nature portfolio

Peer Review File

Human brain organoids record the passage of time over multiple years

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

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
Summary of key results
This work shows that human brain organoids can be cultured in vitro for extended periods, continuing to mature in ways that mimic natural human brain development. These long-term organoids undergo ongoing transcriptional and epigenetic aging. Notably, older neural progenitors retain their developmental history, allowing them to produce late-born neurons without reiterating the early differentiation stages. This underscores the potential of brain organoids to model extended phases of human brain maturation, providing a valuable platform for studying postnatal development and aging processes in vitro. The effort by Dr. Faravelli and colleagues is commendable, particularly in their careful and transparent reporting throughout the manuscript

Originality and significance
This work marks a significant improvement over earlier organoid research by showing that brain organoids can be maintained over extended periods while continuously maturing. Unlike previous studies, such as Hergenreder et al. (Nat Biotechnol, 2024), which relied on pharmacological cocktails to accelerate neuronal development in a significantly shorter timeframe, this study explores whether organoids can naturally maintain pace with time and preserve developmental memory. If fully supported, this method provides a novel and valuable experimental platform for investigating human neurodevelopment.

Data and methodology
The study employs a comprehensive combination of methods, such as single-cell transcriptomics, DNA methylation analysis, electrophysiology, ultrastructural imaging, and chimeric organoid experiments. This multimodal approach is a significant advantage, offering a broad perspective on maturation over time. The authors present the datasets and analytical methods accurately and transparently, demonstrating a commitment to reproducibility and scientific integrity.
One aspect that could be improved is addressing inter-organoid variability, particularly over long culture periods and among different donor lines. Although the authors mention that variability stays within the limits of variation found in endogenous tissue, providing a detailed explanation of how batch effects and donor differences were measured and controlled would be beneficial. Considering the extended duration and natural heterogeneity of organoid systems, including more precise variability metrics at various time points would enhance the robustness of the results.

Appropriate use of statistics
Statistical methods are properly selected. Including biological replicates and donor variation in the models is a strength. However, the reporting of uncertainty could be clearer. Confidence intervals, effect sizes, and measures of variability are sometimes inconsistently reported, making it hard to evaluate the robustness of some results, especially over long culture periods. Sample sizes are reasonable given the technical challenges of long-term culture, but they are relatively small in key experiments like electrophysiology, EM, and chimeric organoids. Providing a clearer justification for sample sizes and increasing transparency in reporting variability across replicates would strengthen and clarify the conclusions.

Conclusions: robustness, validity, and reliability
The main conclusions are generally well-supported by the data presented, with some limitations (see below). Using complementary methods enhances the credibility and strength of the central claims. However, small sample sizes and limited validation at later time points reduce the overall generalizability. Overall, the conclusions are valid within the scope of the data but would be much stronger with more empirical support in the later stages of culture.

Suggested improvements
While the study is ambitious and methodologically sound, several critical gaps need to be addressed to fully support the manuscript's central claims and ensure its suitability for publication.

- While some experiments involve culturing organoids for up to five years, most functional and structural tests, such as electrophysiology, EM, and morphological analysis, have been conducted within 9 to 12 months. Only a small subset of data reaches the five-year mark. To robustly support the claim of long-term maturation, it is crucial to repeat key assays at later stages, such as beyond 2 to 3 years.

- A common issue in long-term organoid culture is hypoxia and necrosis in the core caused by the lack of vasculature. The manuscript does not discuss this issue, especially at later stages. Adding analyses like cleaved caspase-3, TUNEL staining, or hypoxia markers could offer valuable insights into the physiological relevance and structural integrity of aged organoids.

- To better address concerns regarding the viability of the inner core, metabolic profiling methods like Seahorse assays, hypoxia sensors, or reactive oxygen species measurements can offer important insights into energy consumption and stress levels over time in long-term cultures.

- Several key assays, such as MEA recordings, EM, and chimeric organoid experiments, rely on a small number of biological replicates and donor lines. This limits the statistical power and the broader applicability of the results. Increasing the number of replicates, particularly at later time points, or offering stronger statistical justification would improve the robustness of the conclusions.

- Although transcriptomic comparisons are conducted with existing in vivo datasets, the model's validity would be stronger if organoid epigenomic or chromatin accessibility profiles were directly matched to postnatal human brain tissue.

Considering the importance of the claims, conducting these additional experiments and analyses is crucial to support the main argument. Addressing these points would definitively confirm the model's relevance in studying extended human brain development and verify its physiological accuracy.

Clarity, context, and references
The manuscript is clearly written, with a concise and informative abstract and introduction. References are up-to-date and appropriate. Figures are well-constructed overall, but some would benefit from improved labeling and simpler layouts to enhance readability. The discussion could be strengthened by more directly addressing known limitations of the model, such as the absence of vasculature, microglia, or sensory input, as these factors likely influence the fidelity of maturation. A more explicit comparison to other long-term 3D brain models would also help clarify where this approach offers unique advantages and where it falls short.

Referee #2

(Remarks to the Author)
Faravelli et al. present a comprehensive and technically ambitious study extending the culture of human cortical organoids to five years. Building on prior work from the same group (Quadrato et al., 2017), the authors optimize culture conditions using activity-permissive media (APM) to improve neuronal activity, axo-spine interactions, and complexity. Transcriptomic and methylomic profiling suggest temporal trajectories aligned with perinatal brain tissue, highlighting the potential of long-term organoid models to capture features of human brain aging and development.The dataset is expansive and the computational analyses are sophisticated, particularly regarding transcriptional and methylation dynamics over time. Notably, the authors document substantial neuronal loss after one year, and attempt to address this using APM and heterochronic reaggregation strategies. These results represent an important resource for the community, particularly for long-term modeling of human cortical development. This is a well-powered and rigorous study providing a detailed description of human cortical organoids in long-term culture. However, several aspects of the study require clarification or additional experimentation to fully support the manuscript’s conceptual claims and translational relevance.

Major Points:
Comparison to 2D Culture Systems (Line 118) The authors state that “in 2D culture systems, the methylome is more unstable and epigenomic drift is common14–18,” yet none of the cited papers examine iPSC-derived human neurons or glia. Most references focus on cancer or stem cell populations. It is thus speculative to assume iPSC-derived neural cells would not mature in 2D.
• Is the implication that 3D environment or a specialized niche in organoids is uniquely permissive for developmental aging?
• Direct comparison of transcriptional and methylation profiles between 2D and 3D cultures would be more informative than tangential citations.
• Clarifying whether cellular aging is cell-autonomous or niche-dependent would be a technical advance that elevates the

manuscript beyond descriptive observations.
Interpretation of Figure 1 and Longitudinal Analyses Figure 1C shows organoids mapping to fetal scRNA-seq signatures of age-matched human brains, but the presentation is overly simplistic. The manuscript would benefit from:
• Identifying which gene expression programs and factors drive these correlations.
• Adding IHC for cell-type-specific markers over time to assess morphological complexity and cellular interconnectivity.
• Integrating MEA recordings and neuronal tracings in early figures to show whether transcriptional and methylation changes have functional consequences.
Functional Relevance of Epigenetic Changes: Extended fig 1h shows altered methylation of OLIG2. Aspects of myelination are shown in Extended Fig 1g and h. But, this is not quantified. It is unclear whether aspects of myelination increase from 1-4 years, which would support functional maturation and recording of the passage of time. Likewise, do DLX1/2 DMVs correlate with increased late-born inhibitory neuronal populations, such as PV interneurons?
Cell-Type Composition and Neuronal Loss Post-Year 1
• Extended Data Fig. 1b (cell abundance) should be moved to the main figures, as the predominance of glia and loss of neurons beyond 1 year is a major caveat.
• Extended Data Fig. 2f shows neurons only up to year 1, whereas glia/astrocytes are presented up to year 4.
How does the loss of neurons post-year 1, shown in Extended data Fig 2, factor into the analysis shown in figure 1b-k? In the human brain, gliogenesis occurs at specific time points. Given that there are very limited neuronal cells in the year 1+ organoids, is mapping onto fetal datasets just a reflection of increased glial signatures? How does glial diversity change in the organoids over time, and how well does this align with fetal and postnatal development? Do the older organoids model developmental waves or processes that could be leveraged to understand human-specific biology in greater detail? Similarly, does the cellular aging shown in Figure 1 occur in specific cell types or do the transcriptional and epigenetic signatures seen arise from specific populations?
The translational or basic biology value of the aged (post year 1) organoids is unclear. A major caveat to the utility of the organoids is the loss of neurons at year 1 and the predominance of glia. Key questions around this phenomenon remain unaddressed. For example, do the glial population represent healthy homeostatic states, or conversely, do glia harbour cytotoxic signatures that potentially contribute to neuronal loss? It would be valuable to the reader/audience to more clearly illustrate a technical advance or limitations uncovered by this tour de force analysis. Specifically, given the significant loss of neurons and non-physiological abundance of glia, can post-year 1 organoids be applied to investigate developmental, cell-cell interactions, or disease-relevant processes?
Figure 2f shows that APM increases neuronal activity measured by MEA. The TTX controls nicely show that the activity can be attributed to sodium currents. The assumption is that the more electrically permissive environment provided by APM promotes neuronal survival. To fully support this claim, a population analysis, either by scRNAseq or IHC (ideally both), showing an increased number of neurons. It should be noted that this approach was first described by the Gage lab in 2015. Many labs use this approach to promote neuronal maturation and survival. Other studies have shown that transplantation of organoids into an in vivo environment with increased activity promotes neuron morphological and electrical complexity. Therefore, the conceptual novelty of this approach is limited.
In figure 2c, staining needs quantification to support the differences in CFOS and SATB2 staining mentioned on the text.

