. These findings suggest that aberrant Erbb4 expression in excitatory neurons drives early AD pathology and represents a potential therapeutic target.
The study is highly significant and has the potential for major impact in the field, the manuscript is well-written, and the authors have conducted a thorough meticulous analysis. However, there are some comments and concerns:
Major
• A primary limitation of the study is its reliance on amyloid mouse models, specifically APP/PS1 and 5XFAD mice. While these models recapitulate early amyloid pathology, they do not fully mirror the complex, slowly progressive nature of human AD, especially with respect to tau pathology, vascular contributions, and chronic neuroinflammation. This raises some concerns about how well findings related to Erbb4 dysregulation and synapse loss will translate to the broader spectrum of human AD. This limitation is mentioned in the Discussion, but needs some elaboration.
• There are also some concerns with the snRNA-seq analyses. First, authors claim that clusters 5-9 (DIEN) clusters are enriched in 3-mo 5XFAD mice (Fig. 2b); however, no quantitative data is provided to support this enrichment. Second, it is unclear why KEGG was used to identify pathways enriched in DIEN clusters, while REACTOME was used for pathways enriched in the turquoise module. If clusters 5–9 (i.e., DIEN) are truly enriched in 3-month-old 5XFAD mice, it would have been more appropriate to perform a direct marker gene analysis of these clusters rather than relying on WGCNA and module enrichment. Third, it is unclear whether Fig. 2d represents DEGs comparing DIEN clusters to other excitatory subclusters. In Extended Fig. 5e, proportions should be shown rather than absolute counts. Finally, it is unclear how authors concluded from 5e that only signaling by Erbb4 was enriched in DIEN.
• Another concern is the limited analysis of human data. While snRNA-seq identified Erbb4-positive DIENs in mouse models, it is critical to demonstrate Erbb4 dysregulation directly in human AD tissue datasets. The authors show increased ERBB4 expression in excitatory neurons in Extended Fig. 10e using a dataset from the middle temporal gyrus; however, it is unclear why this particular dataset was selected given the availability of over a dozen AD snRNA-seq datasets. Moreover, no quantitative data is presented on this dataset. Quick search of several AD datasets shows very low expression of ERBB4 in excitatory neurons relative to inhibitory neurons.
• The authors show that multiple neurodegenerative disease pathways (AD, ALS, Parkinson's, and Huntington's disease) are enriched in DIEN (Fig 2c). Similar results are presented on human data (Extended Data Fig. 10f, g). Authors show that injection of Aβ oligomer into the CA1 was sufficient to induce ERBB4 expression in excitatory neurons within 2 days (Extended Data Fig. 5h, i). What is the corresponding factor in these other neurodegenerative diseases?
• Authors demonstrate that knockdown of the astrocyte-specific phagocytic receptor Megf10 in 5XFAD mice increased excitatory synapse numbers without affecting inhibitory synapses (130-137). How do the authors interpret the lack of effect on inhibitory synapses? How about the microglia phagocytic activity?
• The Erbb4 deletion experiments convincingly demonstrate that excitatory neuron-intrinsic Erbb4 upregulation initiates synapse loss and glial activation, suggesting that glial hyper-phagocytic activity is not the primary driver of early synapse elimination. However, even with Erbb4 deletion, it remains possible that neuron-glia interactions contribute to the differential elimination of excitatory versus inhibitory synapses. Also, in lines 112-114, the authors probably mean that neuroinflammation and hyper-phagocytic activity of glial cells may not be the primary drivers of synapse loss.

Version 3:

Reviewer comments:

Referee #1

(Remarks to the Author)
The authors have done a careful and compelling revision, resulting in an interesting paper that will likely have strong impact on the Alzheimer's and neurodegenerative disease field.

I only have one minor comment:

Figure 2f: seems to show enrichment of other pathways (e.g. Notch, Erbb2 signaling), apparently contradicting that “only the ERBB4 signaling pathway was enriched in ERENs” (line 172 of manuscript). This should be corrected, or the analysis needs to be made more clear, if I misunderstood the figure.

Referee #2

(Remarks to the Author)
The authors have made improvements relative to the previous submission; however, two substantive concerns remain that require additional work. Of these, the absence of electrophysiological data addressing Comment 1 is particularly concerning and, in its current form, substantially weakens the manuscript.

The authors state that EPSCs and IPSCs were not assessed because results from prior work were variable. This justification is not sufficient. A central premise of the study, demonstrated from the outset in Figure 1, is that excitatory and inhibitory synapses are differentially regulated by glia and by the genetic manipulations of ERBB4 employed throughout the manuscript. Because synaptic changes are the primary readout used to support the proposed mechanism, direct measurements of EPSCs and IPSCs from the relevant neuronal populations are essential. Without these data, the mechanistic framework of the study is incomplete.

Instead, the authors pivot to measurements of intrinsic excitability. This shift is not logically connected to the earlier findings based on synaptic staining and does not address the core question of how excitatory and inhibitory synaptic transmission is altered. As a result, the electrophysiological results do not adequately support the conclusions drawn from the anatomical data. This disconnect represents a major flaw in the study and must be resolved.

Moreover, it is difficult to reconcile the claim that EPSCs and IPSCs were not assessed with the fact that intrinsic excitability was recorded (e.g., Extended Data Fig. 7n–p). These recordings necessarily involve access to synaptic events, including EPSPs and IPSPs. The absence of these data in the manuscript creates the impression that the central synaptic mechanism proposed may not be supported by the experimental results. Publishing the study without these measurements would likely cause confusion among readers and undermine confidence in the conclusions.

A second issue concerns the interpretation of the analysis presented in Figure 5u. The authors use human postmortem data to claim a causal mechanism, which is not justified. This section should be rewritten to clarify that the human data support the relevance of the mouse-identified mechanism in human tissue, without implying causality. All claims of a causal role in humans should be removed. To strengthen relevance to human AD, expression of ERBB4 protein in postmortem AD tissue for the subpopulation of excitatory neurons is needed to accompany Fig 5u.

Beyond these major points, there are multiple areas where the text requires clarification and moderation of claims. For example, the manuscript title is syntactically incorrect and implies that ERBB4 itself is excitatory, rather than being expressed in excitatory neurons. Similar imprecisions occur throughout the text and contribute to confusion and overstatement.

Finally, while the proposed link between ERBB4 and mTOR signaling is acknowledged by the authors to be previously described, the principal novelty of the study appears to be the identification of ERBB4-expressing excitatory neurons. However, key mechanistic links explaining how these neurons drive the reported synaptic and behavioral effects are missing. At a minimum, electrophysiological evidence demonstrating the predicted changes in excitatory and inhibitory synaptic transmission is required.

In summary, without direct measurements of EPSCs and IPSCs and a more restrained interpretation of the human data, the manuscript does not yet meet the standard expected for publication in Nature and would be more appropriate for a specialized journal in its current form.

Referee #3

(Remarks to the Author)
The authors have substantially improved the manuscript through the inclusion of additional data and analyses, including electrophysiology, behavioral experiments, and ROSMAP snRNA-seq analyses. These additions significantly strengthen the study.
Several clarifications and revisions are needed to improve accuracy and interpretability of the 5XFAD mouse snRNA-Seq analyses:
• The statement “Among these, only the turquoise module was strongly correlated with ERENs and significantly upregulated” should be revised. The results indicate that genes in the turquoise module are enriched for EREN marker genes, rather than the turquoise module itself being upregulated.
• The sentence “For further analysis, we selected the top 25% of turquoise module genes based on p-value and expression level” should be revised to clarify that the authors selected the top 25% of EREN marker genes that are members of the turquoise module, based on differential expression p-values comparing ERENs with other excitatory neuron subclusters.
• The legend for Figure 2d should explicitly state that the volcano plot displays differentially expressed genes between the EREN cluster and other excitatory neuron subclusters.
• The statement “GSEA with Reactome Pathway terms indicated that among the top 25% of turquoise module genes, only

the ERBB4 signaling pathway was enriched in ERENs" remains unclear. The authors should clarify that among all enriched pathways, ERBB4 is the only one within the top 25% of turquoise module genes.
• Extended Data Fig. 5a (snRNA-seq analysis): Bar graphs showing the fraction of the EREN population are based on only two samples per group. Error bars should be removed, and individual data points should be shown, with the mean optionally overlaid.
• The legends for Extended Data Fig. 5g, 5h, 5i, and 5j are switched. In addition, Extended Data Fig. 5i and 5j are cited in the text after Extended Data Fig. 5k and should be reordered.
ROSMAP snRNA-seq analysis:
• The authors may consider presenting a larger portion of the ROSMAP snRNA-seq results in the main figures rather than limiting them to the Extended Data.
• Authors should include a citation to the study that generated the snRNASeq dataset from 446 donors of the ROSMAP study.
• Extended Data Fig. 12f and 12g: Individual donor level data should also be presented.
• Extended Data Fig. 12h: Violin plots showing ERBB4 expression in healthy controls and PSP patients (n = 3 per group) require revision. A Wilcoxon rank-sum test is not appropriate, as cells from the same donor cannot be treated as independent observations. Donor-level aggregation or an appropriate mixed-effects model should be used instead.

Version 5:

Reviewer comments:

Referee #1

(Remarks to the Author)
I had only minor comments after the first revision. Those have been satisfied by the second revision.
Overall I believe the second revision is even further improved, and it addresses reasonably the biggest remaining concerns of the reviewers (around electrophysiology).

Referee #2

(Remarks to the Author)
The authors have done a good job of addressing my comments. The inclusion of the new electrophysiology makes the paper stronger. My only minor comment that does not require re-review but should be accommodated is to add some representative traces of synaptic events to ED Fig 7n-F so that future readers can assess the quality of the raw data, especially for iPSCs which were measured at 0 mV.

Referee #3

(Remarks to the Author)
The authors have fully addressed all comments. Two minor issues:
• The sentence "Among these, only the turquoise module score was strongly correlated with ERENs and significantly upregulated" should be revised to: "Amond these, only the turquoise module exhibited a significantly elevated module score in ERENs compared to other excitatory neuron subclusters" as stated in the rebuttal.
• The legend for Fig 5 is very confusing, as it is not in order.

Referee #4

(Remarks to the Author)
The manuscript proposes a novel and interesting conceptual framework of synaptic remodeling by glial cells induced by a previously unrecognized population of Early Responsive Excitatory Neurons (ERENs) with ectopic expression of ERBB4 in AD. The aberrant expression of ERBB4 in excitatory cells drives a complex cascade leading to circuit dysfunction, which includes neuronal hyperexcitability and reduced mEPSCs/synapses, hypoactivity of inhibitory cells, and activity-driven synaptic engulfment of excitatory and inhibitory synapses by glial cells. Thus, instead of the canonical pathway of reactive glia-mediated early synapse loss, the authors propose that ectopic ERBB4 expression directly mediates changes in neuronal activity, which drive differential activity-dependent synaptic remodeling by glial cells of excitatory (increased engulfment) and inhibitory synapses (decreased engulfment), resulting in synaptic imbalance. Importantly, the proposed mechanism also affects downstream pathology and ultimately cognitive impairment.

The authors provide strong evidence to experimentally support this working model in two AD mouse models and in humans with AD, including longitudinal analyses of two AD mouse models, single-nucleus transcriptomics in humans and mice showing ectopic Erbb4 expression in excitatory cells, rescue experiments with Erbb4 knockdown in AD mice, gain-of-function experiments with Erbb4 in WT mice to recapitulate AD-like features, cell type- and activity-dependent modulation of synaptic engulfment, pathology, electrophysiology, and behavioral testing. Altogether, the study supports the notion that aberrant ERBB4 expression in excitatory neurons is both necessary and sufficient to reproduce many hallmark features of AD, including neuronal hyperexcitability and glia-mediated synaptic remodeling. Mechanistically, the work also identifies the ERBB4–mTOR signaling pathway as one potential molecular mediator. Although additional work will ultimately be needed to determine how broadly this mechanism applies to human disease, the evidence presented is compelling and should

stimulate further investigation.

The inclusion of the electrophysiology substantially improves the manuscript. Previously, the work relied heavily on c-Fos as a surrogate for neuronal activity and synaptic morphology as a surrogate for synaptic function. The new recordings resolve this major gap in the functional interpretation of the structural data. The new electrophysiology demonstrates that ectopic ERBB4 expression in WT excitatory neurons increases hyperexcitability and reduces the input of functional excitatory synapses (mEPSC frequency), and that deleting ERBB4 in 5XFAD mice rescues these synaptic and excitability alterations. The decrease in mEPSC frequency and hyperexcitability aligns well with the reduction in excitatory synapse number measured anatomically and with the c-Fos data, providing a much stronger mechanistic foundation for the proposed working model. However, the mIPSC frequency data is difficult to reconcile with the structural data (see below).

Major: The revised manuscript has a couple of major caveats:
1) The clusters of excitatory cells expressing ectopic ERBB4 are very poorly defined (Figure 2; UMAP clusters 5, 6, 7, 8, and 9). All UMAP clusters (0–9) need to be classified and properly labeled. Ideally, the origin of these aberrant cell states should be identified (DG, CA1, CA3, CA2, ProS, SUB, POST, etc.). The identity of these cells needs to be better described.

2) The UMAPs in Figure 5 have the same deficiencies. There is no labeling or identification of the clusters of excitatory cells expressing ectopic ERBB4. All UMAP clusters need to be classified and properly labeled.

3) For Figure 2, UMAP plots showing Erbb4 expression is needed.

4) The mIPSC frequency data strongly suggest that the reduced mIPSC frequency in 5xFAD is independent of the ectopic Erbb4 expression in excitatory cells, as this functional feature is not rescued by the knockdown of Erbb4 in the excitatory cells. Similarly, the expected increase in mIPSC frequency following Erbb4 expression in WT was only a non-significant trend. These results question the proposed model regarding effects on inhibitory cells, as the reduction of functional inhibitory synapses in 5xFAD mice is independent of Erbb4 expression. The model needs to be refined, as it contains strong experimental functional evidence for excitatory, but not inhibitory, synapses.

Minor:
5) The authors equate the mouse models with human AD patients, including expressions referring to the mice. Line 26, 58: "AD progression in mice"; Line 60: "initial stages of AD"; Line 80: "AD glia"; Line 117: "AD brains". The authors should be more careful, as these mouse models do not develop AD.

6) Expression levels in Figure 5f and 5n are binary (0 or 1); they do not capture the dynamic range of the continuous scale (0–1).

7) The mIPSC frequency data are somewhat difficult to reconcile with the rest morphology data. The authors acknowledge that increased inhibitory synapse number does not translate into increased functional inhibition and propose several reasonable explanations (immature or silent synapses). More caution regarding the interpretation of the effects on inhibitory function is needed, as there is no clear experimental support.

Referee #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Dear Referees,

We would like to thank all referees for their thoughtful and constructive comments on our manuscript (2025-03-06021) "Excitatory Neuronal ERBB4 Drives Early Pathophysiology of Alzheimer's Disease" by Lee et al. Based on these excellent suggestions, we have conducted substantial new experiments, expanded analyses, and refined interpretations, which we believe have significantly improved the manuscript.

First, to strengthen the mechanistic basis, we performed additional **electrophysiological experiments**, demonstrating that *Erbb4* overexpression upregulates excitability in WT excitatory neurons, while *Erbb4* deletion rescues hyperexcitability in 5XFAD neurons. These findings provide functional support for a central role of excitatory neuronal ERBB4 in shaping aberrant neuronal hyperactivity observed in AD models.

Second, we broadened the scope of **behavioral analyses**, including the Barnes maze and contextual fear conditioning, and replicated these experiments in an additional amyloid mouse model (APP/PS1). These results consistently showed that *Erbb4* deletion rescues, while overexpression disrupts, cognitive performance.

Third, to address concerns regarding human relevance, we incorporated **extensive snRNAseq analyses** from the ROSMAP cohort, comprising 446 individuals with well-characterized pathological and cognitive measures. We identified ERBB4^{high} excitatory neuronal clusters in human AD brains whose relative abundance positively correlates with plaque severity (CERAD score) and negatively correlates with cognitive performance (MMSE score). In addition, we applied causal inference approaches, including mediation analysis and structural equation modeling, to support both direct and indirect associations between excitatory neuronal *ERBB4* expression and cognitive decline.

Fourth, to validate and expand beyond snRNAseq, we performed **RNAscope FISH**, confirming *Erbb4* mRNA upregulation in excitatory neurons of 5XFAD hippocampal CA1, and reduction upon *Erbb4* deletion.

Fifth, to examine whether the **role of *Erbb4* extends beyond the hippocampus**, we analyzed additional snRNAseq data from the cortex of 7-month-old 5XFAD mice as well as performed ectopic *Erbb4* expression experiments in the WT cortex. In parallel, we integrated human dorsolateral prefrontal cortex (DLPFC) snRNAseq data, altogether demonstrating that ERBB4-associated mechanisms are not restricted to hippocampal circuits but extend to cortical regions in both mouse and human.

Sixth, to explore **downstream mechanisms of ERBB4 signaling**, we investigated the role of the mTOR pathway using targeted genetic manipulation of *Rptor*, an essential component of mTORC1. Deletion of *Rptor* in excitatory neurons of 5XFAD mice rescued multiple AD-related phenotypes, including synaptic alterations, neuronal hyperactivity, and glial activation. Importantly, we further tested this pathway in a complementary context by deleting *Rptor* in WT mice with ectopic *Erbb4* overexpression. In this setting, suppression of mTOR signaling markedly attenuated the pathological effects induced by ERBB4. Together, these experiments establish mTOR signaling as a key downstream mediator through which excitatory neuronal ERBB4 drives AD-related pathophysiology.

In summary, through new experiments, expanded datasets, and careful revisions, we have substantially strengthened both the mechanistic depth and the human relevance of our study. We believe the manuscript has been considerably improved and is now more clearly positioned within the broader context of Alzheimer's disease research.

Please find below our detailed responses to Referees' comments.

Referee #1 (Remarks to the Author):

This is a rather comprehensive study with many intriguing results and some main conclusions that, if true, would represent what the authors call a "paradigm shift" for Alzheimer's disease research. Remarkably, they show evidence in mouse amyloidosis models (mainly 5xFAD) that Abeta induces, early in disease pathogenesis, a class of "Disease Initiating Excitatory Neurons" (DIENs), which are driven by aberrantly high expression of ErbB4 in those excitatory neurons. Elevated ErbB4 activity leads to neuronal hyperactivity, increased phagocytosis of excitatory synapses by microglia and astrocytes, but reduced phagocytosis of inhibitory synapses, and hence corresponding opposite changes in density of excitatory and inhibitory synapses. More amazingly, AAV-CRISPR mediated deletion of Erbb4 from hippocampal excitatory neurons (CA1) rescued neuronal network imbalance, synapse loss, microgliosis, astrogliosis, and even amyloid plaque load and behavioral abnormalities in 5xFAD mice. Moreover, Erbb4 overexpression in a wild-type mouse can recapitulate many of these disease phenotypes. The whole story, which is too wide in scope to summarize here in a short paragraph, is backed up by multiple layers of evidence, some of which could be described/explained in more detail (for instance, the RNAseq data and interpretation is presented in somewhat superficial and summary manner, presumably due to space limitations).

General comments

The strengths of the paper are: its conceptual novelty (especially that Erbb4 and DIENs are the primary

drivers of neuronal network dysregulation and synapse loss, rather than activation of microglia), the broad scope of experiments, and its potential impact on the AD field.

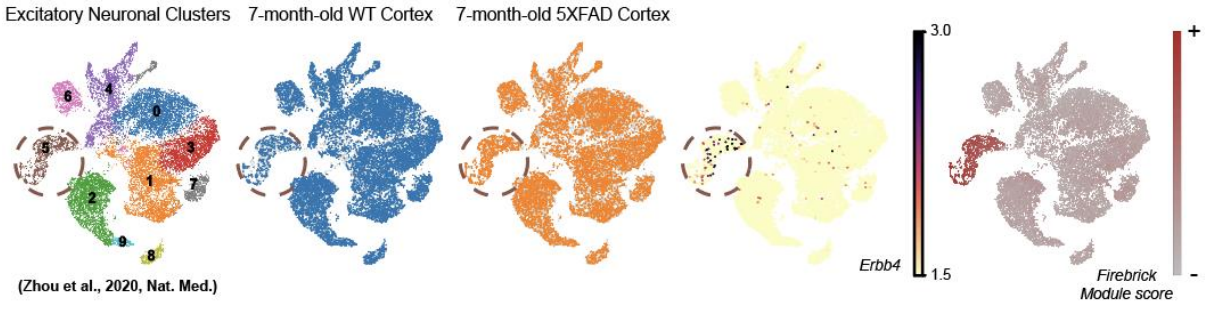
However, there are a number of general weaknesses or gaps; some arise in part because this study is so wide-ranging and far-reaching:

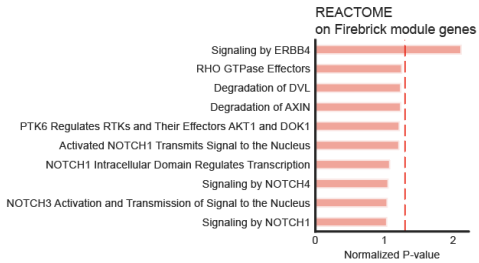
It is difficult to understand, without being told more about the specific details of the RNAseq data and analysis, how the authors homed in on Erbb4 as the gene that drives the formation and dysfunction of DIENs.