In figure 4, authors claim that APM enriches the tissue in more mature cell types such as callosal projection neurons (CPN). They show a clear enrichment in excitatory neurons and reduction in astrocytes and certain types of glia and neuro progenitors. The representation of astrocytes and oligodendroglia is intriguing. Specifically, the reduction of astrocytes is linked with maturation, but it could also mean patterning towards neuronal differentiation rather than gliogenesis. CPN form the largest myelinated tract of the human brain, yet, the oligodendroglia signatures are not represented. Collectively, it is unclear to me whether the lack of certain glial cell types is a result of patterning towards specific neuronal subtypes rather than gliogenesis or if this is actual tissue aging.

SATB2 staining in figure 4j needs quantification.

In figure 5, the authors confronted the proportion of CPN in chimeroids generated by reaggregation of cells derived from young, old or young + old organoids. Authors claim that old progenitors in the heterochronic tissue can respond to “instructive neurogenic signals” to make neurons again and that the neuronal progenitors involved retain developmental memory to generate late-stage neurons faster. The experimental approach here was sophisticated, but the interpretation of the results seemed to overstate fundamental developmental steps without validation. One possible experimental approach to validate the presence of young versus old progenitors is to sort the neural progenitor from young, old and young+old tissue and 1- characterize the expression of NPC markers. What kind of NPC are these? Are they committed to specific CNS regions and does that differ between old and young tissue? This information would guide whether this is aging of the progenitors, or commitment to a different cell type or brain region. 2 – expose these sorted progenitors to differentiation factors in vitro and determine whether they generate glia, neurons and whether neuronal differentiation occurs faster in the group that contains old progenitors exposed to the same factors as the young progenitors. 3 – expose the progenitors to conditioned media of young and old organoids and determine differences in the progeny as above.

In 9-month monochronic chimeroids, there is less CPN than in 9-month organoids before dissociation. Is this due to a developmentally appropriate death of mature neurons and/or to an inability of mature neurons to incorporate in the tissue? In contrast, heterochronic chimeroids have a very high proportion of CPN even when compared with 9-month-old organoids. This was claimed to be a result of the instructive signals from the young progenitors acting on the old developmentally committed progenitors. Were these instructive signals not present in the older tissue while it was developing? Can the authors identify the developmental stage of these old-tissue-derived progenitors and compare with that of younger tissue? One approach would be sorting the neural progenitors from the dissociated organoids and from the chimeroids and staining for markers of different developmental stages. Another approach would be treating old organoids with conditioned media

from the young organoids and analyzing the generation of CPN neurons.

Line 252: The authors mention the lack of statistical differences in synaptic density in 6 and 12 month old organoids cultured in CDM4 versus APM. They try to strengthen the results by highlighting the large variability within conditions, yet do not give reasoning for using such a small sample size (n=3 per condition).

Line 374: The claim that the old MonChr increased in size, and thus contained newly generated progeny, seems subjective. Suggest including either representative Brightfield images of the chimeroids at 1 DAA and 15 DAA and/or average cell counts obtained from dissociations.

Extended data Fig. 3b, c and Fig. 4d, f: They show that APM significantly increases the percentage of late-born neurons at 6 months. Meanwhile, astro and OPC percentages decrease, and no significant module score differences exist for these cell types at this age (Extended data Fig 3b,c). This is one example of many where the authors could expand upon the nuances of glial cell presence and health in these aged systems. If the main takeaway from the study is that organoids are a sufficient model to recapitulate human brain development and maturity, they should provide clearer insight into how the glial populations change over time—do their changes reflect human brain biology in such a compelling way as the neurons seem to? Glia are only mentioned throughout the body of the paper when their percentage makeup is mentioned. Including content about the importance and necessary contributions these cells make to neuronal health and longevity would make for a more comprehensive story of the developing brain and would strengthen the case for using organoids to study diseases of the CNS that do not exclusively affect neurons.

The APM and heterochronic are presumably framed as approaches to rescue defects seen in the post-year 1 organoids. However, this is not directly tested past year 1 where the maturity of neuron loss was observed.

The study is technically impressive and provides an unprecedented long-term dataset on human cortical organoids. However, the current version is largely descriptive and lacks key functional validations.

Referee #3

(Remarks to the Author)
The submitted ms, by a well-established group of high international recognition, reports on a powerful approach to gain further understanding in the relevance and value of cortical organoïds as model systems, focusing on later stages of cortical maturation. The long-term cultures combined with the extensive in-depth bio-informatic analysis constitute an impressive tour de force. The data represent an invaluable and unique resource for all the neuroscience community.
The data are clearly illustrated and thoroughly documented, they rely on the highest standard in bio-informatic analyses and statistics.

The first section of the ms is based on the unprecedented maintenance of cortical organoïds over 5 years- which represents a truly unique achievement- with their molecular and epigenetic / methylation characterization.
The second part which reports on a “functional analysis” of cortical organoïds focuses on a shorter period of 6-12 months, and is based on the analyses of the functional activity in two culture conditions- APM medium vs CDM4. This raises the question of the representativeness of cell types (Figure 1) as documented in > 5 years organoids (in CDM4), which seems to favor neural populations. It therefore appears somehow tricky to link these two sections.
The third section addresses the fascinating question of the maintenance of a cell's memory once it has passed through the various stages of differentiation. The approach which relies on the establishment of chimeric organoids - young vs. ‘old’ – is implemented during the 0-12 month culture period. Therefore, the term “old” used in this section does not correspond to “> 5 years organoids “refered to in the first section. This introduces some confusion.
As a general comment, the discussion is lacking a necessary perspective regarding the differences in kinetics and tempo between long term cortical organoïds analysed in the first section and cortical organoïds used in the two other sections of the ms.

Specific comments:
The title should be modified (remove “in culture”).
Line 140 : the CpG methylation profile appears almost definitively established around the 6 to 9 months transition, therefore not so ‘progressive’ as mentioned. Could the authors comment on this point?
Lines 193-201 : While the differences observed in the distribution of different cell types are documented, the occurrence of proliferation foci/islands vs a uniform distribution of proliferating progenitors in long term organoïds remains to be clarified. For instance, regular monitoring of Ki67 labeling in long term organoids will provide crucial information for understanding how the balance between proliferation and differentiation is maintained in the long run - an intriguing question given that the morphology/size of the organoids changes very little over time (Figure 1a). Similarly, detection of apoptotic or necrotic cells is essential to gain insight into the homeostasy and evolution of this balance over time.
In view of their potentially different impact on proliferation vs. differentiation, the comparison between the two culture media (APM and CDM14) should be provided.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
I believe the authors have effectively addressed the reviewers' concerns, leading to improvements in the manuscript. The revisions are clearly articulated and enhance the overall quality. The paper is now in good shape and ready for publication.

Referee #2

(Remarks to the Author)
Overall, the authors addressed most reviewer's comments. Of improvement, is the addition of the characterization of cell fidelity up to 5 years of age (starting line 248). They are able to align molecular shifts in glia and neurons that occur within this time frame with that of changes that occur in human brain tissue.

The diminished neuron representation at this experiment's endpoint remains a major concern and critical flaw with the dataset given this is a centeral claim. The authors account for this in speculating that neurons are particularly susceptible to dissociation steps in the library preparation for sequencing, but this is not supported with evidence and is not the case for human brain tissue which should be the reference for this study.

Relatedly, I would like to see a more thorough and clearly articulated discussion of Figure 2b. The authors note that a small population of neurons persists at year 5. But this population is not present at any timepoints prior to 5 years, and they do not address this apparent absence of this "neuron" cluster at earlier time points. This raises questions about how this cluster is defined and why it emerges only at later stages. In addition, while several neuronal subtypes are listed in the cell type breakdown, the identity of this broadly labeled "neuron" cluster is unclear does it correspond to excitatory neurons, or another subtype?

I am also concerned about the large "unknown" cell population, which appears to comprise approximately 40% of cells at year 5. The manuscript does not provide sufficient interpretation or characterization of this population, making it difficult to assess its biological relevance.

More broadly, the central claim of the study that extended culture to 5 years yields a well-characterized system that closely tracks human brain development seems well supported up to approximately one year. Beyond this point, however, there appears to be a marked decline in neuronal representation and clarity of cell identity. This raises important concerns about the fidelity and utility of the model at later time points, and it remains unclear what applications these long-term cultures are best suited for in their current form.

Referee #3

(Remarks to the Author)
In our opinion, the authors have provided convincing arguments that address all the points of concern raised during the first round of review.
More, they have remarkably built upon their initial results and therefore significantly strengthened the significance of their work.
In its present form, the submitted work provides unrivalled and remarkable evidence for the ability of organoïds to mimic brain development over extended periods of time.

Referee #4

(Remarks to the Author)
Summary: I was asked to specifically review the WGBS-related analyses in the revised manuscript, which were well done. I had a few suggestions around analyses that could provide additional context, as well as a few minor suggestions for the Methods:

- Estimating biological age with this additional reference profile – derived from prenatal brain tissue - could perhaps be informative: https://pubmed.ncbi.nlm.nih.gov/34174924/ to contrasts the postnatal cortical clock currently in the manuscript (and pan-tissue Horvath clock)

- DNAm signatures are probably a more stable and predictive substrate for estimating underlying cell composition than RNA levels – it would be interesting to perform analogous transfer learning to pre- and post-natal single cell WGBS datasets (eg https://www.nature.com/articles/s41586-024-08030-7) to complement the existing RNA-based analyses

- Methods:
a) It did not seem obvious why the hg19 reference genome was used for the WGBS alignments, given its successor - hg38 – was released over a decade ago. Some statement/clause in the methods offering some rationale for this choice should be provided.
b) The targeted sequencing depth/coverage should for the WGBS should also be provided, eg "Sequencing was conducted on an Illumina NovaSeq 6000 platform, generating 150 base paired-end reads[, targeting XX reads (or YYx coverage) per sample]"

- Andrew Jaffe (please leave my signature in)

RESPONSE TO REVIEWERS:

We thank the Reviewers for their enthusiasm for our work and for their thoughtful comments and constructive feedback for the revisions. We have now addressed all of the comments through extensive new experiments and data analysis, as well as revisions to the text and figures. Here, we first highlight the major changes to the revised manuscript and then address each of the Reviewers' comments point-by-point.

Key new experiments and data in the revised manuscript:

- **Single-cell RNA sequencing datasets**: We added 10 new scRNA-seq datasets comprising 142,214 additional cells, bringing the total to 513,656 cells derived from 129 individual organoids/chimeroids and 4 replicates of pooled nascent chimeroids. These additions extend sampling across exceptionally long culture durations, up to 5 years *in vitro*, and increase biological replication. These experiments represent a substantial body of work, given the difficulty of generating samples reflecting these many years in culture.
- **Mouse chimeroid experiments**: We included an additional independent batch of mouse heterochronic and monochronic experiments. These new data are in agreement with our prior results.
- **Functional analyses**: We have expanded multielectrode array (MEA) recordings across additional genetic backgrounds and later time points, up to 2 years *in vitro*, making this the only study, to our knowledge, reporting functional characterization of circuit activity this late in *in vitro* development. In addition, we performed immunohistochemical quantification of SATB2 and SATB2/C-FOS signals to quantify CPNs and activity markers across CDM4 and APM conditions.
- **Ultrastructural analyses**: We increased the number of samples with EM analysis, doubling the number of 1-year samples, and performed quantitative assessments of synaptic density and spine frequency.
- **Transcriptomic and trajectory analyses**: We have added additional late time points for transcriptomic analysis (including 1.5 years, 2 years, and 5 years), performed updated DIALOGUE analyses integrating additional human endogenous and organoid datasets, refined developmental trajectory characterization using cell cycle scoring, and evaluated progenitor temporal alignment in the heterochronic and monochronic chimeroid systems.
- **Metabolic and tissue health assessment**: We conducted cell type–resolved analyses of stress-associated metabolic programs, including glycolysis, hypoxia, and apoptotic gene sets, and performed hypoxia labeling using pimonidazole. The data shows that enrichment of metabolic stress programs is largely driven by progenitor populations. This observation is consistent with the high metabolic demands of progenitors *in vivo*.
- **Methylome profiling**: We have extended whole-genome methylome profiling and analyses to additional time points, increased numbers of biological replicates, included profiling and analysis of organoids grown in APM media, and performed comparative analyses with human endogenous datasets.

Overall, with the addition of this additional data and analysis, we trust that we have addressed the Reviewers' comments. The new data further reinforces our conclusions on the validity and uniqueness of these long-term organoid cultures as powerful experimental systems to fuel understanding of the largely unexplored mechanisms governing human brain neoteny, maturation, and evolution.

We are very thankful to the Reviewers for their constructive suggestions, which have substantially strengthened the manuscript.

We address the Reviewers' specific comments below.

Referee #1 (Remarks to the Author)

Summary of key results

This work shows that human brain organoids can be cultured in vitro for extended periods, continuing to mature in ways that mimic natural human brain development. These long-term organoids undergo ongoing transcriptional and epigenetic aging. Notably, older neural progenitors retain their developmental history, allowing them to produce late-born neurons without reiterating the early differentiation stages. This underscores the potential of brain organoids to model extended phases of human brain maturation, providing a valuable platform for studying postnatal development and aging processes in vitro. The effort by Dr. Faravelli and colleagues is commendable, particularly in their careful and transparent reporting throughout the manuscript.

We are grateful to the reviewer for appreciating our work and efforts to investigate organoid biology over long-term development.

Originality and significance

This work marks a significant improvement over earlier organoid research by showing that brain organoids can be maintained over extended periods while continuously maturing. Unlike previous studies, such as Hergenreder et al. (Nat Biotechnol, 2024), which relied on pharmacological cocktails to accelerate neuronal development in a significantly shorter timeframe, this study explores whether organoids can naturally maintain pace with time and preserve developmental memory. If fully supported, this method provides a novel and valuable experimental platform for investigating human neurodevelopment.

Data and methodology

The study employs a comprehensive combination of methods, such as single-cell transcriptomics, DNA methylation analysis, electrophysiology, ultrastructural imaging, and chimeric organoid experiments. This multimodal approach is a significant advantage, offering a broad perspective on maturation over time. The authors present the datasets and analytical methods accurately and transparently, demonstrating a commitment to reproducibility and scientific integrity.

One aspect that could be improved is addressing inter-organoid variability, particularly over long culture periods and among different donor lines. Although the authors mention that variability stays within the limits of variation found in endogenous tissue, providing a detailed explanation of how batch effects and donor differences were measured and controlled would be beneficial. Considering the extended duration and natural heterogeneity of organoid systems, including more precise variability metrics at various time points would enhance the robustness of the results.

Appropriate use of statistics

Statistical methods are properly selected. Including biological replicates and donor variation in the models is a strength. However, the reporting of uncertainty could be clearer. Confidence intervals, effect sizes, and measures of variability are sometimes inconsistently reported, making it hard to evaluate the robustness of some results, especially over long culture periods. Sample sizes are reasonable given the technical challenges of long-term culture, but they are relatively small in key experiments like electrophysiology, EM, and chimeric organoids. Providing a clearer justification for sample sizes and increasing transparency in reporting variability across replicates would strengthen and clarify the conclusions.

We agree that these are important points. We have now increased the sample size across experiments and organoid ages for the electrophysiological analyses, by adding an additional genetic background (H1, n=3 organoids for each time point) across all analyzed time points and also extending the analysis up to 2 years in culture (H1, n=4 biological replicates). We also increased the number of EM replicates, including six novel organoids (3 per condition, CDM4 and APM) at 12 months, and included a new batch in the mouse chimeric experiments (replicating the same conditions of the first 2 batches and analyzing monochronic and heterochronic conditions 1 day after aggregation). A full compendium of single-cell data from organoid and chimeroid samples is included in the revised **Supplementary Table 1**.