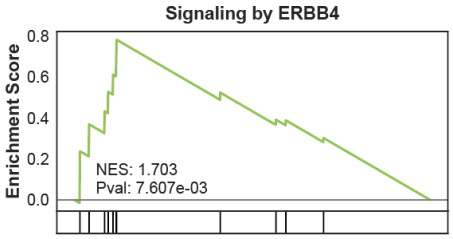
We thank the referee for raising this point. In the original submission, our description of the RNAseq analysis was necessarily abbreviated due to space limitations, which may have obscured the logic by which *Erbb4* emerged as a central candidate. To support our rationale, we have now incorporated additional public snRNAseq data from 7-month-old 5XFAD mice that were previously omitted from the manuscript.

Specifically, we also performed Weighted Gene Correlation Network Analysis (WGCNA) on excitatory neuronal transcriptomes from the cortex of 7-month-old 5XFAD mice and age-matched WT controls. This analysis identified a gene module that was strongly enriched in the EREN (Early-Responsive Excitatory Neuron)-like (previously referred to as DIEN) population (Extended Data Fig. 5e). Importantly, Gene Set Enrichment Analysis (GSEA) of this module revealed a significant and specific enrichment of the Reactome pathway 'Signaling by ERBB4' (Extended Data Fig. 5i, j). Based on the convergence of evidence from early hippocampal (3-month-old) and later-stage cortical (7-month-old) datasets, we selected *Erbb4* as the most plausible gene promoting the formation of ERENs.

We have revised the manuscript to clarify this analytical pipeline and now explicitly describe the inclusion of the 7-month-old 5XFAD snRNAseq data and its WGCNA/GSEA results, thereby strengthening the transparency and rigor of our gene-selection rationale.







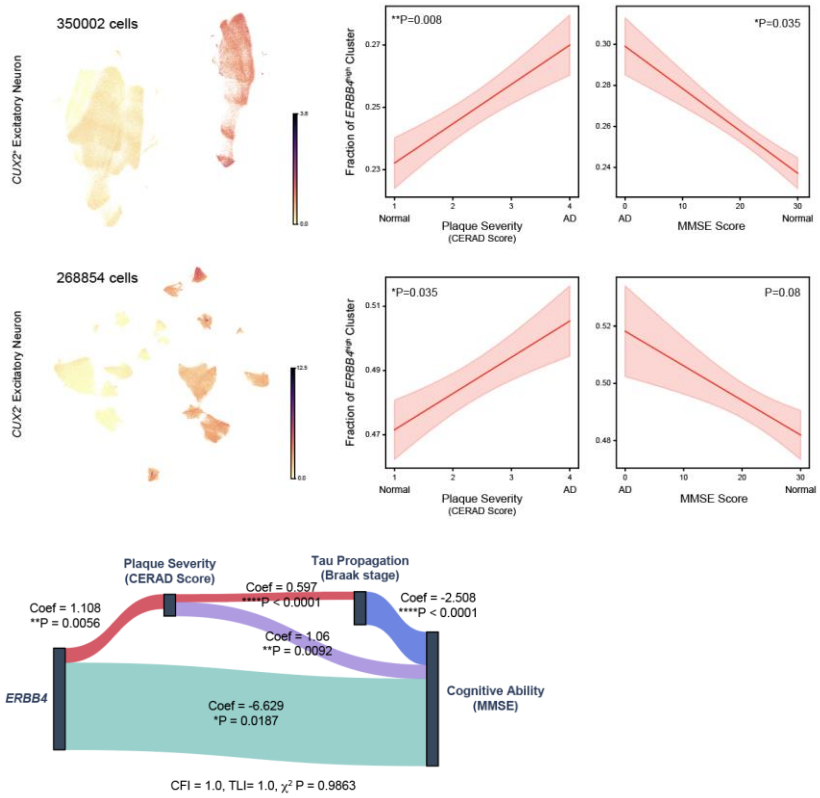
Are the findings of this study relevant to human AD? The whole manuscript is based on mouse models of amyloidosis, and yet the authors often tend to overwrite the conclusions as though they apply to human AD. I'd like to know more about whether Erbb4 is elevated in excitatory neurons of human AD brain tissue (there are many databases out there to query, including early AD from live biopsies from humans [for example, Gazestani V…Macosko E, Cell 2023]. Does Erbb4 have any human genetics association with AD?

In our previous submission, we had shown that excitatory neurons from human AD patients contain an *ERBB4*$^{+}$ cluster. However, we agree with the referee that a more in-depth analysis would be necessary to strongly link our result with human dataset. To strengthen the human relevance of our study, we have now performed a more comprehensive analysis using snRNAseq data from 446 individuals (healthy controls and AD patients) in the Religious Orders Study and Memory and Aging Project (ROSMAP) cohort[1,2].

Specifically, we analyzed *CUX2*$^{+}$ and *CUX2*$^{-}$ cortical excitatory neurons and identified *ERBB4*^{high} excitatory neuronal clusters in human AD brains (Extended Data Fig. 12d, e), similar to our mouse EREN population. Importantly, the fraction of these *ERBB4*^{high}clusters was positively correlated with plaque severity (CERAD score) and negatively correlated with cognitive performance (MMSE) (Extended Data Fig. 12f, g).

To further increase rigor beyond correlation, we applied causal modeling approaches, including mediation analysis and structural equation modeling, within a biologically grounded AD pathological cascade framework. These analyses supported a model in which excitatory neuronal *ERBB4* participates in a pathogenic pathway linking amyloid pathology to tau propagation and consequent cognitive decline (Fig. 5u).

Taken together, these human transcriptomic and causal modeling data strongly support the relevance of our findings to human AD and indicate that *ERBB4* expression in excitatory neurons is not merely a mouse-specific phenomenon, but is significantly associated with disease pathology and cognitive impairment in humans.

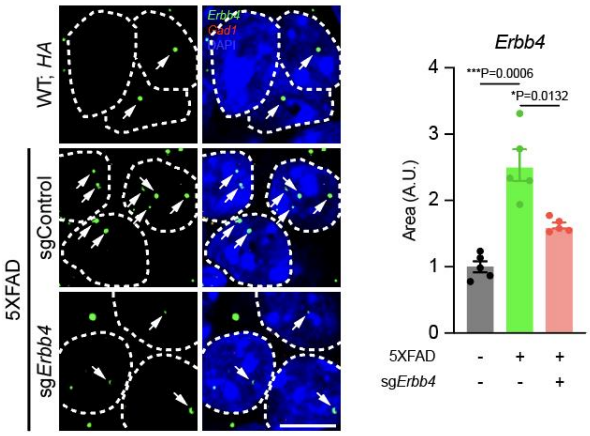


[1]A. Bennett, D., A. Schneider, J., Arvanitakis, Z., & S. Wilson, R. (2012). Overview and findings from the religious orders study. *Current Alzheimer Research*, *9*(6), 628-645.

[2]A Bennett, D., A Schneider, J., S Buchman, A., L Barnes, L., A Boyle, P., & S Wilson, R. (2012). Overview and findings from the rush Memory and Aging Project. *Current Alzheimer Research*, *9*(6), 646-663.

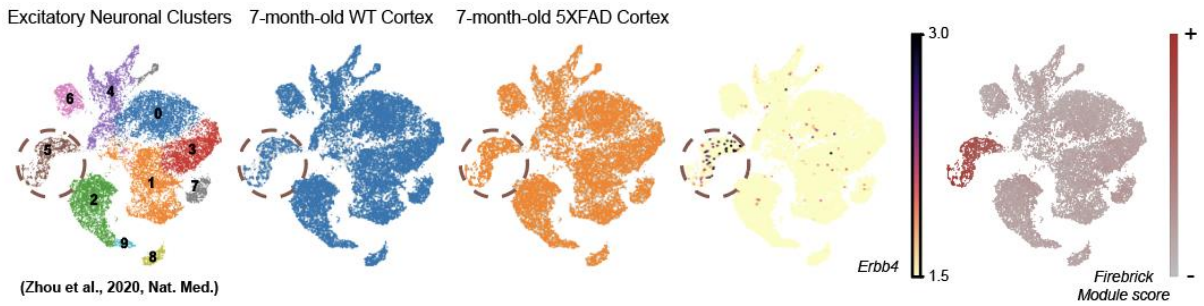
The entire premise of the study comes out of single nucleus RNAseq analysis, which can be misleading because of bias of nucleus isolation, and because snRNAseq changes often do not reflect what is happening in total mRNA in the cell or bulk. At the least, it would good to measure Erbb4 RNA levels in a more naturalistic preparation (in vivo in intact brain)-- using RNAscope or some similar RNA measurement approach that measures all the RNA in a brain section.

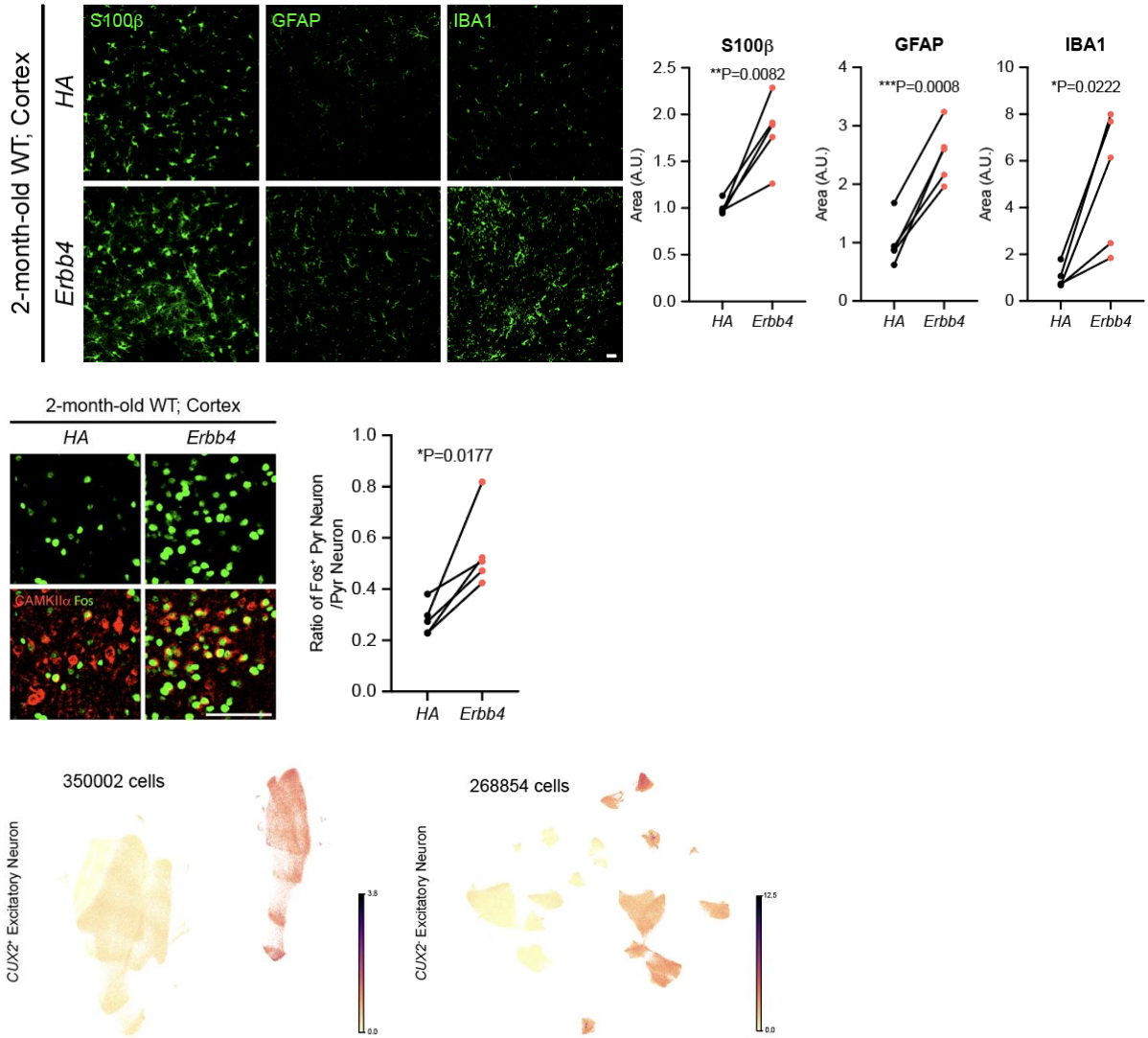
We agree with the referee's concern. Therefore, we performed fluorescence *in situ* hybridization (FISH) using RNAscope (Extended Data Fig. 5g, h), which directly measures RNA transcripts in intact brain sections. Consistent with our snRNAseq findings, we observed that *Erbb4* mRNA was highly expressed in the pyramidal layer of the hippocampal CA1 region in 5XFAD mice compared to WT controls, and that this elevation was markedly reduced upon *Erbb4* deletion using Cas9. These results provide independent validation in a more physiological context and support the robustness of our snRNAseq-based conclusions.



The study is highly focused on hippocampus (in particular, CA1 region), and it is unclear how widely this mechanism can be extrapolated to other parts of the brain.

We appreciate the referee's insightful comment and agree that it is important to determine whether the role of *Erbb4* extends beyond the hippocampus. In the 7-month-old 5XFAD cortex snRNAseq dataset, we identified an *Erbb4*$^{+}$ excitatory neuronal cluster (Extended Data Fig. 5e), suggesting that this phenomenon is not limited to the hippocampal CA1 region. To functionally validate this observation, we ectopically expressed *Erbb4* in the cortex of WT mice using AAV and observed increased expression of S100β, IBA1, and GFAP, along with increased Fos^{+} pyramidal neurons (Extended Data Fig. 9r-u). Moreover, in the revised manuscript, we have incorporated analyses of human snRNAseq data from the ROSMAP cohort, derived from the dorsolateral prefrontal cortex (DLPFC). Similar to our mouse data, we identified *ERBB4*^{high} excitatory neuronal population, whose abundance was associated with increased pathological burden, and cognitive decline (Extended Data Fig. 12d, e). Taken together, these findings indicate that the effects of *Erbb4* are not restricted to CA1, but extend to other brain regions such as cortex in both mouse and human, supporting a broader relevance of this mechanism.





The study is perhaps over-reliant on inferences from RNAseq and from immunostaining of activity markers and counting of inhibitory and excitatory synapses in brain sections. It would be extremely helpful to get some electrophysiology data to support the central role of ErbB4 in excitatory neurons (DIENs) in the disruption of E/I activities.

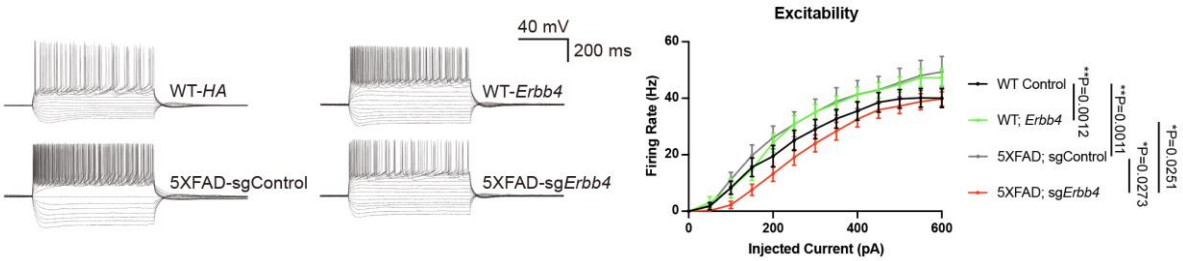
We fully agree with the referee on the importance of electrophysiological validation. While measurements of EPSCs and IPSCs could, in principle, provide information on E/I activities, previous studies using diverse AD mouse models have reported highly variable and sometimes contradictory changes in the frequency and amplitude of EPSCs and IPSCs, even within the same brain region and at comparable ages[1-4]. This variability makes synaptic current measurements a less reliable readout for assessing network-level activity changes in AD models.

Therefore, instead of focusing on EPSCs and IPSCs, we assessed intrinsic neuronal excitability as a more direct and robust functional measure of neuronal activity. Using whole-cell patch-clamp

recordings from CA1 pyramidal neurons, we found that ectopic expression of *Erbb4* in WT excitatory neurons significantly increased neuronal excitability to levels comparable to those observed in 5XFAD mice. Conversely, deletion of *Erbb4* in excitatory neurons of 5XFAD mice reduced excitability to levels comparable to WT controls (Extended Data Fig. 7o, p).

These electrophysiological findings are fully consistent with our activity marker data, in which pyramidal neurons in *Erbb4*-injected WT and 5XFAD exhibited increased Fos expression, indicating hyperactivity (Fig. 4h, j and Extended Data Fig. 6d, f). Moreover, this excitatory neuronal hyperexcitability is accompanied by reduced Fos expression in SST interneurons (Fig. 4i, k and Extended Data Fig. 6e, g), which might be mediated through enhanced recruitment of VIP interneurons (Extended Data Fig. 6b, c), ultimately leading to disruption of the E/I balance.

Together, these electrophysiological data provide direct functional support for a central role of *Erbb4* in excitatory neurons in driving neuronal hyperexcitability and downstream excitatory and inhibitory imbalance.



[1]Li, Y., Zhu, K., Li, N., Wang, X., Xiao, X., Li, L., ... & Zheng, Y. (2021). Reversible GABAergic dysfunction involved in hippocampal hyperactivity predicts early-stage Alzheimer disease in a mouse model. *Alzheimer's Research & Therapy*, *13*(1), 114.

[2]Andersen, J. V., Skotte, N. H., Christensen, S. K., Polli, F. S., Shabani, M., Markussen, K. H., ... & Waagepetersen, H. S. (2021). Hippocampal disruptions of synaptic and astrocyte metabolism are primary events of early amyloid pathology in the 5xFAD mouse model of Alzheimer's disease. *Cell Death & Disease*, *12*(11), 954.

[3]Jo, S., Yarishkin, O., Hwang, Y. J., Chun, Y. E., Park, M., Woo, D. H., ... & Lee, C. J. (2014). GABA from reactive astrocytes impairs memory in mouse models of Alzheimer's disease. *Nature medicine*, *20*(8), 886-896.

[4]Zhou, B., Lu, J. G., Siddu, A., Wernig, M., & Südhof, T. C. (2022). Synaptogenic effect of APP-Swedish mutation in familial Alzheimer's disease. *Science translational medicine*, *14*(667), eabn9380.

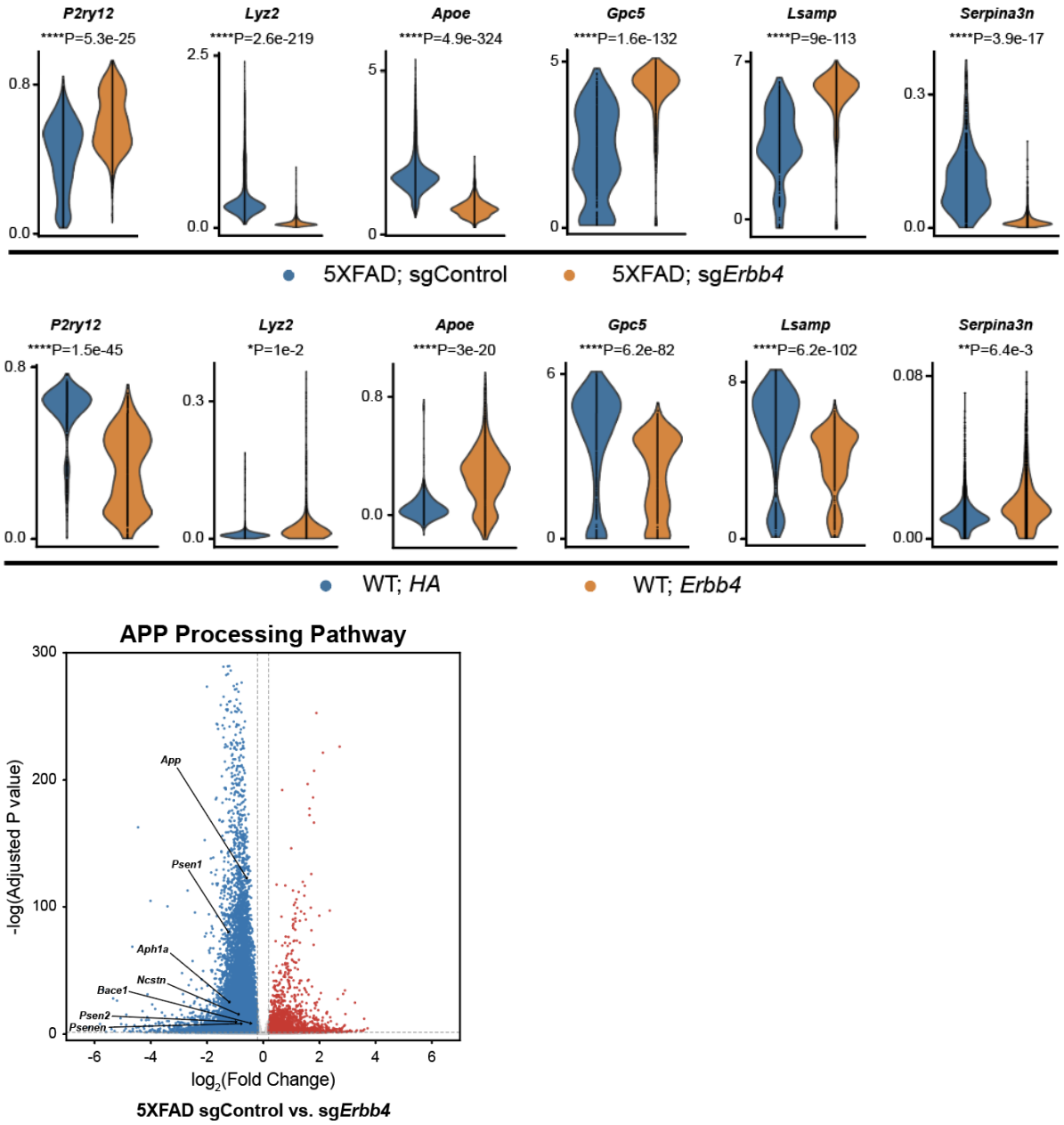
In multiple experiments (which I won't list here), whether the result is positive or negative, the n number appeared to be as low as n =3. This seems to be inappropriate for in vivo experiments, especially ones involving AAV injections (which are known to be highly variable).

We agree with the referee's concern regarding sample size. To address this concern, we increased the number of animals in most experiments to the total number of at least five, thereby improving the statistical robustness and reliability of our findings.

More specific comments:

UMAP plots are not very quantitative ways to display RNA expression changes, or the degree of transcriptional change. For some experiments and specific genes they could be supplemented with bar graphs, or volcano plots.

While UMAP plots are useful for visualizing cell clustering and global expression patterns, we agree that they are not fully quantitative for assessing transcriptional changes of specific genes. To provide more rigorous and quantitative support, we have now supplemented the UMAP visualizations with Violin plots (Fig. 5g, h, o, p) and volcano plot (Extended Data Fig. 12a), which clearly illustrate the magnitude and statistical significance of the observed expression changes.



I think it is not justified to say that this study "implies that neuroinflammation and phagocytic hyperactivity of glial cells may not be the primary cause of synapse elimination". Such interpretations ("chicken and egg") should be softened or removed.

We agree with the referee that determining the exact causal relationship between neuroinflammation and synapse loss can be challenging, often presenting a "chicken and egg" problem. We appreciate the referee pointing out that our previous statement might have been too definitive regarding the primary cause.

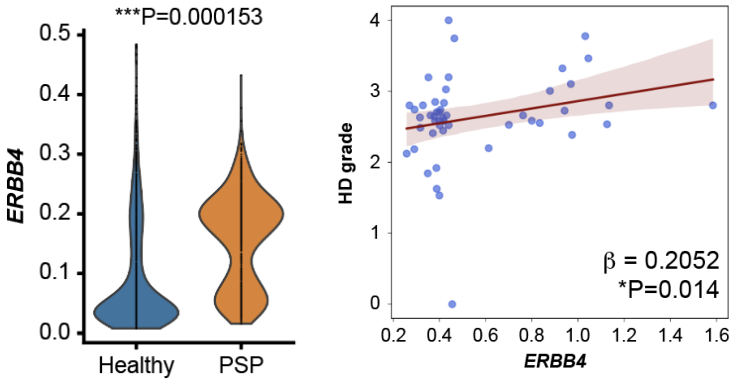
Accordingly, we have softened the interpretation as requested. Instead of ruling out neuroinflammation as a primary cause, we have revised the text to suggest that neuronal state changes may shape glial behavior, thereby offering a more nuanced explanation that avoids unjustified causal claims.
The revised sentence reads:
"Furthermore, the selective elimination of excitatory synapses, coupled with the preservation of inhibitory ones, raises the possibility that intrinsic neuronal states guide glial phagocytic behavior, and that indiscriminate neuroinflammation is unlikely to be the sole driver of synapse elimination in AD."

I find the bioinformatics experiment on patient derived iPSC-neurons quite unconvincing. I would suggest to remove.

We appreciate the referee's feedback and acknowledge the concern regarding the iPSC-neuron bioinformatics analysis. In line with this suggestion, we have removed these data from the revised manuscript. We believe that the overall conclusions of the study remain fully supported by other lines of evidence, particularly the newly added analyses of the ROSMAP cohort.

I am curious whether any toxic protein aggregate (besides Abeta) would induce Erbb4 expression in excitatory neurons.

We were also curious about this possibility and therefore examined whether ERBB4 upregulation in excitatory neurons is specific to amyloid pathology or can be induced by other toxic protein aggregates. To address this, we analyzed an independent snRNAseq dataset derived from patients with progressive supranuclear palsy (PSP), a tauopathy predominantly driven by neurofibrillary tangles[1] with minimal, if any, amyloid accumulation[2,3]. Notably, this analysis revealed that excitatory neurons from PSP patients express significantly higher level of *ERBB4* compared to healthy controls (Extended Data Fig. 12h).
In addition, we analyzed snRNAseq data from patients with Huntington's Disease (HD), a neurodegenerative disorder driven by CAG repeat expansion in the *HTT* gene rather than amyloid or tau aggregation[4]. In this dataset, we also observed a significant linear correlation between *ERBB4* expression level in excitatory neurons and HD grade (Extended Data Fig. 12i).
Together, these findings indicate that ERBB4 upregulation in excitatory neurons is not restricted to amyloid-driven pathology, but may represent a more general neuronal response to diverse neurodegenerative proteinopathies. How excitatory neurons can sense these distinct proteinopathies yet similarly upregulate *ERBB4* transcription remains a critical question that we aim to address in future studies.



[1]Cervos-Navarro, J., & Schumacher, K. (1994). Neurofibrillary pathology in progressive supranuclear palsy (PSP). *Progressive Supranuclear Palsy: Diagnosis, Pathology, and Therapy*, 153-164.

[2]Boxer, A. L., Yu, J. T., Golbe, L. I., Litvan, I., Lang, A. E., & Höglinger, G. U. (2017). Advances in progressive supranuclear palsy: new diagnostic criteria, biomarkers, and therapeutic approaches. *The Lancet Neurology*, *16*(7), 552-563.

[3]Josephs, K. A., Petersen, R. C., Knopman, D. S., Boeve, B. F., Whitwell, J. L., Duffy, J. R., ... & Dickson, D. W. (2006). Clinicopathologic analysis of frontotemporal and corticobasal degenerations and PSP. *Neurology*, *66*(1), 41-48.

[4]MacDonald, M. E., Ambrose, C. M., Duyao, M. P., Myers, R. H., Lin, C., Srinidhi, L., ... & Harper, P. S. (1993). A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell*, *72*(6), 971-983.

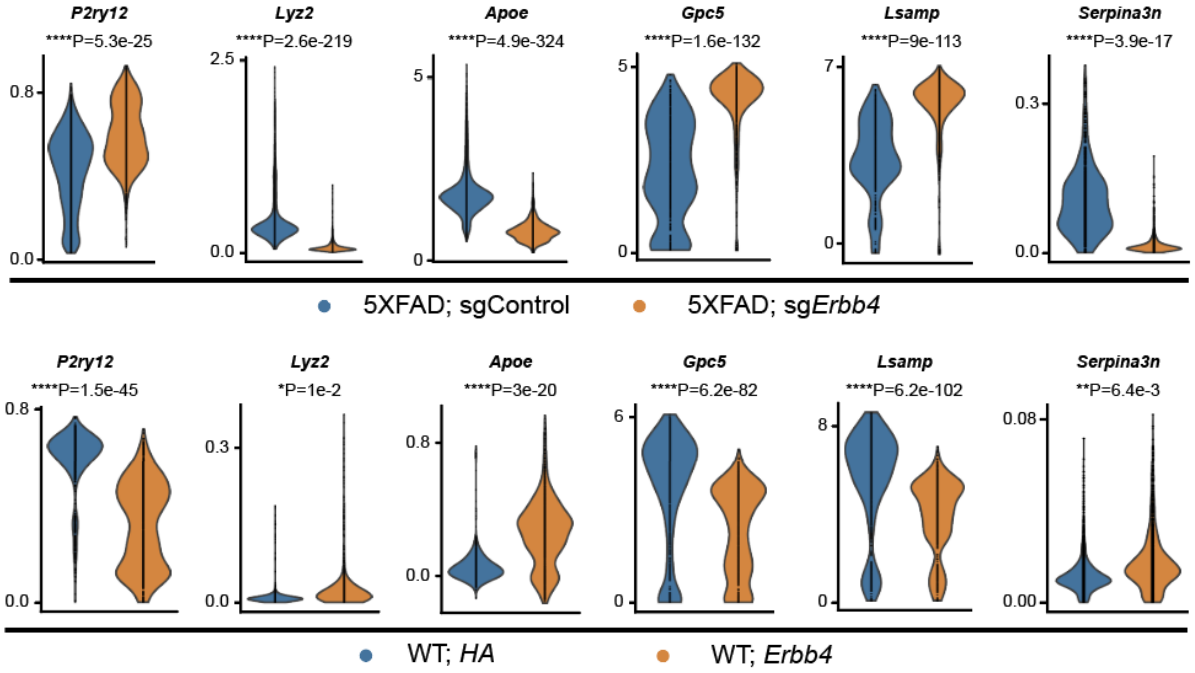
What is the "control guide RNA" for the AAV-CRISPR experiments?

We apologize for not providing sufficient detail regarding the control guide RNA in the original submission. The control guide RNA used in our AAV-CRISPR experiments was designed to lack complementarity to any sequence in the mouse genome, and its inertness has been previously validated[1]. The sequence of the control guide is now provided in the Methods section.

[1]Morgens, D. W., Wainberg, M., Boyle, E. A., Ursu, O., Araya, C. L., Tsui, C. K., ... & Bassik, M. C. (2017). Genome-scale measurement of off-target activity using Cas9 toxicity in high-throughput screens. *Nature communications*, *8*(1), 15178.

In the AAV "rescue" experiments (Fig 3 and related supplemental figures): is it practical to do RNAseq analysis of microglia and astrocytes in response to Erbb4 deletion in excitatory neurons? IHC of a couple of markers (e.g AXL) is not as satisfying as transcriptomic analysis.

We thank the referee for this question. We would like to clarify that we have already performed snRNAseq analyses in both the “rescue” and “phenocopy” experiments, as presented in Fig. 5. These transcriptomic analyses demonstrate that deletion of *Erbb4* in excitatory neurons drives astrocytes and microglia away from inflammatory and damage-associated states, respectively, and back toward homeostatic states (Fig. 5b, c). Conversely, ectopic overexpression of *Erbb4* in excitatory neurons induces transition in the opposite direction (Fig. 5j, k). Representative genes underlying these shifts are detailed in Fig 5d-h for the deletion and Fig.5l-p for the overexpression.



The ideal validation experiment would be to show that whole brain or whole body Erbb4 knockout (or Erbb4 het +/-) would protect mice in some way from the full 5xFAD phenotype (whether it be amyloid load, gliosis, synapse loss etc). I acknowledge this might take a long time, and be beyond the scope of this already large study.

We appreciate the referee’s thoughtful suggestion and acknowledgment of the scope of the study. While whole-brain or whole-body *Erbb4* knockout could in principle be informative, previous studies have shown that global *Erbb4* loss causes schizophrenia-like phenotypes due to dysfunction of PV interneurons[1], in which *Erbb4* is highly enriched at baseline. For this reason, we pursued an excitatory neuron-specific approach using AAVs with CaMKIIα promoter-driven *Erbb4* deletion and overexpression, which allowed us to directly assess the role of excitatory neuronal *Erbb4* in AD-related pathology.

[1]Skirzewski, M., Cronin, M. E., Murphy, R., Fobbs, W., Kravitz, A. V., & Buonanno, A. (2020). ErbB4 null mice display altered mesocorticolimbic and nigrostriatal dopamine levels as well as deficits in cognitive and motivational behaviors. *eneuro*, *7*(3).

Referee #2 (Remarks to the Author):

The paper by Lee and colleagues has identified a population of pyramidal neurons that express ERBB4 in mouse models of amyloidogenesis that may reflect Alzheimer's disease (AD). The data suggest this population is responsible for the emergence of defining features of AD such as neuroinflammation, synapse loss and behavioral changes. The work also suggests that synapse loss due to glia is probably not directly causing such effects but happens downstream of ERBB4 expression in the pyramidal neuron subgroup. The authors arrived at these conclusions using molecular methods and both knockout and overexpression of ERBB4 in AD model and control mice respectively. Overall, this is an interesting study but several points need clarification and additional work.

Among the major comments below are listed points that need to be addressed with experiments. Among the less major points are comments that require additional clarifications or data to make some of the points clearer. If all such comments could be addressed, this could be a valuable contribution to the literature.

Major points

1. Large parts of the study are reliant on snRNAseq data. However, there are no details for the quality control metrics for any of the data. These should be provided. How many cells we assessed in each group? How was contamination accounted for? How many animals were used? How many batches of mice were used? How were the data analyzed? Some of these details are needed in the results. As written, the snRNAseq sections are superficial and appear to lack rigor. This aspect needs to be improved.

We apologize for the insufficient detail regarding the snRNAseq data in the original submission and greatly appreciate the referee's suggestion. In the revised manuscript, we have added information on the number of cells, animals, and batches analyzed in each group, which is now included in the figure legends of the relevant experiments. We have also provided detailed quality control metrics including doublet removal in the Methods section, together with additional details of the analysis pipeline. We believe these additions substantially improve the rigor and transparency of the snRNAseq results.

2. ERBB4 KO improves the AD-related readouts. However, the study lacks mechanism for how this happens. It is understood that the authors tried to perform snRNAseq in the last figure to address this, but this did not show how ERBB4 causes the changes in control mice and how KO of ERBB4 in AD mice rescues the

phenotype. Since the authors are claiming this one gene controls a broad spectrum of AD phenotypes, it is critical that they provide some mechanism for how all these things happen. Such data are needed to support the bold claims that are made and to support the primary importance of ERBB4 that they claim.

We fully agree with the referee that elucidating the mechanism by which ERBB4 exerts such broad effects is critical, particularly given our conclusion that excitatory neuronal ERBB4 controls multiple AD-related phenotypes.

To address this point, we focused on the mTOR signaling pathway, which has been well established as a major downstream effector of ERBB4 signaling[1], and whose hyperactivation in excitatory neurons has been implicated in neurodegenerative phenotypes[2]. We therefore tested whether mTOR signaling mediates the pathological effects of ERBB4 *in vivo*.

Specifically, we deleted *Rptor*, an essential component of the mTORC1 complex, in excitatory neurons of 5XFAD mice using CRISPR-based approaches. Deletion of *Rptor* robustly rescued multiple AD-related phenotypes, including aberrant excitatory and inhibitory synapse phagocytosis, synapse number alterations, and abnormal neuronal activity as assessed by Fos expression (Extended Data Fig. 10). These results indicate that mTOR signaling is required for the manifestation of neuronal and synaptic dysfunction in AD mice.

To further establish a causal relationship between ERBB4 and mTOR signaling, we next combined *Rptor* deletion with ectopic *Erbb4* overexpression in WT mice. Under these conditions, *Rptor* deletion significantly attenuated or abolished the effects of ERBB4 overexpression on synapse number, neuronal hyperactivity, and gliosis (Extended Data Fig. 11). These findings demonstrate that mTOR signaling acts downstream of ERBB4 and is necessary for ERBB4-induced phenotypes.

Together, these loss-of-function experiments provide a mechanistic framework in which aberrant ERBB4 expression in excitatory neurons drives AD-related pathophysiology through activation of the mTOR pathway. While the precise downstream effectors of mTOR that mediate individual phenotypes (e.g., synaptic remodeling and glial activation) remain to be fully elucidated, defining these molecular branches will be an important direction for future studies and is beyond the scope of the current work.

[1]Bernard, C., Exposito-Alonso, D., Selten, M., Sanalidou, S., Hanusz-Godoy, A., Aguilera, A., ... & Marín, O. (2022). Cortical wiring by synapse type–specific control of local protein synthesis. *Science*, *378*(6622), eabm7466.

[2]Kassai, H., Sugaya, Y., Noda, S., Nakao, K., Maeda, T., Kano, M., & Aiba, A. (2014). Selective activation of mTORC1 signaling recapitulates microcephaly, tuberous sclerosis, and neurodegenerative diseases. *Cell reports*, *7*(5), 1626-1639.

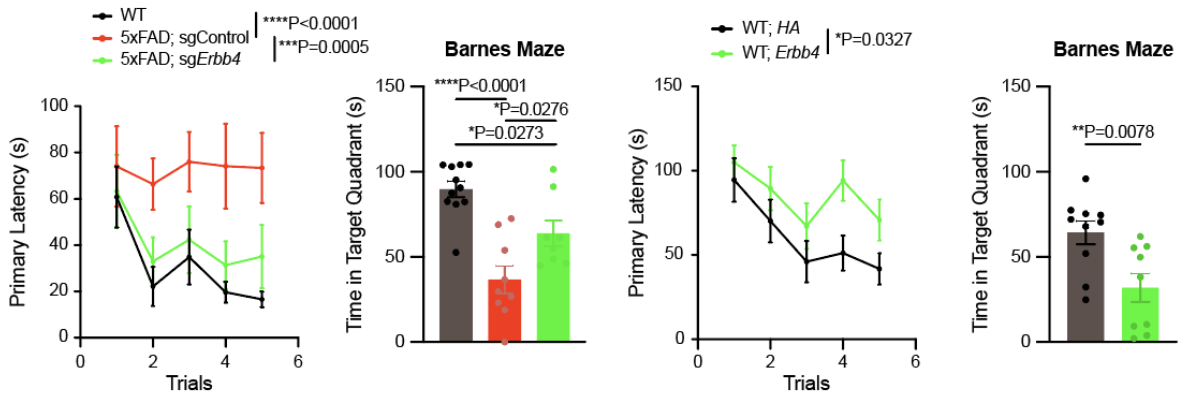
3. The relevance to AD readouts is supported by behavioral analysis in the last figure. However, the behavioral experiments are simplistic and a greater range of AD-related phenotypes need to be assessed.

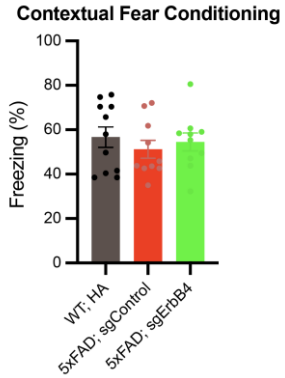
Such evaluations could also include some in vivo electrophysiology to assess sleep wake cycles and LTP. The authors are making an amazing claim that ERBB4 alone in some pyramidal neurons causes all the major behavioral disruptions of AD in mice. This could potentially be important but they must do better by performing far better and more detailed behavior experiments that are relevant to AD.

As the referee pointed out, we were also initially surprised by the strong impact of excitatory neuronal ERBB4 on cognitive impairment. It is indeed well established that vascular dysfunction[1], as well as Aβ-driven direct effects on neuronal dysregulation and reactive gliosis[2,3], contribute to AD progression and behavioral symptoms. In this context, our findings do not argue against these established mechanisms, but rather suggest that an excitatory neuron-intrinsic mechanism mediated by aberrant ERBB4 expression may operate at relatively earlier stages of disease progression and subsequently synergize with these factors to exacerbate neurodegeneration. We have added this point to the discussion.

In AD mouse models, the most representative and widely accepted behavioral dysfunctions are impairments in spatial learning and memory[4,5], typically assessed by Morris water maze or Barnes maze. Following the referee's suggestion, we performed the Barnes maze test and found that *Erbb4* deletion rescued the spatial learning and memory deficits observed in 4-month-old 5XFAD mice, whereas *Erbb4* overexpression in 2-month-old WT mice disrupted these behaviors (Fig. 5s, t)

We also assessed fear memory using contextual fear conditioning. At 6 months of age, however, 5XFAD controls did not show detectable impairment in fear memory, and therefore no rescue effect was observed upon *Erbb4* deletion. For this reason, we did not include this data in our revised manuscript.