In addition, we have now clarified in the **Methods** and corresponding figure legends how batch effects and donor differences were measured and controlled. Specifically, we further detailed the statistical analyses used to account for donor and batch as covariates and describe the criteria applied to assess variability across time points. We employ Aitchison distances between organoids to represent the degree of dissimilarity in cell type composition while accounting for the compositional nature of cell type abundances, but as would be the case with any metric for variability, this metric is most meaningful when values are compared with some reference range. Therefore, to facilitate comparison to endogenous tissue, we have now included Aitchison distances among the expanded perinatal tissue datasets from Velmeshev et al./Wang et al., grouped by age range to ensure that the measurements are meaningfully matched with those of our organoids. We have also incorporated confidence intervals and explicit variability metrics across all relevant statistical analyses throughout the text as well as in the newly-added **Supplementary Table 11**.

Conclusions: robustness, validity, and reliability

The main conclusions are generally well-supported by the data presented, with some limitations (see below). Using complementary methods enhances the credibility and strength of the central claims. However, small sample sizes and limited validation at later time points reduce the overall generalizability. Overall, the conclusions are valid within the scope of the data but would be much stronger with more empirical support in the later stages of culture.

Suggested improvements

While the study is ambitious and methodologically sound, several critical gaps need to be addressed to fully support the manuscript's central claims and ensure its suitability for publication.

- While some experiments involve culturing organoids for up to five years, most functional and structural tests, such as electrophysiology, EM, and morphological analysis, have been conducted within 9 to 12 months. Only a small subset of data reaches the five-year mark. To robustly support the claim of long-term maturation, it is crucial to repeat key assays at later stages, such as beyond 2 to 3 years.

We agree with the reviewer that profiling additional timepoints is important. Despite the limits imposed by the fact that it takes many years to generate the aged samples, we have now been able to repeat several key analyses. We performed additional single-cell RNA sequencing experiments, including 10 new individual samples ranging from 1.5 to 5 years of age. With these additions, the scRNA-seq timecourse now matches the 5 year WGBS methylation timecourse in this study. This new data confirmed our previous results, and informed us on the cell type distribution over the full 5 year time range, including the presence of neurons at 5 years of culture. In addition, we included six additional 1-year-old samples processed for electron microscopy analysis, which validated the original conclusions of the study. Finally, we were able to extend our electrophysiological analyses of APM organoids to 2 years in culture, the oldest samples available thus far for APM cultures; notably, we continued to

observe the presence of neuronal activity at this late age. In each of these cases, we have endeavored to use the oldest available organoid samples for these new analyses. We agree with the reviewer that the addition of these experiments added important validation and critical new information to the study.

- A common issue in long-term organoid culture is hypoxia and necrosis in the core caused by the lack of vasculature. The manuscript does not discuss this issue, especially at later stages. Adding analyses like cleaved caspase-3, TUNEL staining, or hypoxia markers could offer valuable insights into the physiological relevance and structural integrity of aged organoids.
- To better address concerns regarding the viability of the inner core, metabolic profiling methods like Seahorse assays, hypoxia sensors, or reactive oxygen species measurements can offer important insights into energy consumption and stress levels over time in long-term cultures.

We have now examined the metabolic state of the organoids through several assays. We examined cell type–specific variation in metabolic processes relevant to organoid biology over the 5-year time course, using curated gene sets from MSigDB. We observed an overall increase in metabolic signatures over time; however, subsetting the analysis by cell type revealed that this effect was largely driven by progenitor populations (**Extended Data Fig.8** and **Supplementary Table 11).** Other cell types did not show as large or as significant of an increase over time. This observation is consistent with the high metabolic demands of progenitors, which are also expected to decrease in abundance over time under physiological conditions. We note that enrichment of glycolysis and hypoxia gene sets is also observed in a subset of endogenous human fetal progenitors *in vivo* (Trevino et al., *Cell*, 2021; Polioudakis et al., *Neuron*, 2019; Uzquiano et al., *Cell*, 2022). As a complementary approach, we performed hypoxia labeling using the pimonidazole detection reagent. Consistent with our previous findings (Uzquiano *et al.*, *Cell*, 2022), pimonidazole signal was largely restricted to the organoid core, while much of the peripheral tissue remained non-hypoxic, even after 12 months *in vitro.*

- Several key assays, such as MEA recordings, EM, and chimeric organoid experiments, rely on a small number of biological replicates and donor lines. This limits the statistical power and the broader applicability of the results. Increasing the number of replicates, particularly at later time points, or offering stronger statistical justification would improve the robustness of the conclusions.

We have now increased the number of biological replicates across all key assays. Specifically, we added an additional genetic background (H1) across all time points for MEA recordings, with at least three organoids per condition per time point, and extended these analyses to organoids grown in APM for up to 2 years. We included six new samples for electron microscopy analysis, and increased the number of batches analyzed for mouse chimeric organoids. In addition, we increased the number of replicates for whole-genome bisulfite sequencing and methylome analyses by adding a new set of organoids (n=6) also including an additional genetic background for APM/CDM4 comparisons. Finally, we performed additional single-cell RNA-sequencing analyses on organoids aged 1.5, 2, and 5 years.

- Although transcriptomic comparisons are conducted with existing in vivo datasets, the model's validity would be stronger if organoid epigenomic or chromatin accessibility profiles were directly matched to postnatal human brain tissue.

We agree that direct comparison of organoid epigenomic profiles to postnatal human brain tissue strengthens model validation. We have now incorporated such a comparison, using cell-type-resolved, age-associated differentially methylated regions (cdDMRs) derived from the human postnatal cortex (Price *et al*., 2019). In that study, $NeuN^+$ (neuronal) and $NeuN^-$ (non-neuronal) populations were prospectively sorted and profiled by whole-genome bisulfite sequencing, and age-associated DMRs were classified into six categories (c1-c6) reflecting cell-type-specific and shared methylation trajectories.
We systematically mapped these cdDMRs onto our organoid methylomes over extended culture periods and observed concordant, age-directional methylation remodeling, demonstrating that long-term-cultured cortical organoids resemble endogenous, cell-type-specific epigenomic aging signatures (**Extended Data Fig. 1h-k, and Supplementary Table 3**). While broader access to raw postnatal human brain methylomes remains limited due to repository and data protection constraints, the cdDMR framework provides a stringent, cell-type-matched reference for *in vivo* comparison. We have now included this analysis and its rationale in the revised manuscript.

Considering the importance of the claims, conducting these additional experiments and analyses is crucial to support the main argument. Addressing these points would definitively confirm the model's relevance in studying extended human brain development and verify its physiological accuracy.

Clarity, context, and references
The manuscript is clearly written, with a concise and informative abstract and introduction. References are up-to-date and appropriate. Figures are well-constructed overall, but some would benefit from improved labeling and simpler layouts to enhance readability. The discussion could be strengthened by more directly addressing known limitations of the model, such as the absence of vasculature, microglia, or sensory input, as these factors likely influence the fidelity of maturation. A more explicit comparison to other long-term 3D brain models would also help clarify where this approach offers unique advantages and where it falls short.

We thank the reviewer for their positive comments and appreciation of our work. We have now revised both figures and text to include the new data, and to discuss these important points.

Referee #2 (Remarks to the Author):
Faravelli et al. present a comprehensive and technically ambitious study extending the culture of human cortical organoids to five years. Building on prior work from the same group (Quadrato et al., 2017), the authors optimize culture conditions using activity-permissive media (APM) to improve neuronal activity, axo-spine interactions, and complexity. Transcriptomic and methylomic profiling suggest temporal trajectories aligned with perinatal brain tissue, highlighting the potential of long-term organoid models to capture features of human brain aging and development. The dataset is expansive and the computational analyses are sophisticated, particularly regarding transcriptional and methylation dynamics over time. Notably, the authors document substantial neuronal loss after one year, and attempt to address this using APM and heterochronic reaggregation strategies. These results represent an important resource for the community, particularly for long-term modeling of human cortical development. This is a well-powered and rigorous study providing a detailed description of human cortical organoids in long-term culture.

We thank the reviewer for their supportive comments on the impact of the work and its quality.

However, several aspects of the study require clarification or additional experimentation to fully support the manuscript's conceptual claims and translational relevance.