[1]Kovbasyuk, Z., Tefera, E., Li, C., Baete, S. H., & Steriade, C. (2025). Imaging brain inflammation and blood brain barrier permeability in neurological and psychiatric diseases: a review. *Journal of Neuroinflammation*, *22*(1), 275.

[2]Li, S., Jin, M., Koeglsperger, T., Shepardson, N. E., Shankar, G. M., & Selkoe, D. J. (2011). Soluble Aβ oligomers inhibit long-term potentiation through a mechanism involving excessive activation of extrasynaptic NR2B-containing NMDA receptors. *Journal of Neuroscience*, *31*(18), 6627-6638.

[3]Sondag, C. M., Dhawan, G., & Combs, C. K. (2009). Beta amyloid oligomers and fibrils stimulate differential activation of primary microglia. *Journal of neuroinflammation*, *6*(1), 1.

[4]Puzzo, D., Lee, L., Palmeri, A., Calabrese, G., & Arancio, O. (2014). Behavioral assays with mouse models of Alzheimer's disease: practical considerations and guidelines. *Biochemical pharmacology*, *88*(4), 450-467.

[5]Attar, A., Liu, T., Chan, W. T. C., Hayes, J., Nejad, M., Lei, K., & Bitan, G. (2013). A shortened Barnes maze protocol reveals memory deficits at 4-months of age in the triple-transgenic mouse model of Alzheimer's disease. *PloS one*, *8*(11), e80355.

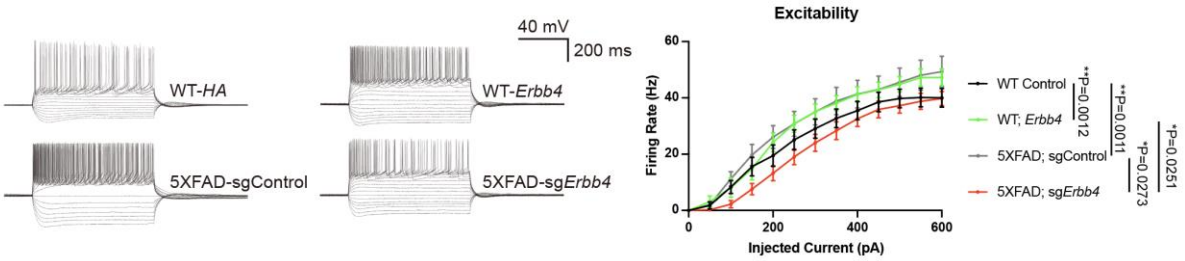
With respect to electrophysiological evaluation, while measurements of EPSCs and IPSCs could, in principle, provide information on E/I balance, previous studies using diverse AD mouse models have reported highly variable and sometimes contradictory changes in the frequency and amplitude of EPSCs and IPSCs, even within the same brain region and at comparable ages[1-4]. This variability makes synaptic current measurements a less reliable readout for assessing network-level activity changes in AD models. Therefore, instead of focusing on EPSCs and IPSCs, we assessed intrinsic neuronal excitability as a more direct and robust functional measure of neuronal activity. Using whole-cell patch-clamp recordings from CA1 pyramidal neurons, we found that ectopic expression of *Erbb4* in WT excitatory neurons significantly increased neuronal excitability to levels comparable to those observed in 5XFAD mice. Conversely, deletion of *Erbb4* in excitatory neurons of 5XFAD mice reduced excitability to levels comparable to WT controls (Extended Data Fig. 7o, p).

These electrophysiological findings are fully consistent with our activity marker data, in which pyramidal neurons in *Erbb4*-injected WT and 5XFAD exhibited increased Fos expression, indicating hyperactivity (Fig. 4h, j and Extended Data Fig. 6d, f). Moreover, this excitatory neuronal hyperexcitability is accompanied by reduced Fos expression in SST interneurons (Fig. 4i, k and

Extended Data Fig. 6e, g), which might be mediated through enhanced recruitment of VIP interneurons (Extended Data Fig. 6b, c), ultimately leading to disruption of the E/I balance.

Together, these electrophysiological data provide direct functional support for a central role of *Erbb4* in excitatory neurons in driving neuronal hyperexcitability and downstream imbalance of excitatory and inhibitory network activity.

Further analyses with sleep cycles as well as LTP/LTD are also excellent suggestions; however, we believe that such analyses would be better addressed in a separate follow-up study.



[1]Li, Y., Zhu, K., Li, N., Wang, X., Xiao, X., Li, L., ... & Zheng, Y. (2021). Reversible GABAergic dysfunction involved in hippocampal hyperactivity predicts early-stage Alzheimer disease in a mouse model. *Alzheimer's Research & Therapy*, *13*(1), 114.

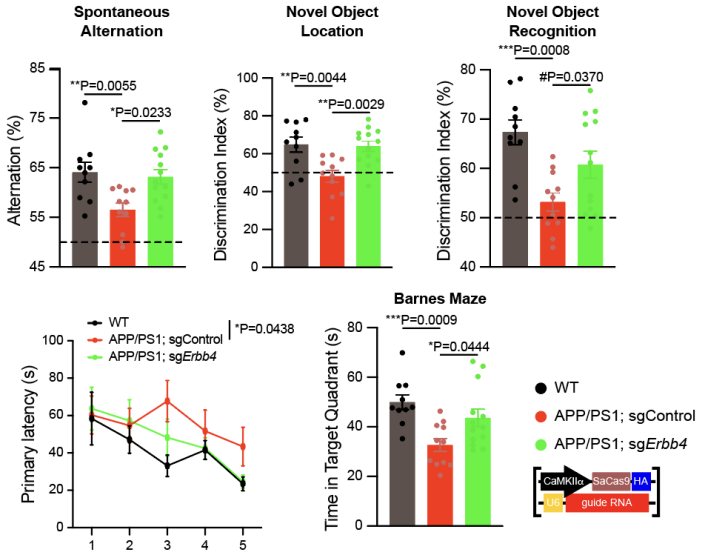
[2]Andersen, J. V., Skotte, N. H., Christensen, S. K., Polli, F. S., Shabani, M., Markussen, K. H., ... & Waagepetersen, H. S. (2021). Hippocampal disruptions of synaptic and astrocyte metabolism are primary events of early amyloid pathology in the 5xFAD mouse model of Alzheimer's disease. *Cell Death & Disease*, *12*(11), 954.

[3]Jo, S., Yarishkin, O., Hwang, Y. J., Chun, Y. E., Park, M., Woo, D. H., ... & Lee, C. J. (2014). GABA from reactive astrocytes impairs memory in mouse models of Alzheimer's disease. *Nature medicine*, *20*(8), 886-896.

[4]Zhou, B., Lu, J. G., Siddu, A., Wernig, M., & Südhof, T. C. (2022). Synaptogenic effect of APP-Swedish mutation in familial Alzheimer's disease. *Science translational medicine*, *14*(667), eabn9380.

4. Key experiments exploring ERBB4 in AD model mice such as behavior should be repeated in at least one additional mouse model. APP/PS1 seems obvious because they used it in Ext Fig 1. I

We were fortunate to secure a sufficient number of APP/PS1 mice for behavioral analysis. With these APP/PS1 mice, as the referee suggested, we repeated the full set of behavioral analyses in 10-month-old APP/PS1 mice. Consistent with our findings in 5XFAD mice, *Erbb4* deletion rescued all tested behavioral assays, including spontaneous alternation, novel object location, novel object recognition, and Barnes maze in APP/PS1 mice (Extended Data Fig. 12b, c). These results further support the robustness and generalizability of our conclusions.



5. The authors have not studied Alzheimer's disease. They have studied a mouse model of amyloidogenesis which may be related to AD. They must change the title and the text to reflect this.

We agree with the referee that 5XFAD and APP/PS1 mice are the most relevant models for amyloid pathology. To suggest the human AD relevance of our study, we have now performed a more comprehensive analysis using snRNAseq data from 446 individuals (healthy controls and AD patients) in the Religious Orders Study and Memory and Aging Project (ROSMAP) cohort[1,2].

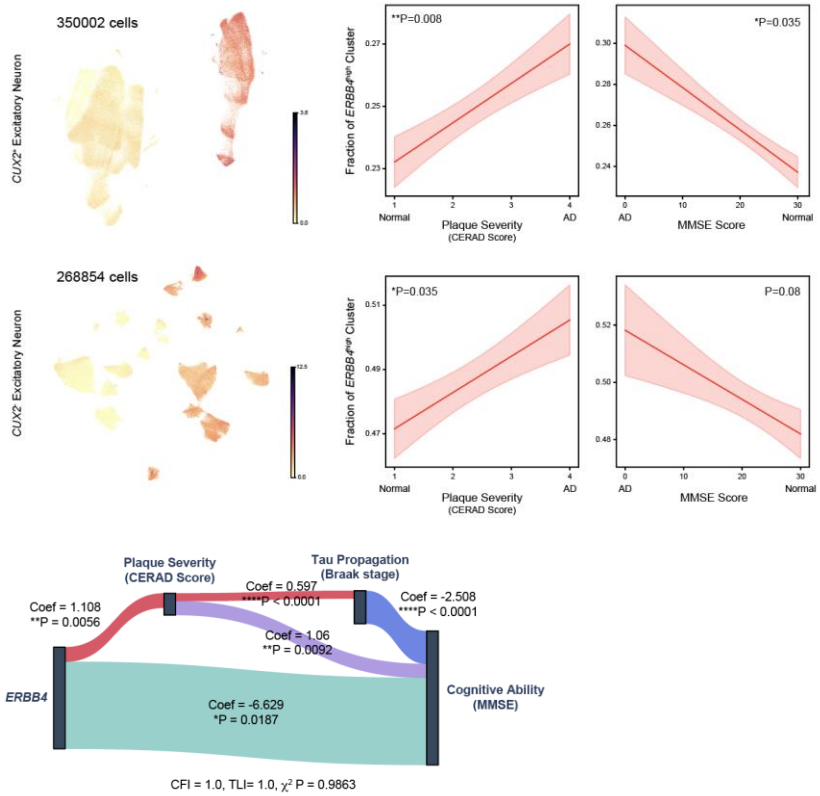
Specifically, we analyzed *CUX2*$^{+}$ and *CUX2*$^{-}$ cortical excitatory neurons and identified *ERBB4*^{high} excitatory neuronal clusters in human AD brains (Extended Data Fig. 12d, e), similar to our mouse EREN population. Importantly, the fraction of these *ERBB4*^{high}clusters was positively correlated with plaque severity (CERAD score) and negatively correlated with cognitive performance (MMSE) (Extended Data Fig. 12f, g).

To further increase rigor beyond correlation, we applied causal modeling approaches, including mediation analysis and structural equation modeling, within a biologically grounded AD pathological cascade framework. These analyses supported a model in which excitatory neuronal *ERBB4* participates in a pathogenic pathway linking amyloid pathology to tau propagation and consequent cognitive decline (Fig. 5u).

Taken together, these human transcriptomic and causal modeling results strongly support the relevance of our mouse findings to human AD and indicate that *ERBB4* expression in excitatory neurons is not merely a mouse-specific phenomenon, but is significantly associated with disease pathology and cognitive impairment in humans.

Given that we have adopted more conservative wording throughout the manuscript (e.g., using "promotes" rather than "drives" in our title) and have now incorporated direct analyses of human AD patient transcriptomic data, we believe that retaining reference to Alzheimer's disease in the title is

appropriate. Nonetheless, we remain open to further adjustment to the title if deemed necessary by the referee or the editor.



[1]A. Bennett, D., A. Schneider, J., Arvanitakis, Z., & S. Wilson, R. (2012). Overview and findings from the religious orders study. *Current Alzheimer Research*, *9*(6), 628-645.

[2]A Bennett, D., A Schneider, J., S Buchman, A., L Barnes, L., A Boyle, P., & S Wilson, R. (2012). Overview and findings from the rush Memory and Aging Project. *Current Alzheimer Research*, *9*(6), 646-663.

6. The authors must explore how ERBB4 and the snRNAseq data changes in the last figure are related to the vast amounts of data for human AD. This is critical to do. Are the mechanisms they find seen in humans? Much deeper analysis is needed.

We agree with the referee that it is critical to place our snRNAseq findings and proposed mechanisms within the broader context of human AD datasets. As described in our response to point 5, we have addressed this by performing extensive analyses of human snRNAseq data from the ROSMAP cohort, encompassing 446 individuals with well-characterized pathological and cognitive measures.

In addition to demonstrating the presence of *ERBB4*^{high} excitatory neuronal populations in human AD brains, we would like to emphasize that we went beyond descriptive transcriptomic overlap by applying causal

modeling approaches grounded in established AD pathological frameworks. Specifically, mediation analysis and structural equation modeling were used to test whether *ERBB4* expression in excitatory neurons participates in a mechanistic pathway linking amyloid pathology, tau propagation, and cognitive decline in humans. These analyses support a model in which *ERBB4* is not merely correlated with disease state, but is statistically consistent with a pathogenic role within the human AD cascade.

Thus, while our mechanistic perturbation experiments were necessarily conducted in mice, the convergence of human transcriptomic signatures with causal modeling provides evidence that the *ERBB4*-associated mechanisms identified in our study are relevant to human AD. We believe this integrative strategy, combining experimental causality in mouse models with causal inference in large-scale human datasets, represents a rigorous and appropriate approach to bridge species and datasets.

7. The statistics section needs to be expanded and additional details provided such as how and why certain statistical tests were chosen. Why is a student's t test always the best test? Was there never any none normally distributed data in the project? Or did they assume normal distributions in all the data? Such details are needed for the behavior experiments.

In the revised manuscript, we have expanded the 'Sofware and statistical analysis' section to provide detailed descriptions of the tests used and the rationale for their selection. Specifically, normality of the data was first assessed using the Shapiro-Wilk test. Student's *t*-tests were applied only when data followed a normal distribution and consisted of two groups. One-way ANOVA was used when normally distributed data involved more than two groups with a single factor, and two-way ANOVA was used for normally distributed data with two factors (e.g., genotype and viral injection). For data not meeting the assumption of normality, non-parametric tests (e.g., Mann–Whitney *U*) were applied as appropriate. We have now clarified these criteria and the tests applied in each experiment, including behavioral analyses, in the Methods section.

Less major points

8. Line 24. The word <contribute> is too strong. It is more accurate to say neuroinflammation and synapses loss cognitive decline. Is there any evidence that neuroinflammation causes the effects directly or synergistically? What is that evidence?

We appreciate the referee's point regarding the wording. While several studies have suggested that neuroinflammation can promote synaptic dysfunction and cognitive impairment in AD, these studies do not directly quantify synapse loss, and therefore do not establish a definitive causal or synergistic

relationship. There is also evidence that neuroinflammation can be associated with network-level dysfunction and cognitive decline independently of overt neuronal loss[1].

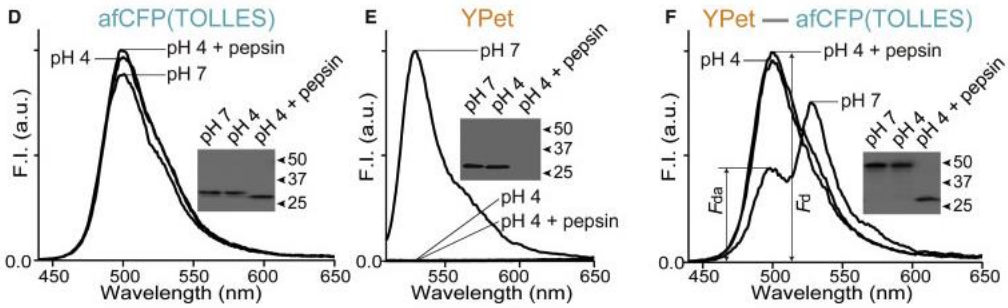
Based on the referee's suggestion, we have revised the sentence to adopt a more cautious and accurate wording that avoids implying direct causality. The revised sentence now reads:

"Neuroinflammation and synapse loss are highly associated with cognitive decline in Alzheimer's disease (AD)."

[1]Leng, F., Hinz, R., Gentleman, S., Hampshire, A., Dani, M., Brooks, D. J., & Edison, P. (2023). Neuroinflammation is independently associated with brain network dysfunction in Alzheimer's disease. *Molecular psychiatry*, *28*(3), 1303-1311.

9. Line 79. Is it the relative instability of EGFP or the relative intensity of EGFP. This needs to be clarified.

We thank the referee for raising this important point. In this context, the key issue is the relative instability of EGFP compared to mCherry under lysosomal conditions, rather than differences in fluorescence intensity per se. As demonstrated in Figure 1D–F of Katayama et al. (Cell, 2020), jellyfish-derived fluorescent proteins, including Aequorea GFP variants such as EGFP (and YPet), exhibit pronounced irreversible acid- and protease-dependent instability, resulting in structural denaturation and degradation in low pH environments such as lysosome. In contrast, coral-derived fluorescent proteins, exemplified in this study by afCFP (TOLLES) and also including mCherry, are substantially more resistant to both acidic conditions and lysosomal proteases. Consistent with this framework, previous studies have shown that EGFP is preferentially degraded under acidic and proteolytic conditions, whereas mCherry remains relatively stable at the protein level[1]. We have therefore revised the text and cited relevant papers to clarify that the observed differences primarily reflect protein stability and degradation fate in acidic compartments, rather than differences in fluorescence intensity.



[1]Katayama, H., Hama, H., Nagasawa, K., Kurokawa, H., Sugiyama, M., Ando, R., ... & Miyawaki, A. (2020). Visualizing and modulating mitophagy for therapeutic studies of neurodegeneration. *Cell*, *181*(5), 1176-1187.

10. Near the section at line 94. It should be described what the phagocytic index is in the graphs. This seems to be some unitless number. This needs to be defined in the results or the graphs need to be changed to have real values that represent phagocytosis per cell, for example. This is needed so that people can interpret and repeat the experiments.

We have now provided a more detailed explanation of the definition and calculation of the phagocytic index in Extended Data Fig. 1a.
Briefly, the phagocytic index is calculated as a unitless value that corresponds to the proportion of phagocytosed synapses ($mCherry^{+}EGFP^{-}$ puncta) relative to the total population of virus-infected synapses ($EGFP^{+}$ puncta). Because viral infection efficiency and the number of infected cells can vary across experiments, this value is normalized to the area of $EGFP^{+}$ signal rather than reported as an absolute count per cell.
Although some variability can still arise due to differences in viral expression levels and imaging conditions, we believe that this normalized, unitless index provides a more interpretable and reproducible measure for comparing relative changes in synapse phagocytosis across conditions, and is therefore appropriate for the purposes of this study.

11. The section between lines 105 and 114 is too strong and should be toned down. Opposing changes in excitation and inhibition could be important. The brain does not simply reflect the aggregate balance between excitation and inhibition. This section needs to be rewritten and comes across as simple minded.

We agree with the referee that our original statement was overly strong and may have oversimplified the complex dynamics of E/I balance. We acknowledge that the brain does not simply operate on an aggregate balance of excitation and inhibition, and concluding that neuroinflammation is not a cause solely based on opposing trends was a leap in logic.
Therefore, we have rewritten the section to be more precise. Instead of making broad claims about neuroinflammation, we now focus on the 'selectivity' of the phenomenon. We have revised the text to suggest that the differential vulnerability of excitatory vs. inhibitory synapses implies that neuronal state determines glial engagement, rather than glial hyper-activity acting alone.
The revised sentence reads:
"Furthermore, the selective elimination of excitatory synapses, coupled with the preservation of inhibitory ones, raises the possibility that intrinsic neuronal states guide glial phagocytic behavior, and that indiscriminate neuroinflammation is unlikely to be the sole driver of synapse elimination in AD."

12. The results section for snRNAseq must report numbers of cells and mice

We now explicitly report the number of cells and the number of mice used for each snRNAseq analysis in the legend of the corresponding figure.

13. Near line 154, they should name at least some of the genes

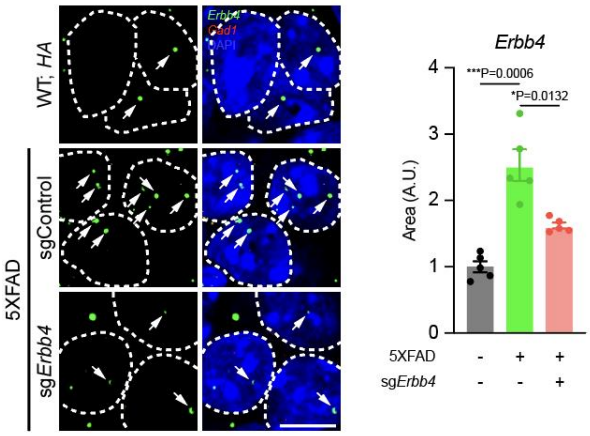
In the revised manuscript, we now list representative genes from this analysis in the Results section.

14. Line 157 it seems very premature to define the neurons as disease initiating excitatory neurons. This must be stated only when the field has had time to repeat the work. Disease-related excitatory neurons seem better.

We agree with the referee's point. In response, we have revised the terminology. While "disease-related excitatory neurons" is more conservative, we felt it does not sufficiently capture the early emergence of these cells in our snRNAseq data. To balance accuracy with appropriate caution, we now refer to this population as "Early Responsive Excitatory Neurons (ERENs)" throughout the manuscript. This terminology avoids overstatement while emphasizing their early responsiveness in AD models.

15. Near line 168-174. Some MERSCOPE or in situ hybridization may be needed to show ERBB4 mRNA is found in the somata of pyramidal neurons.

We agree with the referee's concern. Therefore, to address this, we performed fluorescence *in situ* hybridization (FISH) using RNAscope (Extended Data Fig. 5g, h), which directly measures RNA transcripts in intact brain sections. Consistent with our snRNAseq findings, we observed that *Erbb4* mRNA was highly expressed in the pyramidal layer of the hippocampal CA1 region in 5XFAD mice compared to WT controls, and that this elevation was markedly reduced upon *Erbb4* deletion using Cas9. These results provide independent validation in a more physiological context and support the robustness of our snRNAseq-based conclusions.



16. Near lines 181-185. The data mentioned for humans could not be found in Fig 5j. This analysis should be in Fig 2. As stated earlier the human analysis needs to be expanded.

The data that referee mentioned was originally presented in Extended Fig. 5j, not Fig. 5j. However, based on the suggestion of referee #1, we decided to remove this data in our revised manuscript. Instead, as mentioned above, we have expanded human analysis through ROSMAP data and is presented in Extended Data Fig.12d-g of the revised manuscript.

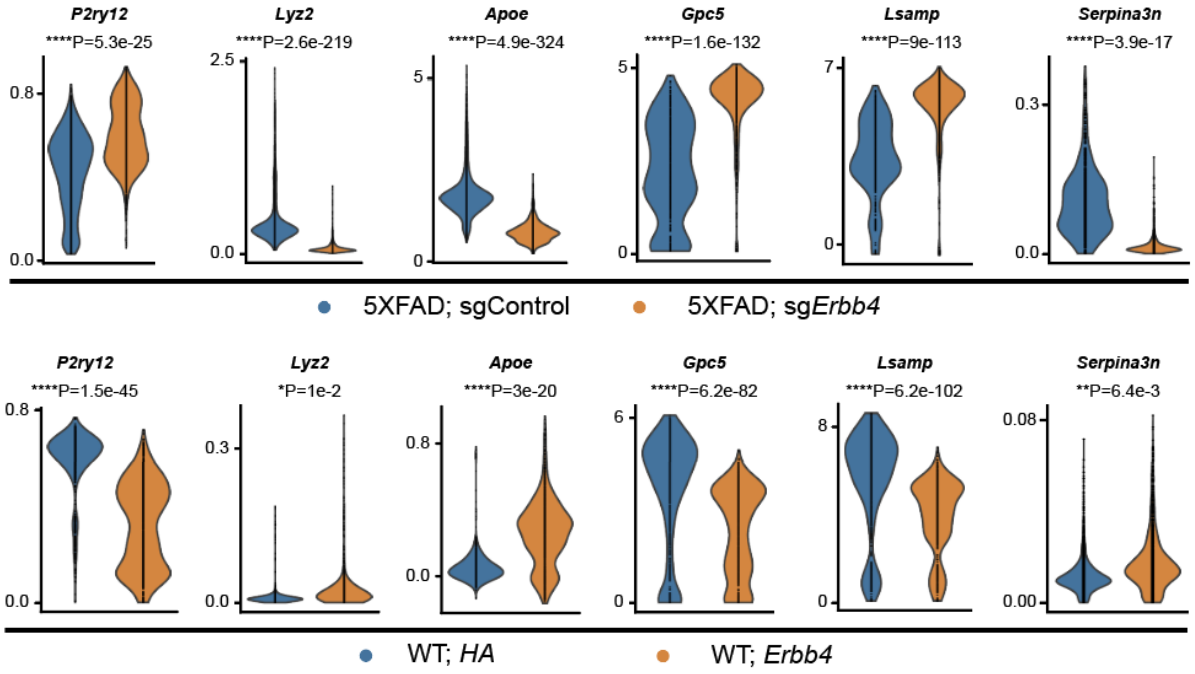
17. Lines 188-198. It is not clear how it was possible to reduce expression of ERBB4 only in those neurons that showed an increase. How was this done? This must be stated.

We did not selectively target ERBB4 expressing excitatory neurons with our CRISPR approach. Instead, we used the CaMKIIα promoter to delete *Erbb4* broadly in 5XFAD pyramidal neurons.
This strategy was feasible and specific in the disease context because *Erbb4* expression is largely absent in WT excitatory neurons and is ectopically induced in excitatory neurons specifically in the 5XFAD background (Fig. 2g, h). As a result, CaMKIIα-driven deletion effectively targets the pathological upregulation of *Erbb4* in 5XFAD mice while having minimal impact on WT excitatory neurons.
Consistent with this interpretation, deletion of *Erbb4* in WT pyramidal neurons did not produce detectable changes in synapse phagocytosis or gliosis (Extended Data Fig. 6r-w), supporting the notion that our approach selectively disrupts disease-associated *Erbb4* expression rather than basal physiological function.

18. Lines 215-229. The assessment of neuroinflammation using three markers is superficial. Neuroinflammation must be evaluated broadly.

We appreciate the referee's comment. In response, we have revised the wording in the Results section to describe these immunohistochemical data more conservatively as "gliosis," rather than broadly referring to "neuroinflammation."

In addition, to evaluate inflammatory changes in a more comprehensive and unbiased manner, we performed snRNAseq analyses in both *Erbb4* deletion and overexpression conditions (Fig. 5a-p). These analyses allowed us to assess broader transcriptional programs characteristic of disease-associated microglia (DAM) and inflammatory reactive astrocytes (Fig. 5b-h, j-p). In the revised manuscript, we now highlight representative marker genes for these states and have added violin plots to more clearly illustrate the extent of these transcriptional changes (Fig. 5g, h, o, p).



19. Near lines 259-266. The data for the late reduction of ERBB4 should be included in the main manuscript

We thank the referee for this helpful suggestion and agree that the data demonstrating the effects of late-stage reduction of ERBB4 are particularly important from a therapeutic perspective. Indeed, one of the strengths of our study is that ERBB4 reduction is effective not only at early stages but also when applied after disease progression has already begun, highlighting its potential therapeutic relevance.

In the current version of the manuscript, these data are included in the Extended Data Fig. 8i-p due to space limitations. However, since we agree that these data would further strengthen the main narrative, we would be happy to move the late-stage ERBB4 reduction data into the main figures if the editor agrees that this is appropriate.

20. Line 265. The authors should stop using poor language such as "preventative care". They have assessed mice. They need to tone things down.

We agree that the wording "preventative care" is inappropriate in the context of mouse studies. We have therefore removed this term and replaced it with more conservative language that accurately reflects the scope of our experiments.
Specifically, we have revised the sentence as follows:
"…indicating that deletion of *Erbb4* in ERENs remains effective even when implemented well after disease onset in this mouse model, leading to significant reductions in multiple AD-related pathologies."
This change removes clinical language and ensures that our conclusions are appropriately framed within the context and limitations of preclinical mouse studies.

21. Lines 269-296. For the ectopic expression experiments how did they ensure the number of neurons expressing ERBB4 and its expression levels was like in AD mice? This must be clarified. Obviously simply overexpressing ERBB4 in all neurons is not a very good experiment.

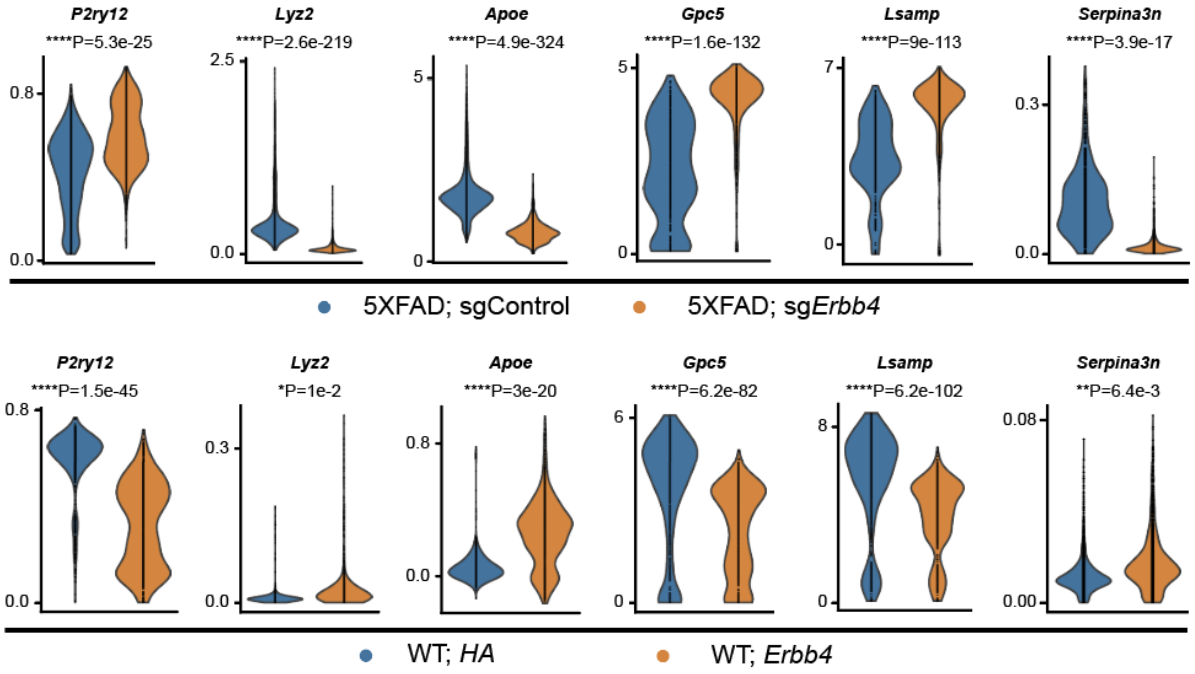
We appreciate the referee's comment and agree that uncontrolled overexpression of ERBB4 in all neurons may not be directly relevant to AD pathology. In our experiments, the proportion of *Erbb4*$^{+}$ excitatory neurons in 5XFAD mice was approximately 5-15% (Fig. 3c). For the ectopic expression experiments, we therefore carefully titrated the viral dose such that the fraction of *Erbb4*$^{+}$ excitatory neurons was centered around 15% (Fig. 4c), corresponding to the upper range observed in 5XFAD mice. In addition, AAV-mediated ERBB4 overexpression was spatially restricted to the CA1 region, rather than being induced globally across all excitatory neurons (Fig. 4b). Thus, our overexpression paradigm was deliberately designed to approximate the extent and distribution of pathological ERBB4 induction observed in AD mouse models, rather than to drive uniform or supraphysiological levels of ERBB4 expression.
We have clarified these points in the revised manuscript.

22. Lines 307-310. That section needs to be expanded more and as written is superficial.

We thank the referee for this helpful comment. In response, we have expanded this section to provide a more detailed description. Specifically, we now list representative disease-associated microglia (DAM) module genes (e.g., *Cst7, Lpl, Lyz2,* and *Apoe*) and inflammatory reactive astrocyte-related genes (e.g., *Vim, Gfap, Serpina3n,* and *Cp*) whose expression was reduced upon *Erbb4* deletion in excitatory neurons of 5XFAD mice. Conversely, we highlight homeostatic microglial genes (e.g., *P2ry12, Cx3cr1,*

and *Tmem119*) and astrocytic genes (e.g., *Gpc5*, *Luzp2*, and *Lsamp*) whose expression was increased following *Erbb4* deletion. In contrast, *Erbb4* overexpression induced the opposite pattern, with increased DAM and inflammatory astrocyte-related gene expression and decreased homeostatic gene expression.

To better visualize and support these conclusions, we have added violin plots illustrating these transcriptional changes in microglia and astrocytes (Fig. 5g, h, o, p). Together, these revisions provide a more comprehensive and mechanistic view of how *Erbb4* deletion and overexpression shift glial populations toward homeostatic and inflammatory states, respectively.



23. Lines 311-313. I do not understand what they mean when they state overexpression of ERBB4 resulted in the emergence of a population of novel neurons expressing ERBB4. Of course, this happened because they overexpressed it. Do they mean something more? Or is this another example of exaggeration?

We appreciate the referee's comment and agree that the original wording could be interpreted as implying the generation of an entirely new neuronal identity. Accordingly, we have revised the manuscript to replace the phrase "novel excitatory neuronal population" with "a clearly separated excitatory neuronal subcluster."

We would like to also clarify that our original intention was to emphasize the magnitude of the transcriptional impact of *Erbb4* overexpression. In general, overexpression of a single gene is rarely

sufficient to produce a clearly separable cluster in UMAP space, particularly within an otherwise homogeneous excitatory neuronal population. The emergence of a distinct *Erbb4*-expressing subcluster therefore reflects the strong and coordinated transcriptional program induced by ERBB4, rather than the creation of a new neuronal type. We believe that the revised wording accurately conveys this point while avoiding overstatement.

24. Line 357. I don't think the work is a paradigm shift. It is a valuable study. They should tone down their language.

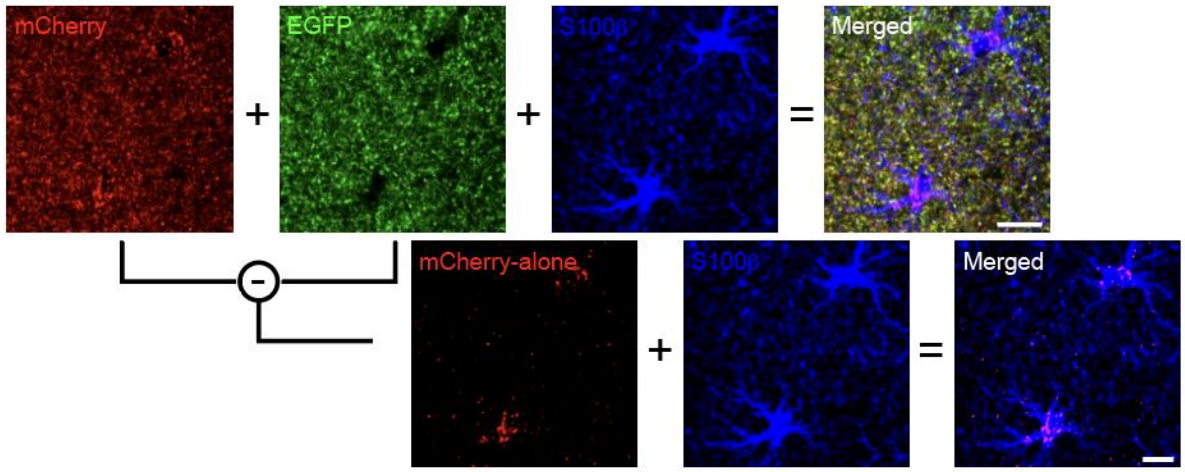
In the revised manuscript, we have removed the phrase "paradigm shift" and replaced it with more measured language, describing our findings as providing "key mechanistic insight" into the role of excitatory neurons in Alzheimer's disease pathogenesis.

25. Are there differences in the phagocytic index between Fig 1 and 3? It would be very useful to have real values on the y-axis rather than some arbitrary index that is hard to compare between figures and other studies.

As noted in our response to Comment 10, normalization to the $EGFP^+$ area is essential to account for variability in the extent of viral infection across animals, and therefore the phagocytic index is necessarily expressed in arbitrary units. Because such variability cannot be fully eliminated, direct quantitative comparison between independent experimental groups or across figures is not appropriate. Accordingly, the phagocytic index is intended for within-experiment comparisons, rather than for direct comparison across separate experimental cohorts or between studies.

26. Throughout the figures, the images showing green synaptic blobs inside astrocytes based on reconstructions of astrocytes (eg Fig 1a and others) could be replaced with detailed colocalization analysis. This would be more valuable to the field.

The procedure for our colocalization analysis was described in Extended Data Fig. 1a, but we agree that the explanation was not sufficiently detailed. In the revised manuscript, we have expanded this section to more clearly describe how colocalization was quantified, including the criteria used to define synaptic puncta within astrocytic volumes and the normalization strategy applied. We believe this expanded description will make the methodology clearer and more reproducible, thereby addressing the referee's concern.



27. Check the naming of the figures in the text because this was confusing.

We have carefully checked all figure citations throughout the manuscript and corrected any inconsistencies or errors to ensure that the figure naming is clear and consistent.

Referee #3 (Remarks to the Author):

This study by Lee et al. shows that during Alzheimer's disease (AD) progression, astrocytes and microglia increase phagocytosis of excitatory synapses but reduce phagocytosis of inhibitory synapses. Also, astrocyte-mediated synapse elimination was altered earlier than microglial one, in both APP/PS1 and 5xFAD mice. Using single-nucleus RNA sequencing, the researchers identified "Disease-Initiating Excitatory Neurons" (DIENs) marked by increased Erbb4 expression as an early event in AD mouse models. Deleting Erbb4 in excitatory neurons substantially prevented multiple AD symptoms, such as excitatory neuronal hyperactivity, inhibitory neuronal hypoactivity, abnormal synapse elimination, reactive gliosis, and even amyloid beta (Ab) accumulation, leading to significant recovery of cognitive functions. On the other hand, overexpressing Erbb4 in wild-type neurons mimicked AD phenotypes without plaques. These findings suggest that aberrant Erbb4 expression in excitatory neurons drives early AD pathology and represents a potential therapeutic target.
The study is highly significant and has the potential for major impact in the field, the manuscript is well-written, and the authors have conducted a thorough meticulous analysis. However, there are some comments and concerns:

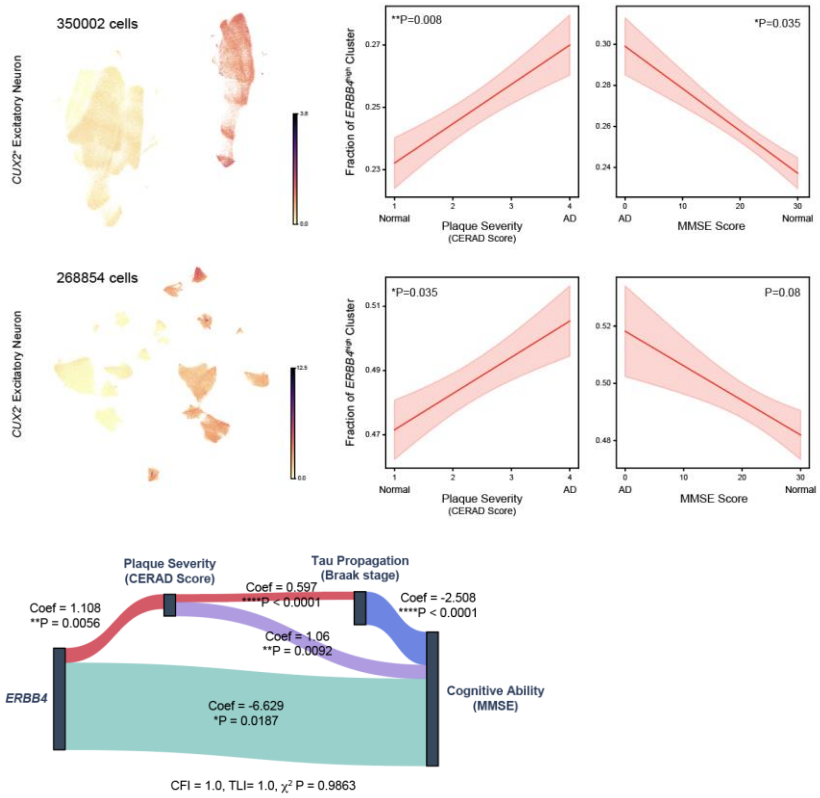
Major
• A primary limitation of the study is its reliance on amyloid mouse models, specifically APP/PS1 and 5XFAD mice. While these models recapitulate early amyloid pathology, they do not fully mirror the

complex, slowly progressive nature of human AD, especially with respect to tau pathology, vascular contributions, and chronic neuroinflammation. This raises some concerns about how well findings related to Erbb4 dysregulation and synapse loss will translate to the broader spectrum of human AD. This limitation is mentioned in the Discussion, but needs some elaboration.

In our previous submission, we had shown that excitatory neurons from human AD patients contain an *ERBB4*$^{+}$ cluster. However, we agree with the referee that a more in-depth analysis would be necessary to link our result with human dataset. To strengthen the human relevance of our study, we have now performed a more comprehensive analysis using snRNAseq data from 446 individuals (healthy controls and AD patients) in the Religious Orders Study and Memory and Aging Project (ROSMAP) cohort[1,2]. Sepcifically, we analyzed *CUX2*$^{+}$ and *CUX2*$^{-}$ cortical excitatory neurons and identified *ERBB4*^{high} excitatory neuronal clusters in human AD brains (Extended Data Fig. 12d, e), similar to our mouse EREN population. Importantly, the fraction of these *ERBB4*^{high}clusters was positively correlated with plaque severity (CERAD score) and negatively correlated with cognitive performance (MMSE) (Extended Data Fig. 12f, g).