Major Points:

Comparison to 2D Culture Systems (Line 118) The authors state that "in 2D culture systems, the methylome is more unstable and epigenomic drift is common14–18," yet none of the cited papers examine iPSC-derived human neurons or glia. Most references focus on cancer or stem cell populations. It is thus speculative to assume iPSC-derived neural cells would not mature in 2D.

• Is the implication that 3D environment or a specialized niche in organoids is uniquely permissive for developmental aging?

• Direct comparison of transcriptional and methylation profiles between 2D and 3D cultures would be more informative than tangential citations.

• Clarifying whether cellular aging is cell-autonomous or niche-dependent would be a technical advance that elevates the manuscript beyond descriptive observations.

The reviewer is correct that we were referring to studies using cancer or pluripotent stem cell lines. Given the lack of long-term studies in 2D iPSC-derived neural cultures, we have revised the text to remove over-broad statements regarding epigenomic instability in 2D cultures. Addressing the relative contributions of cell-autonomous versus niche-dependent mechanisms of epigenomic aging is an important question, but given the current unavailability of long-term 2D datasets for comparison, it would unfortunately require culture and profiling of 2D models over multiple years.

Interpretation of Figure 1 and Longitudinal Analyses Figure 1C shows organoids mapping to fetal scRNA-seq signatures of age-matched human brains, but the presentation is overly simplistic. The manuscript would benefit from:

• Identifying which gene expression programs and factors drive these correlations.

We have now expanded the reference datasets to include first-trimester human samples, and extended our organoid dataset to 16 distinct ages, spanning from 15 days to 5 years. This allowed us to run DIALOGUE for each specific cell population, in order to identify multicellular processes (MCPs) that correlate with age in a cell type–specific manner. Using these results, we identified a MCP associated with aging in endogenous human samples (MCP4). We now provide a supplementary table reporting the full list of genes underlying MCP4 (**Supplementary Table 2**), thus providing extensive information on the gene expression programs driving the observed correlations. In addition, we have now added highlighted representative genes with known roles in human brain development that contribute to MCP4 in the relevant plots (**Fig.1f and Extended Data Fig.4a).**

• Adding IHC for cell-type-specific markers over time to assess morphological complexity and cellular interconnectivity.

We have performed immunohistochemical analyses for multiple cell type–specific markers in organoids up to 9 months of culture, as presented in the current manuscript (**Fig. 1d, Fig. 4e** and **Extended Data Fig. 2i, 4c**) and in previous publications from our laboratory (Velasco et al., *Nature*, 2019, Uzquiano et al., *Cell*, 2022). In general, we have observed that after about 9 months in culture, there is a reduction in staining specificity, with increased background signal, despite extensive optimization efforts. Similar technical limitations have been reported for immunostaining of aged human brain tissue (Piña et al., *Int J Mol Sci.*, 2022, Park et al., *Mol Cells*. 2024). For these reasons, we prioritized alternative approaches better suited for the

analysis of long-term cultures in our hands. These methodological considerations are now clarified in the **Methods** section of the revised manuscript.
As an alternative approach, we implemented a fresh-frozen tissue immunostaining protocol (see **Methods**) to label the pan-neuronal marker NEUN. This strategy did result in robust nuclear staining for this antibody in 2- and 3-year-old CDM4 samples, and 2-year APM samples, indicating the presence of neuronal populations at late time points (**Extended Data Fig. 2i** and **Fig. 4e**).

• Integrating MEA recordings and neuronal tracings in early figures to show whether transcriptional and methylation changes have functional consequences.

We have now incorporated additional analysis of the APM organoids, as this model maintains neuronal representation and activity over longer periods. Following the reviewer's suggestion, we have now profiled APM organoids by whole-genome bisulfite sequencing to determine whether the morphological and functional differences we observed were associated with corresponding epigenomic changes. We found that differentially-methylated regions identified in APM-cultured organoids are enriched at neuron-associated and maturation-related genes, consistent with the increased neuronal number and activity in this condition. Importantly, these differences reflect modulation of locus-specific methylation states rather than a disruption of the overall directionality or temporal progression of age-associated epigenomic remodeling. This data is now presented in **Extended Data Fig.9 and Supplementary Table 12.**

Functional Relevance of Epigenetic Changes: Extended fig 1h shows altered methylation of OLIG2. Aspects of myelination are shown in Extended Fig 1g and h. But, this is not quantified. It is unclear whether aspects of myelination increase from 1-4 years, which would support functional maturation and recording of the passage of time. Likewise, do DLX1/2 DMVs correlate with increased late-born inhibitory neuronal populations, such as PV interneurons?

We observe coordinated, locus-specific methylation remodeling across multiple oligodendrocyte- and myelination-associated genes, including *OLIG2* and other developmentally regulated loci, as shown in the manuscript (**Extended Data Fig. 2c, d**). These epigenetic trajectories parallel the transcriptomic maturation of glial populations observed in our single-cell RNA-seq analyses (**Extended Data Fig. 3e, f**), which together support age-associated glial development in long-term cortical organoids. These findings are consistent with the spontaneous emergence of myelin structures interspersed within the tissue at later time points (after 4–6 months), which demonstrates the intrinsic capacity of these organoids to initiate myelination programs (**Extended Data Fig. 4b, c**). However, it was not possible to perform a sufficient number of EM reconstructions at multiple ages to be able to establish a robust trajectory of changes in myelination. We therefore do not make any claim about this point, despite its importance for understanding myelination *in vitro*.
In regard to INs, we detect progressive methylation changes at differentially-methylated valleys (DMVs) associated with *DLX1/2* genes, which play key roles in inhibitory neuronal differentiation (**Extended Data Fig. 2c, d**). However, the cortical organoids used in this study are dorsally patterned and do not generate ventrally-derived interneuron lineages, including parvalbumin-positive (PV) interneurons, as confirmed by our single-cell transcriptomic analyses. Accordingly, we do not interpret DLX-associated methylation changes as evidence for the emergence of specific interneuron populations. Rather, we interpret them as evidence of continued maturation of inhibitory neuronal identity over extended culture periods.

Cell-Type Composition and Neuronal Loss Post-Year 1

• Extended Data Fig. 1b (cell abundance) should be moved to the main figures, as the predominance of glia and loss of neurons beyond 1 year is a major caveat.

Following the reviewer's suggestion, we moved **Extended Data Fig. 1b** to main Figure (**2b**).

• Extended Data Fig. 2f shows neurons only up to year 1, whereas glia/astrocytes are presented up to year 4.
How does the loss of neurons post-year 1, shown in Extended data Fig 2, factor into the analysis shown in figure 1b-k? In the human brain, gliogenesis occurs at specific time points. Given that there are very limited neuronal cells in the year 1+ organoids, is mapping onto fetal datasets just a reflection of increased glial signatures? How does glial diversity change in the organoids over time, and how well does this align with fetal and postnatal development? Do the older organoids model developmental waves or processes that could be leveraged to understand human-specific biology in greater detail? Similarly, does the cellular aging shown in Figure 1 occur in specific cell types or do the transcriptional and epigenetic signatures seen arise from specific populations?
The translational or basic biology value of the aged (post year 1) organoids is unclear. A major caveat to the utility of the organoids is the loss of neurons at year 1 and the predominance of glia. Key questions around this phenomenon remain unaddressed. For example, do the glial population represent healthy homeostatic states, or conversely, do glia harbour cytotoxic signatures that potentially contribute to neuronal loss? It would be valuable to the reader/audience to more clearly illustrate a technical advance or limitations uncovered by this tour de force analysis. Specifically, given the significant loss of neurons and non-physiological abundance of glia, can post-year 1 organoids be applied to investigate developmental, cell-cell interactions, or disease-relevant processes?

We acknowledge that the reduced neuronal representation observed after 1 year in culture represents a limitation of the CDM4 system. In this revised manuscript, we demonstrate the presence of NeuN+ neurons at 2 and 3 years in CDM4 culture, and extend our single-cell RNA sequencing to 5 years, at which time small numbers of neurons are still detectable. It is possible that the low representation of neurons may reflect the fact that the dissociation required to perform scRNA-seq may lead to differential death of neurons. Alternatively, this reduction may reflect limitations of the culture conditions, which motivated the implementation of APM medium. Using this optimized condition, we were able to detect preservation of neuronal populations and sustained neuronal activity in organoids cultured for up to 2 years (the latest timepoint that we have generated to date in this media), supporting the capacity of this model to capture functional aspects of long-term maturation and to investigate the passage of time *in vitro*.