To further increase rigor beyond correlation, we applied causal modeling approaches, including mediation analysis and structural equation modeling, within a biologically grounded AD pathological cascade framework. These analyses supported a model in which excitatory neuronal *ERBB4* participates in a pathogenic pathway linking amyloid pathology to tau propagation and consequent cognitive decline (Fig. 5u).

Taken together, these human transcriptomic and causal modeling results strongly support the relevance of our mouse findings to human AD and indicate that *ERBB4* expression in excitatory neurons is not merely a mouse-specific phenomenon, but is significantly associated with disease pathology and cognitive impairment in humans.



[1]A. Bennett, D., A. Schneider, J., Arvanitakis, Z., & S. Wilson, R. (2012). Overview and findings from the religious orders study. *Current Alzheimer Research*, *9*(6), 628-645.

[2]A Bennett, D., A Schneider, J., S Buchman, A., L Barnes, L., A Boyle, P., & S Wilson, R. (2012). Overview and findings from the rush Memory and Aging Project. *Current Alzheimer Research*, *9*(6), 646-663.

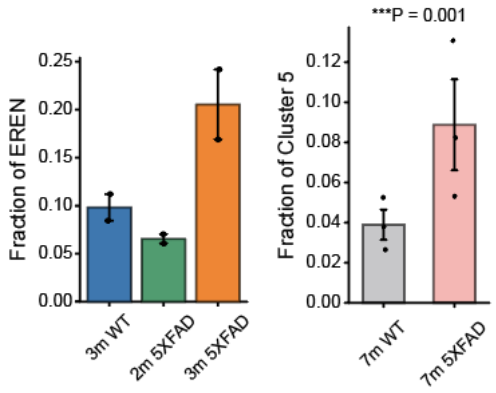
• There are also some concerns with the snRNA-seq analyses. First, authors claim that clusters 5-9 (DIEN) clusters are enriched in 3-mo 5XFAD mice (Fig. 2b); however, no quantitative data is provided to support this enrichment.

We thank the referee for this helpful comment. In the revised manuscript, we now provide quantitative data to support the enrichment of clusters 5–9 (ERENs, formerly DIENs) in 3-month-old 5XFAD mice by reporting the fraction of cells assigned to these clusters across genotypes and ages, presented as bar graphs in Extended Data Fig. 5a.

We note, however, that the 3-month hippocampal dataset was derived from a limited number of biological replicates (n = 2 per group), which precluded formal statistical testing. To further support the robustness of this observation, we analyzed an independent snRNA-seq dataset from the cortex of 7-month-old 5XFAD mice. Although the number of biological replicates in this dataset (n = 3 per group)

remains modest, we nonetheless observed a consistent enrichment of an EREN-like excitatory neuronal cluster in 5XFAD mice compared with controls (Extended Data Fig. 5f).

Together, these results demonstrate consistent enrichment of EREN/EREN-like clusters across independent datasets, ages, and brain regions, providing quantitative and statistically supported evidence for their association with AD pathology.



Second, it is unclear why KEGG was used to identify pathways enriched in DIEN clusters, while REACTOME was used for pathways enriched in the turquoise module. If clusters 5–9 (i.e., DIEN) are truly enriched in 3-month-old 5XFAD mice, it would have been more appropriate to perform a direct marker gene analysis of these clusters rather than relying on WGCNA and module enrichment.

We thank the referee for raising this important point regarding our pathway analysis strategy. The use of KEGG and REACTOME was motivated by distinct analytical objectives at different stages of the study.

For the EREN clusters (formerly DIEN; clusters 5–9), we initially applied KEGG pathway enrichment to assess whether these clusters were associated with pathways broadly relevant to Alzheimer's disease, thereby providing a first-pass validation of their disease relevance. In contrast, for the turquoise gene module identified by WGCNA, our goal was to interrogate signaling pathways enriched within a tightly co-expressed gene network. In this context, REACTOME was better suited due to its more detailed curation of signaling pathways and finer functional resolution.

With respect to the referee's suggestion to perform direct marker gene analysis of clusters 5–9, we carefully considered this approach. However, given the large number of differentially expressed genes within these clusters, a direct enrichment analysis would likely yield a very broad and heterogeneous set of pathways, increasing the risk of spurious associations. We therefore adopted a module-based strategy, which allowed us to focus on a coherent gene network and to identify biologically interpretable signaling pathways with greater robustness.

Third, it is unclear whether Fig. 2d represents DEGs comparing DIEN clusters to other excitatory subclusters.

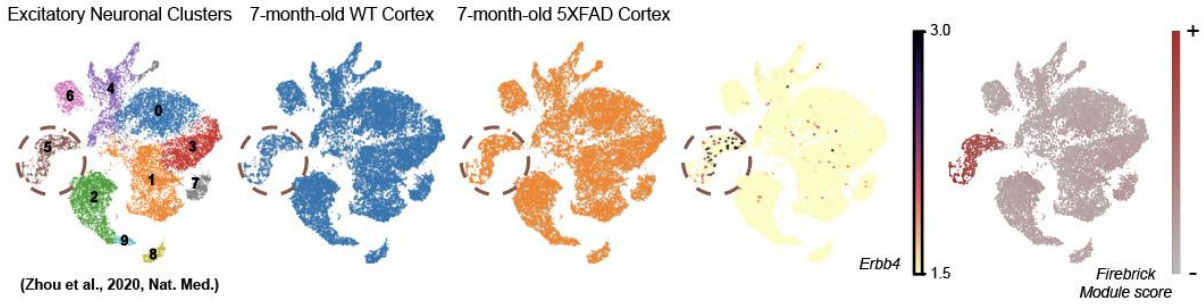
We thank the referee for pointing this out. The referee is correct that the DEGs shown in Fig. 2d represent a comparison between the EREN cluster and other excitatory subclusters. We have now clarified this explicitly in the figure legend of the revised manuscript to avoid any confusion.

In Extended Fig. 5e, proportions should be shown rather than absolute counts.

We thank the referee for this suggestion. In the revised figure, we now present the data as proportions for easier interpretation. Accordingly, we reanalyzed the data using generalized estimating equation, which is well suited for comparing proportions, and confirmed that the results remain consistent with our original conclusions.

Finally, it is unclear how authors concluded from 5e that only signaling by Erbb4 was enriched in DIEN.

We thank the referee for this helpful comment. As the referee notes, other signaling pathways were also detected in the enrichment analysis. However, when we narrowed down the candidates based on log fold change and *p*-value (top ~25% of genes), *Erbb4* was the only gene that remained in this range; other signaling-related genes, such as *Erbb2*, *Notch*, or *Nlr* family members, were not represented among the top candidates (Fig. 2e, f). Importantly, when we repeated the same analysis pipeline in 7-month-old 5XFAD data, now included in Extended Data, *Erbb4* again emerged as the consistent candidate (Extended Data Fig. 5e, i, j). For these reasons, we focused our attention on signaling by *Erbb4*. We have clarified this rationale more explicitly in the revised manuscript.



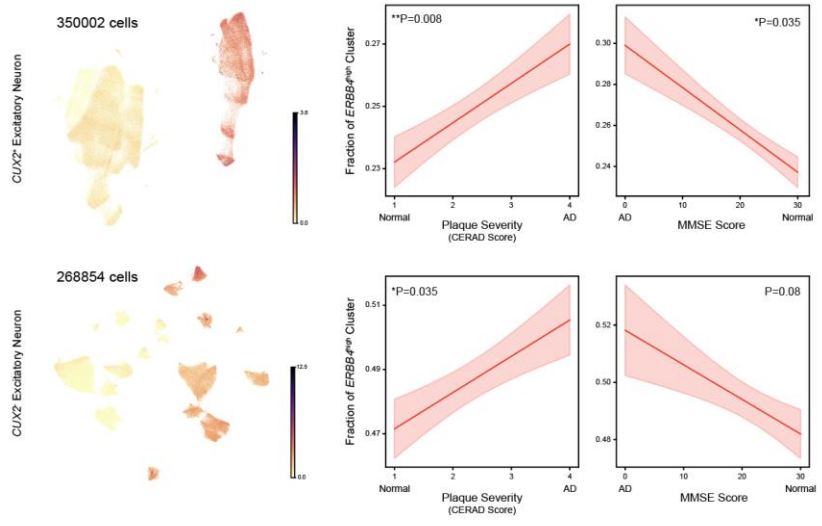
• Another concern is the limited analysis of human data. While snRNA-seq identified Erbb4-positive DIENs in mouse models, it is critical to demonstrate Erbb4 dysregulation directly in human AD tissue datasets. The authors show increased ERBB4 expression in excitatory neurons in Extended Fig. 10e

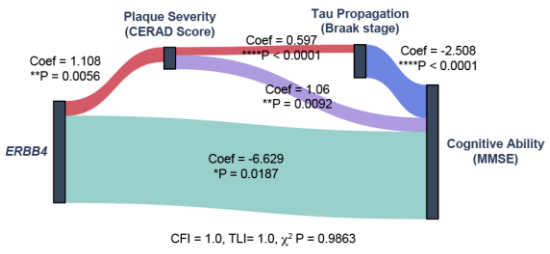
using a dataset from the middle temporal gyrus; however, it is unclear why this particular dataset was selected given the availability of over a dozen AD snRNA-seq datasets. Moreover, no quantitative data is presented on this dataset. Quick search of several AD datasets shows very low expression of ERBB4 in excitatory neurons relative to inhibitory neurons.

As described in our response to the earlier comment regarding the relevance of our findings to human AD, we have substantially expanded our analysis of human snRNAseq datasets in the revised manuscript to directly address *ERBB4* dysregulation in human disease.

Briefly, we now include a comprehensive analysis of the ROSMAP cohort (446 individuals), in which we identify an *ERBB4*^{high} excitatory neuronal cluster whose relative abundance is positively correlated with amyloid plaque severity (CERAD score) and negatively correlated with cognitive performance (MMSE score) (Extended Data Fig. 12d-g). In addition, causal modeling analyses support a model in which ERBB4 expression in excitatory neurons is associated with cognitive decline both directly and indirectly through amyloid plaques and tau propagation (Fig. 5u)

With respect to the referee's point regarding relative expression levels, we agree that *ERBB4* expression is highest in inhibitory neurons in both mouse and human datasets, while expression in excitatory neurons is comparatively lower. Importantly, however, our data indicate that the subset of excitatory neurons expressing *ERBB4* plays a particularly important role in AD pathogenesis, as evidenced by their strong correlations with pathology and cognition.





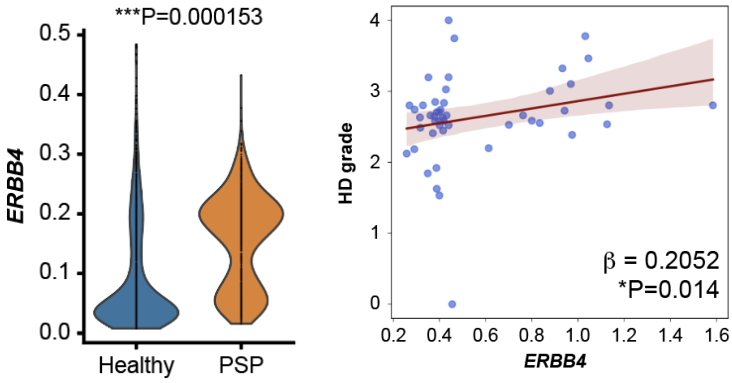
• The authors show that multiple neurodegenerative disease pathways (AD, ALS, Parkinson's, and Huntington's disease) are enriched in DIEN (Fig 2c). Similar results are presented on human data (Extended Data Fig. 10f, g). Authors show that injection of Aβ oligomer into the CA1 was sufficient to induce ERBB4 expression in excitatory neurons within 2 days (Extended Data Fig. 5h, i). What is the corresponding factor in these other neurodegenerative diseases?

We thank the referee for this insightful question. Our current working hypothesis is that aggregated protein-induced neuronal stress may act as a convergent upstream factor driving *Erbb4* upregulation across multiple neurodegenerative diseases. In Alzheimer's disease, we directly demonstrated that Aβ oligomers are sufficient to induce *Erbb4* expression in excitatory neurons. However, the corresponding ERBB4-inducing factors in other neurodegenerative conditions such as ALS, Parkinson's disease, and Huntington's disease remain to be fully defined.

To begin addressing this question, we analyzed additional human snRNAseq datasets from neurodegenerative diseases with distinct primary pathologies. Specifically, we examined data from patients with progressive supranuclear palsy (PSP), a tauopathy characterized predominantly by neurofibrillary tangles with minimal or no amyloid accumulation[1-3]. In this dataset, excitatory neurons from PSP patients exhibited significantly elevated ERBB4 expression compared with healthy controls (Extended Data Fig. 12h).

In addition, we analyzed snRNA-seq data from Huntington's disease (HD) patients, in which pathology is driven by CAG repeat expansion in the *HTT* gene rather than amyloid or tau aggregation[4]. In this dataset, *ERBB4* expression levels in excitatory neurons showed a significant linear correlation with HD disease grade (Extended Data Fig. 12i).

Together, these findings indicate that ERBB4 upregulation in excitatory neurons is not restricted to amyloid-driven pathology, but may represent a more general neuronal response to diverse neurodegenerative proteinopathies. We agree with the referee that these data do not yet fully elucidate the precise mechanisms by which ERBB4 expression is induced in each disease context. Identifying the disease-specific aggregated proteins or stress pathways that trigger ERBB4 dysregulation is an important and intriguing direction for future investigation, which we are actively pursuing.



[1]Cervos-Navarro, J., & Schumacher, K. (1994). Neurofibrillary pathology in progressive supranuclear palsy (PSP). *Progressive Supranuclear Palsy: Diagnosis, Pathology, and Therapy*, 153-164.
[2]Boxer, A. L., Yu, J. T., Golbe, L. I., Litvan, I., Lang, A. E., & Höglinger, G. U. (2017). Advances in progressive supranuclear palsy: new diagnostic criteria, biomarkers, and therapeutic approaches. *The Lancet Neurology*, *16*(7), 552-563.
[3]Josephs, K. A., Petersen, R. C., Knopman, D. S., Boeve, B. F., Whitwell, J. L., Duffy, J. R., ... & Dickson, D. W. (2006). Clinicopathologic analysis of frontotemporal and corticobasal degenerations and PSP. *Neurology*, *66*(1), 41-48.
[4]MacDonald, M. E., Ambrose, C. M., Duyao, M. P., Myers, R. H., Lin, C., Srinidhi, L., ... & Harper, P. S. (1993). A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell*, *72*(6), 971-983.

• Authors demonstrate that knockdown of the astrocyte-specific phagocytic receptor Megf10 in 5XFAD mice increased excitatory synapse numbers without affecting inhibitory synapses (130-137). How do the authors interpret the lack of effect on inhibitory synapses? How about the microglia phagocytic activity?

We thank the referee for this important question. As we previously reported in our *Nature* paper[1], astrocytic MEGF10 functions as a phagocytic receptor that is specific to excitatory synapses. In that study, we demonstrated that *Megf10* deletion selectively increased excitatory synapse numbers without affecting inhibitory synapses, and that *Megf10* deletion did not alter microglial phagocytic activity. Consistent with those findings, in the present work we again observed that *Megf10* knockdown in 5XFAD mice led to changes in excitatory but not inhibitory synapses.

[1]Lee, J. H., Kim, J. Y., Noh, S., Lee, H., Lee, S. Y., Mun, J. Y., ... & Chung, W. S. (2021). Astrocytes phagocytose adult hippocampal synapses for circuit homeostasis. *Nature*, *590*(7847), 612-617.

• The Erbb4 deletion experiments convincingly demonstrate that excitatory neuron-intrinsic Erbb4 upregulation initiates synapse loss and glial activation, suggesting that glial hyper-phagocytic activity is not the primary driver of early synapse elimination. However, even with Erbb4 deletion, it remains possible that neuron-glia interactions contribute to the differential elimination of excitatory versus

inhibitory synapses. Also, in lines 112-114, the authors probably mean that neuroinflammation and hyper-phagocytic activity of glial cells may not be the primary drivers of synapse loss.

We appreciate the referee's positive assessment of our *Erbb4* deletion experiments. We essentially agree with the referee's interpretation. Our intention was indeed to suggest that while glial neuroinflammation contributes to the process, it may not be the primary initiator of the specific synapse elimination patterns we observed.

As suggested, we have revised the text to clarify that neuron-glia interactions likely still play a role, but clearly state that neuroinflammation is likely not the sole driver.

The revised sentence reads:

"Furthermore, the selective elimination of excitatory synapses, coupled with the preservation of inhibitory ones, raises the possibility that intrinsic neuronal states guide glial phagocytic behavior, and that indiscriminate neuroinflammation is unlikely to be the sole driver of synapse elimination in AD."

Dear Referees,

We sincerely appreciate the opportunity to respond to the referees' comments on our manuscript, "Aberrant ERBB4 Expression in Excitatory Neurons Promotes Early Pathophysiology of Alzheimer's Disease." We are encouraged by the referees' positive evaluations and their recognition of the potential impact and significance of our study.

In response to the remaining comments, we have carefully considered the referees' suggestions and incorporated the following additional revisions into the manuscript:

• Addressing Referees #1 and #3:

We have implemented all minor suggestions to further improve clarity and presentation.

• Addressing Referee #2:

1. Revision of the title and manuscript flow: We have revised the title and main text flow to more precisely reflect our findings.
2. Tempering of the human data interpretation: We have revised the text to frame the human data as clinically relevant associations, rather than implying direct causality.
3. Addition of electrophysiology data: We have performed mEPSC and mIPSC recordings from CA1 pyramidal neurons in WT mice, WT mice with *Erbb4* overexpression, 5XFAD mice, and 5XFAD mice with *Erbb4* deletion.
4. Further gene expression comparison between the ERBB4 and mTOR axis: We have expanded the gene expression analysis to further compare ERBB4-associated transcriptional changes with the mTOR axis.
5. *ERBB4* mRNA detection in human AD samples: We have added analysis of *ERBB4* mRNA expression in human AD samples.

Below, we provide our point-by-point responses to the referees' comments. We hope that the revised manuscript will be deemed acceptable for publication by the referees and editors.

Referee #1 (Remarks to the Author):

The authors have done a careful and compelling revision, resulting in an interesting paper that will likely have strong impact on the Alzheimer's and neurodegenerative disease field.

I only have one minor comment:

Figure 2f: seems to show enrichment of other pathways (e.g. Notch, Erbb2 signaling), apparently contradicting that "only the ERBB4 signaling pathway was enriched in ERENs" (line 172 of manuscript). This should be corrected, or the analysis needs to be made more clear, if I misunderstood the figure.

We apologize for the ambiguous description. To clarify, our analysis involved two complementary approaches: (1) identification of the top 25% of differentially expressed genes (DEGs) in ERENs, and (2) gene set enrichment analysis (GSEA), which revealed enrichment of multiple pathways including ERBB4, ERBB2, and Notch signaling. Critically, when we cross-referenced the top 25% DEGs with these GSEA-enriched pathways, *Erbb4* was the only gene present among the top 25% DEGs, while genes associated with ERBB2 or Notch signaling were absent from this high-confidence gene set. We therefore focused on ERBB4 signaling as the most robustly supported pathway in ERENs. We have revised the manuscript to clarify this point.

Referee #2 (Remarks to the Author):

The authors have made improvements relative to the previous submission; however, two substantive concerns remain that require additional work. Of these, the absence of electrophysiological data addressing Comment 1 is particularly concerning and, in its current form, substantially weakens the manuscript.

The authors state that EPSCs and IPSCs were not assessed because results from prior work were variable. This justification is not sufficient. A central premise of the study, demonstrated from the outset in Figure 1, is that excitatory and inhibitory synapses are differentially regulated by glia and by the genetic manipulations of ERBB4 employed throughout the manuscript. Because synaptic changes are the primary readout used to support the proposed mechanism, direct measurements of EPSCs and IPSCs from the relevant neuronal populations are essential. Without these data, the mechanistic framework of

the study is incomplete.

Instead, the authors pivot to measurements of intrinsic excitability. This shift is not logically connected to the earlier findings based on synaptic staining and does not address the core question of how excitatory and inhibitory synaptic transmission is altered. As a result, the electrophysiological results do not adequately support the conclusions drawn from the anatomical data. This disconnect represents a major flaw in the study and must be resolved.

Moreover, it is difficult to reconcile the claim that EPSCs and IPSCs were not assessed with the fact that intrinsic excitability was recorded (e.g., Extended Data Fig. 7n–p). These recordings necessarily involve access to synaptic events, including EPSPs and IPSPs. The absence of these data in the manuscript creates the impression that the central synaptic mechanism proposed may not be supported by the experimental results. Publishing the study without these measurements would likely cause confusion among readers and undermine confidence in the conclusions.

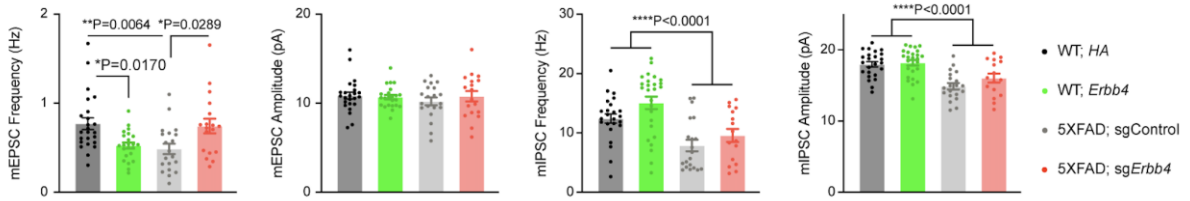
We thank the referee for emphasizing this important point. In our previous response, we focused on intrinsic excitability because conventional EPSC/IPSC measurements in AD models have been reported to vary depending on age, model, brain region, and recording conditions. We therefore considered intrinsic excitability to be a more direct readout of the neuronal hyperactivity observed in our c-Fos analysis. However, we agree that direct assessment of EPSCs and IPSCs provides important information for determining how the structural synaptic changes we observed affects synaptic transmission within the circuit.

To address this concern, we performed additional whole-cell-clamp recordings of mEPSCs and mIPSCs from CA1 pyramidal neurons (Extended Data Fig. 7n–q). We designed these experiments to focus on the early disease/manipulation window, at 4 months of age in 5XFAD mice, when ERBB4-dependent structural synapse alterations first become evident. Under these conditions, we observed a consistent reduction in mEPSC frequency in 5XFAD mice, which was strongly rescued by excitatory neuron-specific *Erbb4* deletion (Extended Data Fig. 7n). Conversely, *Erbb4* overexpression in WT excitatory neurons reduced mEPSC frequency to levels comparable to those observed in 5XFAD mice (Extended Data Fig. 7n), whereas mEPSC amplitude remained unchanged (Extended Data Fig. 7o). These findings provide direct functional support for a role of ERBB4 in regulating excitatory synaptic input number.

Although inhibitory pre-synaptic density was increased in the SLM of 5XFAD mice (Fig. 1i), the frequency and amplitude of mIPSCs recorded from 5XFAD CA1 pyramidal neurons were reduced compared with WT controls (Extended Data Fig. 7p). In addition, *Erbb4* modulation in 5XFAD mice

did not produce statistically significant changes in either mIPSC frequency or amplitude (Extended Data Fig. 7p, q). Notably, however, *Erbb*4 overexpression in WT excitatory neurons showed a trend toward increased mIPSC frequency, consistent with the increased inhibitory synapse number observed under this condition (P=0.0504, Unpaired Student's *t*-test). Thus, the absence of a comparable increase in mIPSC frequency in 5XFAD mice, despite increased inhibitory presynaptic density, suggests that structural inhibitory contacts in the 5XFAD circuit may not necessarily translate into increased functional inhibition. One possibility is that the structurally increased inhibitory contacts observed in 5XFAD mice may be immature, silent, or otherwise functionally ineffective. Thus, unlike the excitatory synaptic phenotype, changes in inhibitory synaptic transmission may reflect broader AD-associated circuit-level remodeling or compensatory mechanisms that influence the functional state of inhibitory synapses. Future studies examining interneuron subtype- and dendritic compartment-specific inhibition across disease stages will be important for understanding how inhibitory circuit remodeling is coordinated with excitatory neuronal dysfunction.

Although our mEPSC recordings validate ERBB4-associated changes in excitatory synaptic input, we interpret these changes as a functional consequence of ERBB4-dependent synapse remodeling rather than as the primary initiating event. Our data support a model in which aberrant ERBB4 expression first increases the intrinsic excitability of 5XFAD excitatory neurons, thereby initiating abnormal synapse elimination and leading to subsequent changes in synapse number and corresponding mEPSC frequency. Consistent with this model, we show that chemogenetic induction of neuronal hyperactivity through Gq signaling in WT neurons is sufficient to induce synapse elimination, whereas suppression of neuronal activity through Gi signaling in WT and 5XFAD neurons reduces synapse elimination (Extended Data Fig. 3, 7a-i).

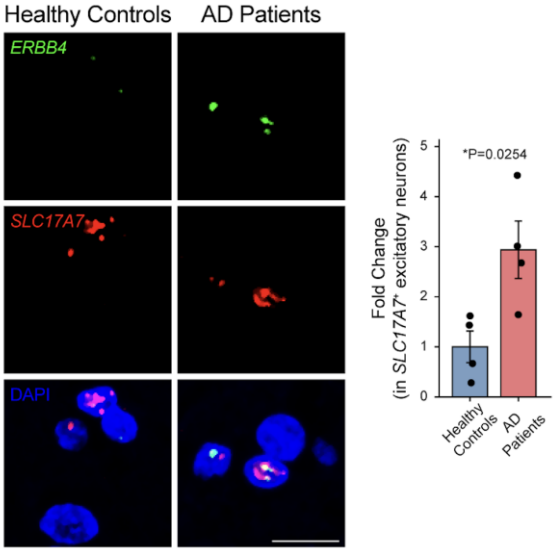


A second issue concerns the interpretation of the analysis presented in Figure 5u. The authors use human postmortem data to claim a causal mechanism, which is not justified. This section should be rewritten to clarify that the human data support the relevance of the mouse-identified mechanism in human tissue, without implying causality. All claims of a causal role in humans should be removed. To strengthen relevance to human AD, expression of ERBB4 protein in postmortem AD tissue for the subpopulation of excitatory neurons is needed to accompany Fig 5u.

We agree that human postmortem datasets are observational in nature and therefore may not, by themselves, establish a causal role for *ERBB4* in human AD. Our intention was to use mediation and structural equation modeling to test whether the relationships among excitatory neuronal *ERBB4* expression, amyloid pathology, tau pathology, and cognitive decline were statistically consistent with the mechanism identified in our mouse experiments. However, we recognize that the previous wording may have implied a stronger causal conclusion than is warranted from human postmortem data.

Accordingly, we have substantially toned down this section throughout the revised manuscript. We have removed statements claiming a causal role for *ERBB4* in human AD and now describe the human analyses as supporting the relevance and consistency of the mouse-identified ERBB4-associated mechanism in human tissue.

To further strengthen the human relevance of our findings, we performed RNAscope fluorescence *in situ* hybridization on human AD tissue and found increased *ERBB4* signal in *SLC17A7*$^{+}$ excitatory neurons compared with healthy control tissue (Extended Data Fig. 12e, f). These new histological data provide cell type-specific evidence that *ERBB4* expression is elevated in excitatory neurons in human AD tissue, supporting the relevance of the *Erbb4*-expressing excitatory neuronal state identified in our mouse models.



Beyond these major points, there are multiple areas where the text requires clarification and moderation of claims. For example, the manuscript title is syntactically incorrect and implies that ERBB4 itself is excitatory, rather than being expressed in excitatory neurons. Similar imprecisions occur throughout

the text and contribute to confusion and overstatement.

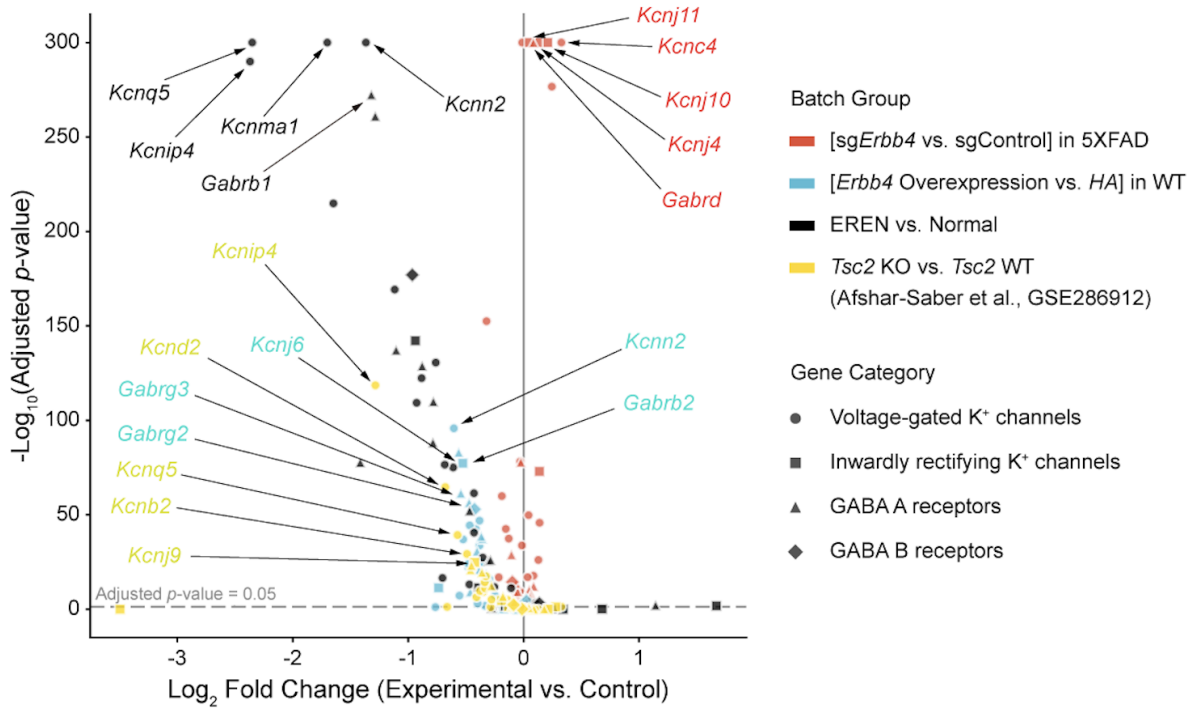
To avoid this ambiguity, we have revised the manuscript title to "Aberrant ERBB4 Expression in Excitatory Neurons Promotes Early Pathophysiology of Alzheimer's Disease." We have also carefully reviewed the manuscript and moderated similar wording throughout the text to more precisely distinguish *ERBB4* expression in excitatory neurons from properties of *ERBB4* itself.

Finally, while the proposed link between ERBB4 and mTOR signaling is acknowledged by the authors to be previously described, the principal novelty of the study appears to be the identification of ERBB4-expressing excitatory neurons. However, key mechanistic links explaining how these neurons drive the reported synaptic and behavioral effects are missing. At a minimum, electrophysiological evidence demonstrating the predicted changes in excitatory and inhibitory synaptic transmission is required.

In summary, without direct measurements of EPSCs and IPSCs and a more restrained interpretation of the human data, the manuscript does not yet meet the standard expected for publication in Nature and would be more appropriate for a specialized journal in its current form.

To address the reviewer's concern, we have expanded the downstream mechanistic analysis of the ERBB4-mTOR axis. ERENs and excitatory neurons from *Erbb4*-overexpressing WT mice showed transcriptional changes that resembled those seen in *Tsc2*-deficient neurons (Extended Data Fig. 12a). Notably, these overlapping changes involved genes related to neuronal excitability, including those encoding voltage-gated K^+ channels, inwardly rectifying K^+ channels, GABAA receptors, and GABAB receptors. In contrast, excitatory neurons from *Erbb4*-deficient 5XFAD mice showed opposing changes in these gene sets (Extended Data Fig. 12a). Given that these ion channels and inhibitory receptors are critical for regulating neuronal excitability, our findings suggest that aberrant ERBB4-mTOR signaling may promote excitatory neuronal hyperactivity, at least in part, by disrupting transcriptional programs that normally constrain neuronal excitability.

Identifying the precise molecular links between ERBB4/mTOR-dependent transcriptional changes and specific electrophysiological phenotypes remains an important next step. However, defining which individual ion channels or receptor subunits causally mediate these functional changes would require systematic molecular perturbation and rescue experiments, which we believe are beyond the scope of the present study.



Referee #3 (Remarks to the Author):

The authors have substantially improved the manuscript through the inclusion of additional data and analyses, including electrophysiology, behavioral experiments, and ROSMAP snRNA-seq analyses. These additions significantly strengthen the study.

Several clarifications and revisions are needed to improve accuracy and interpretability of the 5XFAD mouse snRNA-Seq analyses:

• The statement "Among these, only the turquoise module was strongly correlated with ERENs and significantly upregulated" should be revised. The results indicate that genes in the turquoise module are enriched for EREN marker genes, rather than the turquoise module itself being upregulated.

We agree that our original phrasing was imprecise, as it implied that the turquoise module itself was upregulated. We have revised the sentence to state that among the WGCNA modules, only the turquoise module exhibited a significantly elevated module score in ERENs compared to other excitatory neuron subclusters. We believe this more accurately reflects our analytical approach, in which module-level scores, rather than individual gene expression, were used to assess the association between co-expression modules and the EREN population.

• The sentence "For further analysis, we selected the top 25% of turquoise module genes based on p-

value and expression level" should be revised to clarify that the authors selected the top 25% of EREN marker genes that are members of the turquoise module, based on differential expression p-values comparing ERENs with other excitatory neuron subclusters.

We thank the referee for pointing out this ambiguity. We have revised the sentence to clarify this selection criterion.

The revised manuscript now reads:

"For further analysis, among the turquoise module genes that were differentially expressed between EREN clusters and other excitatory neuronal clusters, we selected the top 25% based on adjusted p-value and expression level."

• The legend for Figure 2d should explicitly state that the volcano plot displays differentially expressed genes between the EREN cluster and other excitatory neuron subclusters.

We thank the reviewer for this suggestion. We have revised the Figure 2d legend to explicitly state that the volcano plot displays differentially expressed genes between the EREN cluster and other excitatory neuronal clusters.

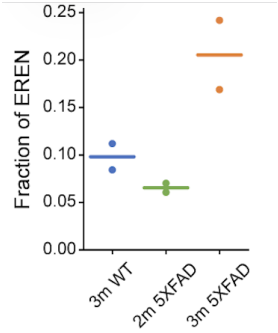
• The statement "GSEA with Reactome Pathway terms indicated that among the top 25% of turquoise module genes, only the ERBB4 signaling pathway was enriched in ERENs" remains unclear. The authors should clarify that among all enriched pathways, ERBB4 is the only one within the top 25% of turquoise module genes.

We apologize for the ambiguous description. We have revised the manuscript to clarify this point.

• Extended Data Fig. 5a (snRNA-seq analysis): Bar graphs showing the fraction of the EREN population are based on only two samples per group. Error bars should be removed, and individual data points should be shown, with the mean optionally overlaid.

We agree with the referee's point. As suggested, we have replaced the bar graph in Extended Data Fig. 5a with dot plot to better represent the small sample size. The revised plot shows individual data points

with an overlaid mean line.



• The legends for Extended Data Fig. 5g, 5h, 5i, and 5j are switched. In addition, Extended Data Fig. 5i and 5j are cited in the text after Extended Data Fig. 5k and should be reordered.

We appreciate your keen observation. As suggested, we have corrected the legends and reordered the figures to ensure the layout aligns with the citation order in the text.

ROSMAP snRNA-seq analysis:

• The authors may consider presenting a larger portion of the ROSMAP snRNA-seq results in the main figures rather than limiting them to the Extended Data.

We sincerely appreciate referee for acknowledging the significance of our ROSMAP data. We will discuss the feasibility of this reorganization with the editor and proceed accordingly.

• Authors should include a citation to the study that generated the snRNASeq dataset from 446 donors of the ROSMAP study.

We have added the appropriate reference for the ROSMAP snRNAseq study.

[Mathys, H. *et al.* Single-cell atlas reveals correlates of high cognitive function, dementia, and resilience to Alzheimer's disease pathology. *Cell* **186**, 4365-4385.e27 (2023).]

• Extended Data Fig. 12f and 12g: Individual donor level data should also be presented.

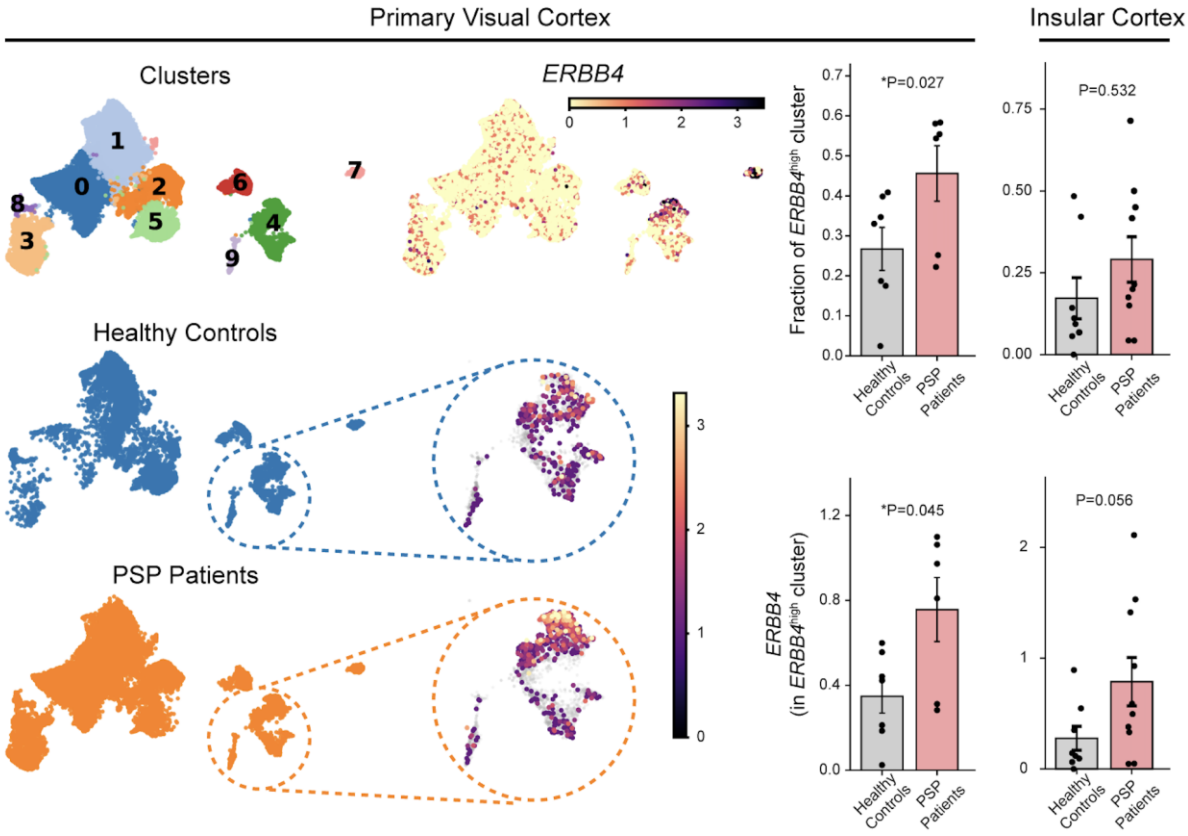
As referee suggested, we revised Extended Data Fig. 12f and 12g to include individual donor level data and now moved to Extended Data Fig. 12i and 12j.

• Extended Data Fig. 12h: Violin plots showing ERBB4 expression in healthy controls and PSP patients (n = 3 per group) require revision. A Wilcoxon rank-sum test is not appropriate, as cells from the same donor cannot be treated as independent observations. Donor-level aggregation or an appropriate mixed-effects model should be used instead.

We thank the reviewer for pointing out this important statistical issue. We agree that treating individual nuclei as independent observations is not appropriate for comparing *ERBB4* expression between diagnostic groups. Accordingly, we no longer use a cell-level Wilcoxon rank-sum test for this analysis.

In response to this concern, we first reanalyzed the original PSP dataset using donor-level pseudobulk aggregation. Although this analysis showed a similar trend, the difference did not reach statistical significance, likely due to the small number of donors in the original dataset. We therefore searched for a larger publicly available PSP snRNAseq dataset[1] and repeated the analysis using donor-aware quantification.

In this larger dataset, we identified an EREN-like *ERBB4*^{high} excitatory neuronal cluster in the primary visual cortex, similar to the EREN-like population observed in our mouse AD models. Both *ERBB4* expression within this cluster and the fraction of excitatory neurons assigned to this cluster were increased in PSP patients compared with healthy controls (Extended Data Fig. 12k, l). We also detected an *ERBB4*^{high} excitatory neuronal cluster in the insular cortex; however, although *ERBB4* expression showed an increasing trend in PSP patients, the fraction of this cluster was comparable between PSP patients and controls (Extended Data Fig. 12m). We therefore interpret these findings cautiously and now discuss the possibility that *ERBB4*-associated excitatory neuronal states may vary across brain regions.