Our epigenomic analyses rely on whole-genome sequencing–based approaches, which provide substantial depth and sensitivity but do not allow deconvolution at single-cell resolution. As a result, these data capture global epigenetic signatures of long-term cultures rather than cell type–specific contributions. However, importantly, all of the transcriptomic analyses in this study were performed at single-cell resolution, allowing us to explicitly account for changes in cellular composition over time, and to evaluate transcriptional signatures of aging in individual cell types (**Extended Data Fig. 3c, d**). In the APM condition, we were able to build more extensive maturation trajectories for excitatory neurons, showing that they continue to mature in culture (**Fig. 3i-k**)
Following the reviewer's suggestions, we have now examined glial populations in greater detail. Gene set enrichment analyses (GSEA) revealed that astrocytes in long-term cultures share maturation-associated gene expression programs with *in vivo* human tissue (**Extended**

Data Fig. 3g). In addition, mapping of *in vitro* glial age revealed a progressive increase that mirrors the passage of time observed *in vivo* (**Extended Data Fig. 3e**). From a metabolic perspective, stress-associated programs were predominantly driven by progenitor populations, whereas glial populations did not show as large or as significant of an increase over time (**Extended Data Fig. 8**).

Figure 2f shows that APM increases neuronal activity measured by MEA. The TTX controls nicely show that the activity can be attributed to sodium currents. The assumption is that the more electrically permissive environment provided by APM promotes neuronal survival. To fully support this claim, a population analysis, either by scRNAseq or IHC (ideally both), showing an increased number of neurons. It should be noted that this approach was first described by the Gage lab in 2015. Many labs use this approach to promote neuronal maturation and survival. Other studies have shown that transplantation of organoids into an in vivo environment with increased activity promotes neuron morphological and electrical complexity. Therefore, the conceptual novelty of this approach is limited.

The reviewer is correct that we are building on the seminal work from the Gage laboratory and others demonstrating that increased neuronal activity promotes neuronal maturation and survival *in vitro*, much as it does *in vivo* (Ghosh et al., *Science*, 1994, Voigt et al., *Eur J Neurosci* 1997, Warm et al., *Front Cell Dev Bio* 2022). Here, we take advantage of these principles to test whether supporting neuronal activity can foster the maintenance of neuronal populations over very long time periods in a three-dimensional human organoid system.
In this revised version of the manuscript, we have now profiled single APM organoids by scRNA-seq at up to 1.5 years in culture. This extends the APM scRNA-seq data from the initial submission showing increased proportions of corticofugal projection neurons (CFuPNs) and callosal projection neurons (CPNs) in APM-treated organoids, starting from as early as 6 months. The new single-cell RNA-seq samples at 1.5 years of age show that the increased proportions of CFuPNs, CPNs, Neural precursors (NPr), and Astrocytes persist under APM conditions until at least this age (**Fig. 3b and Extended Data Fig. 6**). In addition, we now include quantitative immunohistochemical analyses for the CPN marker SATB2, providing independent, population-level validation of increased neuronal representation in APM-treated organoids (**Fig. 2d**). Because these cultures were started more recently than the CDM4 experiments, we do not yet have APM organoids that were maintained past 2 years. A small number of 2-year APM organoids were available, which we chose to use to test neuronal and circuit activity at this time point. APM favored sustained neuronal activity in 3D cultures for up to 2 years, representing an exceptional duration for an *in vitro* system (**Fig. 4d and Extended Data Fig. 10e**).

In figure 2c, staining needs quantification to support the differences in CFOS and SATB2 staining mentioned on the text.

We agree with the reviewer and have now quantified the immunostaining shown in **Fig. 2c**. Quantitative analyses reveal a significant increase in the number of $SATB2^+$ CPNs in APM-treated organoids, as well as a significant increase in $SATB2^+$/C-FOS^+ double-positive cells. These results are consistent with an increased number of CPNs and an increased proportion of active CPNs under APM conditions and are now included in the revised manuscript (**Fig. 2d**).

In figure 4, authors claim that APM enriches the tissue in more mature cell types such as callosal projection neurons (CPN). They show a clear enrichment in excitatory neurons and reduction in astrocytes and certain types of glia and neuro progenitors. The representation of astrocytes and oligodendroglia is intriguing. Specifically, the reduction of astrocytes is linked

with maturation, but it could also mean patterning towards neuronal differentiation rather than gliogenesis. CPN form the largest myelinated tract of the human brain, yet, the oligodendroglia signatures are not represented. Collectively, it is unclear to me whether the lack of certain glial cell types is a result of patterning towards specific neuronal subtypes rather than gliogenesis or if this is actual tissue aging.

In our experiments, the initial patterning phase is the same for both APM and CDM4 culture conditions; organoids are transferred to APM media only after this patterning phase is complete. Therefore, the differences observed between these conditions in later stages are unlikely to reflect altered early fate specification. Instead, the changes in cellular composition observed under APM conditions most likely reflect proportional shifts driven by increased neuronal survival and maintenance. Specifically, the enrichment of CPNs in APM-treated organoids, especially at 6 months, results in a relative decrease in the proportion of other cell types, including astrocytes and certain glial populations. However, at later stages (1 year and 1.5 years), the proportions of glial progenitors are no longer significantly different between the two conditions. Moreover, oligodendrocyte precursor cells, neural precursors, and astrocytes are, in fact, more represented in APM-treated organoids at 1 year and 1.5 years respectively, indicating that these populations emerge and are retained in APM conditions (**Fig. 3** and **Extended Data Fig. 6d-i**).

Together, these findings suggest that the differences observed in **Fig. 3** primarily reflect changes in relative cellular proportions driven by neuronal enrichment and differential proliferative dynamics, rather than a suppression of gliogenesis. While oligodendroglial maturation remains limited *in vitro*, consistent with other organoid systems and the relatively young age of the tissue compared to human development, the presence and later increase of OPCs and astrocytes under APM conditions support the interpretation that APM long-term cultures retain the capacity to support glial lineages alongside neuronal maturation.

SATB2 staining in figure 4j needs quantification.

We have now quantified SATB2 immunostaining corresponding to this panel (now **Fig. 5j**), and included these analyses in the revised manuscript. Quantification shows an increase in $SATB2^+$ cells under heterochronic conditions ($P = 0.0519$), supporting the conclusions described in the text.

In figure 5, the authors confronted the proportion of CPN in chimeroids generated by reaggregation of cells derived from young, old or young + old organoids. Authors claim that old progenitors in the heterochronic tissue can respond to “instructive neurogenic signals” to make neurons again and that the neuronal progenitors involved retain developmental memory to generate late-stage neurons faster. The experimental approach here was sophisticated, but the interpretation of the results seemed to overstate fundamental developmental steps without validation. One possible experimental approach to validate the presence of young versus old progenitors is to sort the neural progenitor from young, old and young+old tissue and 1- characterize the expression of NPC markers. What kind of NPC are these? Are they committed to specific CNS regions and does that differ between old and young tissue? This information would guide whether this is aging of the progenitors, or commitment to a different cell type or brain region. 2 – expose these sorted progenitors to differentiation factors in vitro and determine whether they generate glia, neurons and whether neuronal differentiation occurs faster in the group that contains old progenitors exposed to the same factors as the young progenitors. 3 – expose the progenitors to conditioned media of young and old organoids and determine differences in the progeny as above.

We thank the reviewer for raising this important point. We have now clarified our approach further in the revised manuscript.
Because our chimeroid approach uses young and old input organoids generated from different donor lines (genetically distinct individuals), the young and old progenitors (and their progeny) are genetically distinguishable in the chimeroid by applying SNP detection to the scRNA-seq data (Antón-Bolaños et al., *Nature*, 2024). This allows us to demultiplex cells computationally, and unambiguously assign each cell to descendants of either the young (15DIV) or old (9-month) input organoid. We have previously validated this approach in our published chimeroid study (Antón-Bolaños et al., *Nature*, 2024). This strategy obviates the need for physical sorting, which could potentially introduce additional biases.
This genetic strategy allowed us to disentangle old progenitors and their progeny from the young progenitors and their progeny within the same heterochronic (HetChronic; old + young) cultures. This allows a direct comparison of transcriptomic profiles between young and old progenitors and progenies in heterochronic and monochronic chimeroids. Consistent with the dorsal cortical patterning conditions applied at the beginning of organoid differentiation (Velasco et al., *Nature*, 2019), progenitors across all conditions similarly express canonical telencephalic markers, including FOXG1, along with markers defining specific progenitor subtypes such as apical radial glia, intermediate progenitors, and glial progenitors. Differentially-expressed genes defining each progenitor subtype are provided in **Supplementary Table 5**.
These analyses indicate that the observed differences reflect changes in progenitor state, rather than shifts in regional identity or commitment to alternative CNS lineages. Moreover, this chimeroid paradigm can functionally test cell responsiveness within a shared microenvironment. In the heterochronic condition, old cells are exposed to the same instructive cues as young cells, allowing direct assessment of their neurogenic competence under identical differentiation conditions.