[1]Rexach, J. E. *et al.* Cross-disorder and disease-specific pathways in dementia revealed by single-cell genomics. *Cell* **187**, 5753-5774.e28 (2024).

Referees' comments:

Referee #1 (Remarks to the Author):

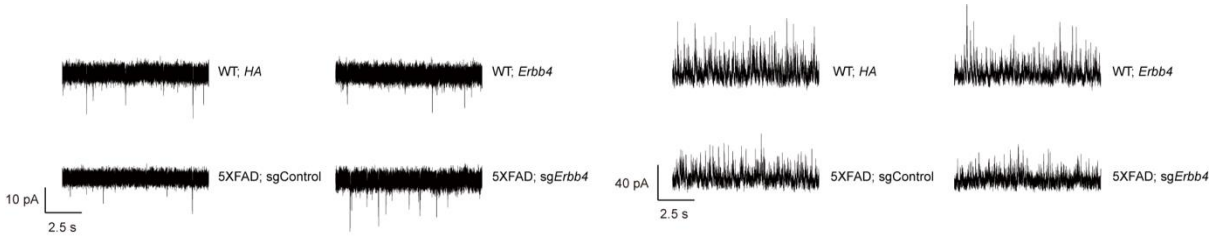
I had only minor comments after the first revision. Those have been satisfied by the second revision. Overall I believe the second revision is even further improved, and it addresses reasonably the biggest remaining concerns of the reviewers (around electrophysiology).

We thank the referee for the positive assessment and for the constructive comments provided throughout the review process.

Referee #2 (Remarks to the Author):

The authors have done a good job of addressing my comments. The inclusion of the new electrophysiology makes the paper stronger. My only minor comment that does not require re-review but should be accommodated is to add some representative traces of synaptic events to ED Fig 7n-F so that future readers can assess the quality of the raw data, especially for iPSCs which were measured at 0 mV.

We thank the referee for this helpful suggestion. We have added representative traces of mEPSCs and mIPSCs to the revised Extended Data Fig. 7n-s. The corresponding scale bars and recording conditions, including the holding potentials used for mEPSC and mIPSC measurements, are now specified in the Materials and Methods.



Referee #3 (Remarks to the Author):

The authors have fully addressed all comments. Two minor issues:

• The sentence "Among these, only the turquoise module score was strongly correlated with ERENs and significantly upregulated" should be revised to: "Amond these, only the turquoise module exhibited a significantly elevated module score in ERENs compared to other excitatory neuron subclusters" as stated in the rebuttal.

We thank the referee for the constructive comments provided throughout the review process. We revised the sentence as follows: "Among these, ERENs exhibited a significantly elevated module score only for the turquoise module"

• The legend for Fig 5 is very confusing, as it is not in order.

We apologize for the confusing organization. We removed redundant descriptions and clarified the flow for better readability.

Referee #4 (Remarks to the Author):

The manuscript proposes a novel and interesting conceptual framework of synaptic remodeling by glial cells induced by a previously unrecognized population of Early Responsive Excitatory Neurons (ERENs) with ectopic expression of ERBB4 in AD. The aberrant expression of ERBB4 in excitatory cells drives a complex cascade leading to circuit dysfunction, which includes neuronal hyperexcitability and reduced mEPSCs/synapses, hypoactivity of inhibitory cells, and activity-driven synaptic engulfment of excitatory and inhibitory synapses by glial cells. Thus, instead of the canonical pathway of reactive glia-mediated early synapse loss, the authors propose that ectopic ERBB4 expression directly mediates changes in neuronal activity, which drive differential activity-dependent synaptic remodeling by glial cells of excitatory (increased engulfment) and inhibitory synapses (decreased engulfment), resulting in synaptic imbalance. Importantly, the proposed mechanism also affects downstream pathology and ultimately cognitive impairment.

The authors provide strong evidence to experimentally support this working model in two AD mouse models and in humans with AD, including longitudinal analyses of two AD mouse models, single-nucleus transcriptomics in humans and mice showing ectopic Erbb4 expression in excitatory cells, rescue experiments with Erbb4 knockdown in AD mice, gain-of-function experiments with Erbb4 in WT mice to recapitulate AD-like features, cell type- and activity-dependent modulation of synaptic engulfment, pathology, electrophysiology, and behavioral testing. Altogether, the study supports the notion that aberrant ERBB4 expression in excitatory neurons is both necessary and sufficient to

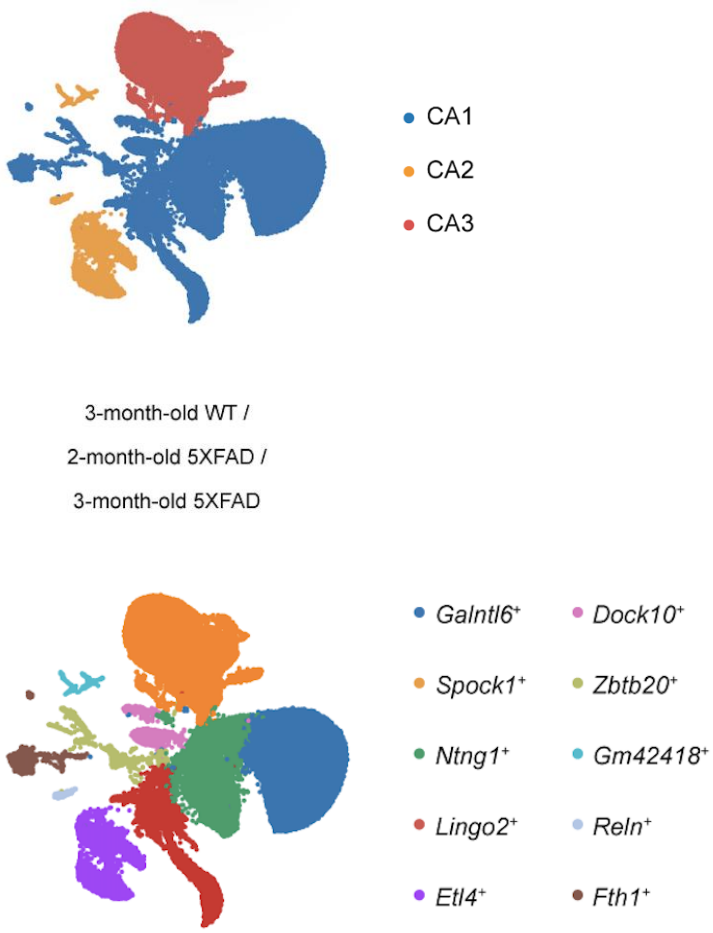
reproduce many hallmark features of AD, including neuronal hyperexcitability and glia-mediated synaptic remodeling. Mechanistically, the work also identifies the ERBB4–mTOR signaling pathway as one potential molecular mediator. Although additional work will ultimately be needed to determine how broadly this mechanism applies to human disease, the evidence presented is compelling and should stimulate further investigation.

The inclusion of the electrophysiology substantially improves the manuscript. Previously, the work relied heavily on c-Fos as a surrogate for neuronal activity and synaptic morphology as a surrogate for synaptic function. The new recordings resolve this major gap in the functional interpretation of the structural data. The new electrophysiology demonstrates that ectopic ERBB4 expression in WT excitatory neurons increases hyperexcitability and reduces the input of functional excitatory synapses (mEPSC frequency), and that deleting ERBB4 in 5XFAD mice rescues these synaptic and excitability alterations. The decrease in mEPSC frequency and hyperexcitability aligns well with the reduction in excitatory synapse number measured anatomically and with the c-Fos data, providing a much stronger mechanistic foundation for the proposed working model. However, the mIPSC frequency data is difficult to reconcile with the structural data (see below).

We thank the referee for the thoughtful assessment of our study and for recognizing that the new electrophysiological experiments substantially strengthened the functional basis of our model.

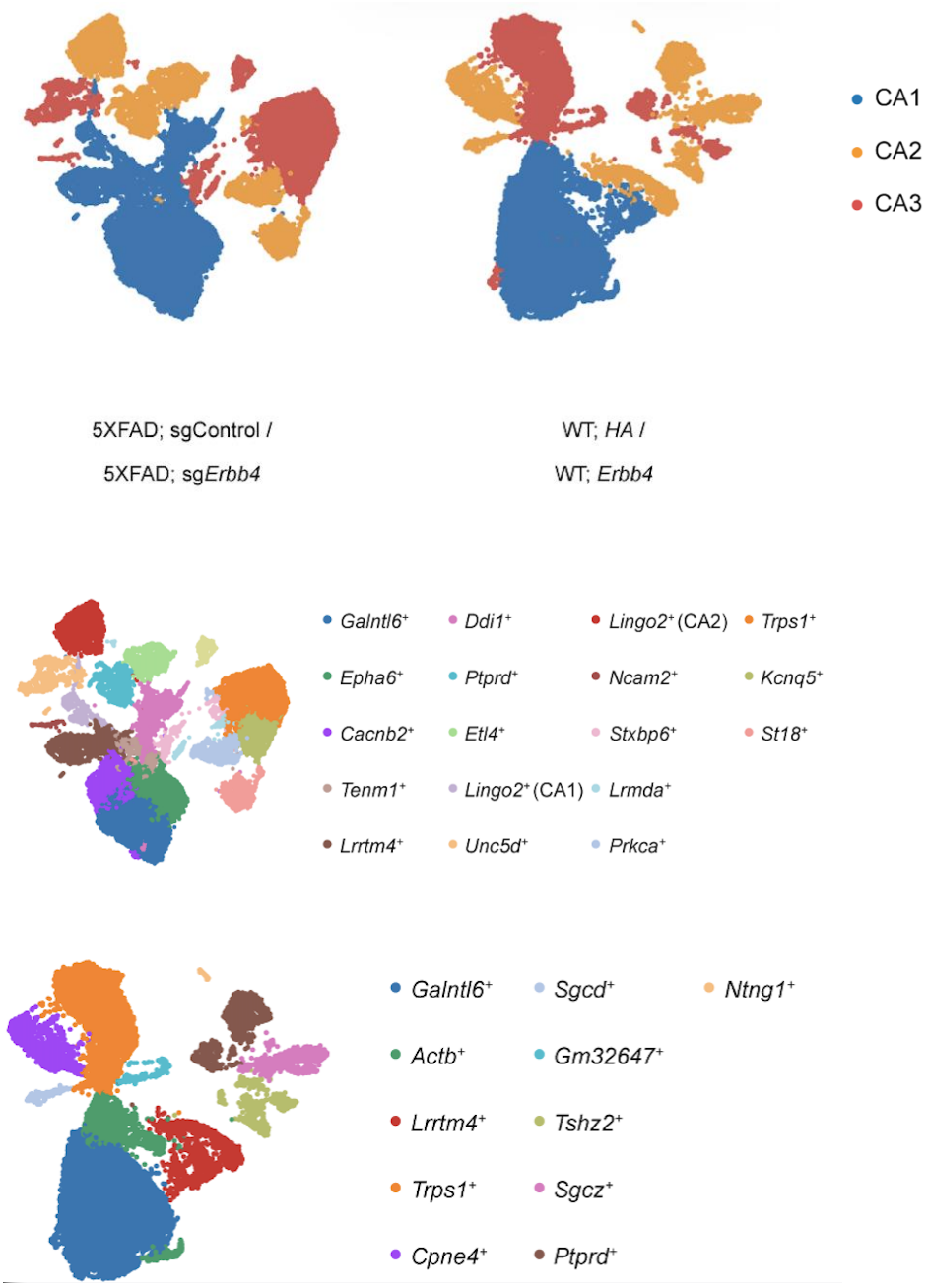
Major: The revised manuscript has a couple of major caveats:
1) The clusters of excitatory cells expressing ectopic ERBB4 are very poorly defined (Figure 2; UMAP clusters 5, 6, 7, 8, and 9). All UMAP clusters (0–9) need to be classified and properly labeled. Ideally, the origin of these aberrant cell states should be identified (DG, CA1, CA3, CA2, ProS, SUB, POST, etc.). The identity of these cells needs to be better described.

We thank the referee for this important suggestion. To address this point, we added a new Supplementary Fig. 1a, b providing marker-gene-based annotations for all excitatory neuronal subclusters (clusters 0-9) and their inferred hippocampal subregional identities. This analysis provides inferred subregional identities and representative markers of the EREN clusters (clusters 5-9), thereby providing a clearer description of their molecular and anatomical identities.



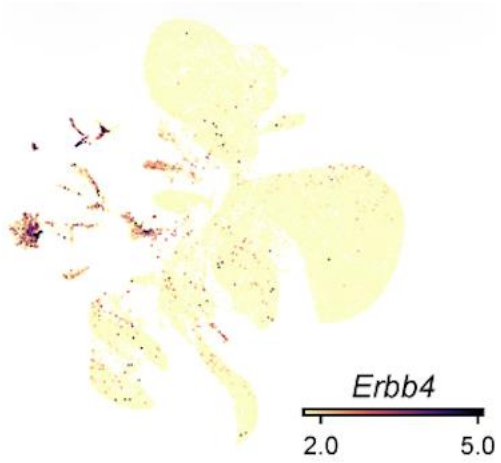
2) The UMAPs in Figure 5 have the same deficiencies. There is no labeling or identification of the clusters of excitatory cells expressing ectopic ERBB4. All UMAP clusters need to be classified and properly labeled.

Similarly, we provided marker-gene-based annotations and inferred regional identities for all excitatory neuronal subclusters in the sgControl- and sg*Erbb4*-injected 5XFAD and *HA*- and *Erbb4*-injected WT datasets (Supplementary Fig. 1a, b).



3) For Figure 2, UMAP plots showing Erbb4 expression is needed.

We added a UMAP feature plot showing *Erbb4* expression in Supplementary Fig. 1c.



4) The mIPSC frequency data strongly suggest that the reduced mIPSC frequency in 5xFAD is independent of the ectopic Erbb4 expression in excitatory cells, as this functional feature is not rescued by the knockdown of Erbb4 in the excitatory cells. Similarly, the expected increase in mIPSC frequency following Erbb4 expression in WT was only a non-significant trend. These results question the proposed model regarding effects on inhibitory cells, as the reduction of functional inhibitory synapses in 5xFAD mice is independent of Erbb4 expression. The model needs to be refined, as it contains strong experimental functional evidence for excitatory, but not inhibitory, synapses.

We agree that mIPSC frequency and amplitude in 5XFAD excitatory neurons were not significantly changed by excitatory neuron-specific *Erbb4* deletion in our recording conditions. However, we think that this does not necessarily mean that inhibitory circuit alterations are independent of excitatory ERBB4, due to the following reasons:

First, mIPSC frequency reflects both functional synapse number and presynaptic release probability, and does not always correlate directly with anatomical density. Indeed, 5XFAD mice showed increased inhibitory presynaptic density despite reduced somatostatin (SST) activity, whereas *Erbb4* deletion pruned these excess synapses and restored SST activity. This dissociation indicates that structural synapse abundance and functional interneuron activity can diverge. Similarly, mIPSC amplitude can be obscured by homeostatic changes in postsynaptic receptor composition and dendritic filtering.

Second, somatic whole-cell recordings suffer from spatial bias, preferentially capturing proximal perisomatic inputs (primarily from parvalbumin [PV] interneurons) while underrepresenting distal dendritic inputs, such as those from SST interneurons in the stratum lacunosum-moleculare (SLM). Because of dendritic filtering, compartment-specific rescue of distal SST inputs that we observed could

have been masked at the soma by the persistent dysfunction of PV populations in 5XFAD mice.

Third, recordings were performed only three weeks after *Erbb4* manipulation in 4-month-old mice. While we targeted this specific window to minimize downstream compensatory mechanisms within the excitatory neurons themselves, the recovery of the wider inhibitory network relies on indirect, non-cell-autonomous plasticity. Although three weeks may suffice to rescue cell-autonomous excitatory excitability, downstream structural and functional remodeling of inhibitory circuits likely operates on a slower temporal scale, which warrants longitudinal analysis at later time points.

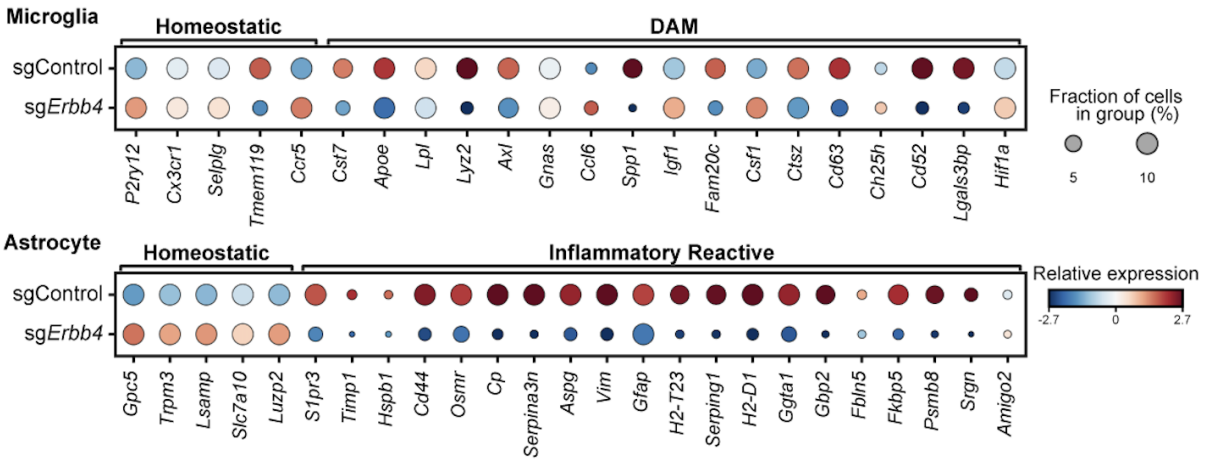
Minor:

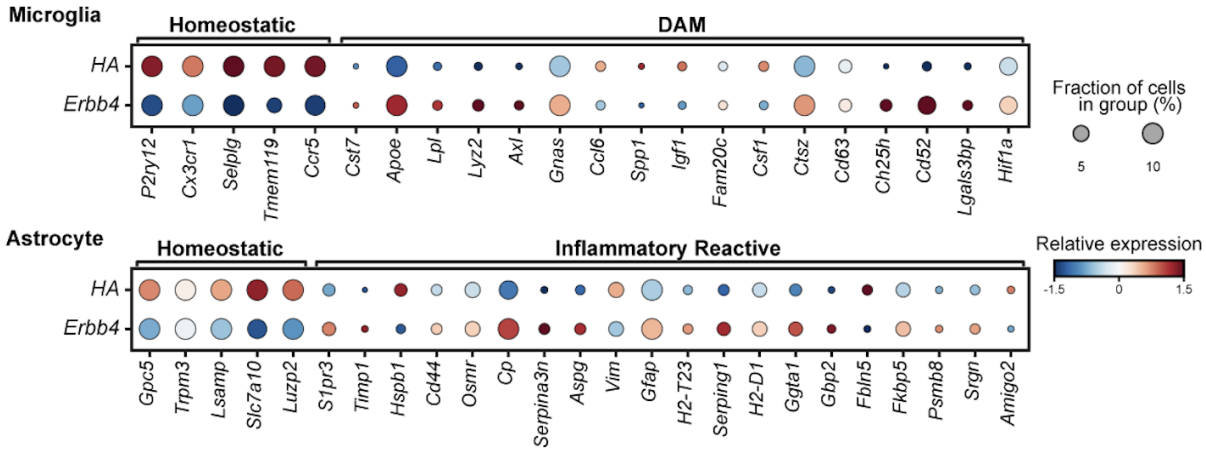
5) The authors equate the mouse models with human AD patients, including expressions referring to the mice. Line 26, 58: "AD progression in mice"; Line 60: "initial stages of AD"; Line 80: "AD glia"; Line 117: "AD brains". The authors should be more careful, as these mouse models do not develop AD.

We have revised the terminology throughout the manuscript to clearly distinguish findings in 5XFAD mice from those in human AD, avoiding language that equates the mouse model with the human disease.

6) Expression levels in Figure 5f and 5n are binary (0 or 1); they do not capture the dynamic range of the continuous scale (0–1).

We revised the dot plots in Fig. 5f, n to display marker-gene expression levels using a continuous color scale, rather than binary values, thereby better representing the dynamic range of gene expression across cell populations.





7) The mIPSC frequency data are somewhat difficult to reconcile with the rest morphology data. The authors acknowledge that increased inhibitory synapse number does not translate into increased functional inhibition and propose several reasonable explanations (immature or silent synapses). More caution regarding the interpretation of the effects on inhibitory function is needed, as there is no clear experimental support.

We fully agree that caution is necessary. As noted in the manuscript, in addition to the immature synapse hypothesis, we discussed that broader AD-associated dysfunction of PV or other interneuron populations could suppress inhibitory transmission and mask the functional readouts.

Referee #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.