In 9-month monochronic chimeroids, there is less CPN than in 9-month organoids before dissociation. Is this due to a developmentally appropriate death of mature neurons and/or to an inability of mature neurons to incorporate in the tissue? In contrast, heterochronic chimeroids have a very high proportion of CPN even when compared with 9-month-old organoids. This was claimed to be a result of the instructive signals from the young progenitors acting on the old developmentally committed progenitors. Were these instructive signals not present in the older tissue while it was developing? Can the authors identify the developmental stage of these old-tissue-derived progenitors and compare with that of younger tissue? One approach would be sorting the neural progenitors from the dissociated organoids and from the chimeroids and staining for markers of different developmental stages. Another approach would be treating old organoids with conditioned media from the young organoids and analyzing the generation of CPN neurons.

In our prior work, we demonstrated that human chimeroid cultures immediately after remix are predominantly composed of progenitors, with few differentiated cells persisting through dissociation and reaggregation (Antón-Bolaños et al., *Nature*, 2024). In this revised manuscript, to directly address whether neurons persist through reaggregation, we leveraged our mouse chimeroid platform. Endogenous mouse chimeroids were infected with AAV PHP.eB, which exhibits established neuronal tropism, bearing a GFP reporter; at three weeks of culture (the "old" state for these fast-developing mouse cultures), the labelled chimeroids were dissociated and reaggregated to create "old" Monochronic mouse chimeroids. Presumptive neurons positive for the GFP^+ viral signal were readily detectable before dissociation, but were no longer detectable in "old" monochronic mouse chimeroids after re-aggregation, indicating that

few if any pre-existing neurons persist through dissociation and reaggregation (**Extended Data Fig. 11e-f**). This finding is consistent with our published results in human chimeroids, supporting the interpretation that the reduced CPN proportion in MonoChronic cultures compared to the input organoids reflects neuronal loss during dissociation and reaggregation, consistent with the increased fragility of neurons during the dissociation process compared to other cell types.

Regarding the developmental stage of old-tissue-derived progenitors, we leveraged our single-cell datasets to compare progenitor maturation states across conditions. Using the DIALOGUE-based maturation module scoring strategy described in the manuscript, we examined how progenitors reorganize multicellular gene programs across the distinct culture systems. This approach allowed us to assess temporal alignment in an unbiased, genome-wide manner while tracking donor identity (and thus time-in-culture) through the SNP detection approach described above, without relying on preselected markers or sorting strategies that could potentially skew progenitor composition. We observed that the old progenitor component within HetChronic (old + young) cultures exhibited low maturation scores, whereas MonoChronic (old) progenitors aligned with later developmental time points (**Extended Data Fig. 11j**). To further define this shift, we performed gene set enrichment analysis (GSEA) using genes that change during aging from 6 to 9 months in canonical cortical organoids—a developmental interval immediately preceding our HetChronic (old+young) and MonoChronic (old) systems and during which CPNs remain abundant. We found that old HetChronic progenitors shared a greater proportion of molecular programs with 6-month progenitors compared to aged MonoChronic progenitors (**Extended Data Fig. 11k**).
These results indicate that old progenitors are still plastic after 9 months in culture and can respond to instructive neurogenic signals to make neurons again; however, they have recorded the passage of time and retain a memory of the developmental steps already performed, as demonstrated by the fact that in only 2 weeks they can generate late stage neurons (CPNs), while their young progenitor counterparts undergo differentiation into much earlier cell fates within the same chimeric organoid.

Line 252: The authors mention the lack of statistical differences in synaptic density in 6 and 12 month old organoids cultured in CDM4 versus APM. They try to strengthen the results by highlighting the large variability within conditions, yet do not give reasoning for using such a small sample size (n=3 per condition).

We acknowledge the reviewer's concern regarding sample size for the electron microscopy analyses. Electron microscopy experiments require extensive tissue processing, high-resolution image acquisition, and in-depth analysis, which typically rely on smaller tissue volumes and limit the number of samples that can be processed per condition. Despite these constraints, we have now substantially expanded the dataset by doubling the number of 1-year samples. This increase in sample size improves statistical power and corroborates our original findings of increased synaptic density in APM conditions. These updated analyses are now included in the revised manuscript (**Fig. 2e-g**).

Line 374: The claim that the old MonChr increased in size, and thus contained newly generated progeny, seems subjective. Suggest including either representative Brightfield images of the chimeroids at 1 DAA and 15 DAA and/or average cell counts obtained from dissociations.

To support the interpretation of potential progenitor expansion, we examined proliferative signatures in our single-cell dataset. At 15 DAA (days after aggregation), MonoChronic old cultures contain a distinct cluster of cycling progenitors expressing canonical cell-cycle

markers, including MKI67, TOP2A, and KIF2C, indicating retained proliferative capacity (**Supplementary Table 5, clusters 9 and 13**). These data support the interpretation that progenitor proliferation contributes to the observed increase in tissue size. We also identified a typographical error in **Fig. 5b**, which we have corrected. The image shown in **Fig. 5b** corresponds to MonoChronic old organoids 2 months after aggregation; organoids at 14 DAA are shown in **Fig. 5c**.

Extended data Fig. 3b, c and Fig. 4d, f: They show that APM significantly increases the percentage of late-born neurons at 6 months. Meanwhile, astro and OPC percentages decrease, and no significant module score differences exist for these cell types at this age (Extended data Fig 3b,c). This is one example of many where the authors could expand upon the nuances of glial cell presence and health in these aged systems. If the main takeaway from the study is that organoids are a sufficient model to recapitulate human brain development and maturity, they should provide clearer insight into how the glial populations change over time—do their changes reflect human brain biology in such a compelling way as the neurons seem to? Glia are only mentioned throughout the body of the paper when their percentage makeup is mentioned. Including content about the importance and necessary contributions these cells make to neuronal health and longevity would make for a more comprehensive story of the developing brain and would strengthen the case for using organoids to study diseases of the CNS that do not exclusively affect neurons.

We agree that understanding glial presence, health, and developmental state is very important for exploring human developmental processes. Because all transcriptomic analyses in this study were performed at single-cell resolution, we were able to specifically examine glial populations independently of changes in overall cellular composition, following the reviewer's suggestion. To further assess glial health and maturation, we performed gene set enrichment analyses specifically within astrocyte populations. These analyses reveal that astrocytes in long-term cultures share gene expression programs with maturing astrocytes *in vivo*, supporting the presence of biologically-relevant astrocytic states despite differences in relative abundance. Moreover, mapping of *in vitro* glial age demonstrates a progressive increase over time that parallels the passage of developmental time *in vivo* (**Extended Data Fig. 3e-g**). With respect to oligodendroglial lineages, while mature myelinating oligodendrocytes remain limited—as is common across current organoid systems and appropriate due to their very late postnatal development in human cortex—we observe that OPCs are more represented in APM-treated organoids compared to CDM4 at 1 year, suggesting preserved oligodendroglial potential.
Finally, we have examined markers of metabolic health across individual cell populations, including astrocytes and OPCs, and find that stress-associated transcriptional programs are largely driven by progenitor populations, while other cell types did not show as large or as significant of an increase over time. We have included these findings in the revised version of the manuscript (**Extended Data Fig. 8**).

The APM and heterochronic are presumably framed as approaches to rescue defects seen in the post-year 1 organoids. However, this is not directly tested past year 1 where the maturity of neuron loss was observed.

We here present the heterochronic cultures primarily as a platform to functionally test how developmental time is recorded *in vitro*. However, we agree with the reviewer that direct testing of APM effects on neurons beyond 1 year is important. We have now extended our analyses of APM-treated organoids to later time points. Specifically, we performed additional single-cell RNA-sequencing experiments at 1.5 years of age, which demonstrate a sustained increase in CPN proportions under APM conditions compared to canonical medium (**Fig. 3b and**

Extended Data Fig. 6). In addition, we extended MEA recordings of APM cultures to 2 years of age and observed preserved neuronal activity at this stage (**Fig. 4d and Extended Data Fig. 10e**). At this time, these represent the oldest APM cultures we have generated to date. Together, these results provide direct evidence that APM supports neuronal maintenance and functional activity beyond the time point at which neuronal loss is observed in canonical conditions.

The study is technically impressive and provides an unprecedented long-term dataset on human cortical organoids. However, the current version is largely descriptive and lacks key functional validations.

We thank the reviewer for their positive assessment of the uniqueness and impact of our work. With the integration of the extensive new data included in this revision, we have further strengthened the manuscript along multiple lines, including additional functional validations across exceptionally long timeframes, as detailed above. Of note, we provide extensive electrophysiological validation of neuronal and circuit function using multielectrode array (MEA) recordings, including the demonstration that these organoids maintain functionally-active circuitry for at least 2 years in culture, at a time point that is unprecedented in human *in vitro* systems. The integration of the new data with our existing analyses shows that we are able to generate organoids which develop a variety of neuronal and glial populations, which follow endogenous maturation programs, maintain metabolic health, and participate in circuit wiring and activity over very extended timelines. We show that over years in culture, progenitors in these organoids change their functional properties, progressively restricting their fate potential as we demonstrate with the heterochronic chimeroid perturbations. We greatly appreciate the constructive suggestions of the reviewer, which have helped shaping this revised, stronger version of the manuscript.

Referee #3 (Remarks to the Author):

The submitted ms, by a well-established group of high international recognition, reports on a powerful approach to gain further understanding in the relevance and value of cortical organoïds as model systems, focusing on later stages of cortical maturation. The long-term cultures combined with the extensive in-depth bio-informatic analysis constitute an impressive tour de force. The data represent an invaluable and unique resource for all the neuroscience community.
The data are clearly illustrated and thoroughly documented, they rely on the highest standard in bio-informatic analyses and statistics.

We are grateful to the review for recognizing the value and impact of our work, and for the constructive suggestions for the revisions.

The first section of the ms is based on the unprecedented maintenance of cortical organoïds over 5 years- which represents a truly unique achievement- with their molecular and epigenetic / methylation characterization.
The second part which reports on a “functional analysis” of cortical organoïds focuses on a shorter period of 6-12 months, and is based on the analyses of the functional activity in two culture conditions- APM medium vs CDM4. This raises the question of the representativeness of cell types (Figure 1) as documented in > 5 years organoids (in CDM4), which seems to favor neural populations. It therefore appears somehow tricky to link these two sections.
The third section addresses the fascinating question of the maintenance of a cell's memory once it has passed through the various stages of differentiation. The approach which relies on the establishment of chimeric organoids - young vs. ‘old’ – is implemented during the 0-12 month

culture period. Therefore, the term “old” used in this section does not correspond to “> 5 years organoids “refered to in the first section. This introduces some confusion.
As a general comment, the discussion is lacking a necessary perspective regarding the differences in kinetics and tempo between long term cortical organoïds analysed in the first section and cortical organoïds used in the two other sections of the ms.

We thank the reviewer for raising this important point. The first section of the manuscript describes the longest cortical organoid trajectory reported to date, extending up to five years in culture. In contrast, the organoids used in the subsequent sections were generated under APM culture conditions, which were introduced to increase excitatory neuronal representation. This approach was implemented at a later stage, and samples collected for cultures up to now; as of yet we have not generated samples past 2 years in culture for this model.
Following the reviewer’s suggestion, we extended the analysis of APM organoids to explore a more direct comparison of developmental kinetics between media. Specifically, we performed functional recordings up to two years in culture and transcriptomic analyses up to 1.5 years. Within this time frame, APM organoids showed a developmental progression that closely resembled that of organoids grown in canonical media. In particular, they displayed a positive correlation with time for the same gene module (MCP4) identified by DIALOGUE along the *in vivo* maturation trajectory and CDM4 organoid developmental timeline (**Fig. 3h-k**).
Analysis of cycling dynamics and proliferation scores revealed differences between APM and CDM4 conditions. APM increased the proportion of cycling apical radial glia (aRG) cells, whereas CDM4 preferentially enhanced proliferation in interneuron progenitors, even though all cell types were still represented, indicating that both media support the development of neuronal and glial populations (**Extended Data Fig.6**).

Specific comments:

The title should be modified (remove “in culture”).

We have edited the title following the reviewer’s suggestion.

Line 140 : the CpG methylation profile appears almost definitively established around the 6 to 9 months transition, therefore not so ‘progressive’ as mentioned. Could the authors comment on this point?

The reviewer is correct that global CpG methylation levels in cortical organoids largely stabilize by the 6-9-month time window, consistent with early establishment of the global cortical methylome, as we mention in the text. However, this stabilization does not preclude continued epigenetic maturation at specific genomic loci. As shown in **Extended Data Fig. 2**, differentially methylated regions and developmentally-regulated loci continue to undergo gradual, directional methylation remodeling over subsequent years in culture. This kind of sustained, locus- and time-specific epigenetic change is similar to mechanisms of epigenetic regulation employed during endogenous development. We therefore use the term “progressive” to refer to these more specific epigenetic changes, rather than to ongoing global shifts in CpG methylation.

Lines 193-201 : While the differences observed in the distribution of different cell types are documented, the occurrence of proliferation foci/islands vs a uniform distribution of proliferating progenitors in long term organoïds remains to be clarified. For instance, regular monitoring of Ki67 labeling in long term organoids will provide crucial information for understanding how the balance between proliferation and differentiation is maintained in the

long run - an intriguing question given that the morphology/size of the organoids changes very little over time (Figure 1a). Similarly, detection of apoptotic or necrotic cells is essential to gain insight into the homeostasy and evolution of this balance over time.
In view of their potentially different impact on proliferation vs. differentiation, the comparison between the two culture media (APM and CDM14) should be provided.

Following the reviewer's suggestion, we have now compared cell-cycle dynamics along the developmental trajectory between APM and CDM4 conditions, by computing cycling-related Seurat module scores across individual progenitor populations in the scRNA-seq datasets. This data is now included in (**Extended Data Fig. 6k and Supplementary Table 11**).
To assess the health of the cells, we have now examined the metabolic state of the organoids through several assays. We examined cell type–specific variation in metabolic processes relevant to organoid biology, including apoptosis, hypoxia, and glycolysis, over the 5 year timecourse, using curated gene sets from MSigDB. We observed an overall increase in metabolic signatures over time; however, subsetting the analysis by cell type revealed that this effect was largely driven by progenitor populations (**revised Extended Data Fig. 8).** Other cell types did not show a comparable significant increase over time. This observation is consistent with the high metabolic demands of progenitors. We note that enrichment of glycolysis and hypoxia gene sets is also observed in a subset of endogenous human fetal progenitors *in vivo* (Trevino et al., *Cell*, 2021; Polioudakis et al., *Neuron*, 2019; Uzquiano et al., *Cell*, 2022).
As a complementary approach, we performed hypoxia labeling using the pimonidazole detection reagent. Consistent with our previous findings (Uzquiano *et al.*, Cell, 2022), pimonidazole signal was largely restricted to the organoid core, while much of the peripheral tissue remained non-hypoxic, even after 12 months *in vitro* (**Extended Data Fig. 8e**).

RESPONSE TO REVIEWERS:
We thank the Reviewers for their enthusiasm for our work and for their thoughtful comments and constructive feedback. We have now addressed all of the final comments and revised the text and figures accordingly. Here, we first highlight the major changes to the revised manuscript and then address each of the Reviewers' comments point-by-point.

Key new experiments and data in the revised manuscript:

- **Extended characterization of long-term organoids up to 5.8 years**: we added additional immunohistochemistry samples at 4 years (n=2 organoids, NeuN IHC) and 5.8 years (n=1 organoid, SATB2 and NeuN IHC), as well as additional neuronal marker analyses in bulk RNA-seq datasets, thereby validating long-term neuronal persistence.

- **Methylome analysis**: We have extended whole-genome methylome analysis to further define the developmental state of the organoids at the DNA methylation level. Specifically, we applied the fetal brain clock (FBC) developed by *Steg et al. (2021)*, an epigenetic clock specifically trained on human prenatal brain samples spanning gestational weeks (GW) 8-40.

Reviewer#1
I believe the authors have effectively addressed the reviewers' concerns, leading to improvements in the manuscript. The revisions are clearly articulated and enhance the overall quality. The paper is now in good shape and ready for publication.

We thank the reviewer for the positive evaluation of our work and for recognizing the improvements made during the revision process. We greatly appreciate the constructive feedback, which helped strengthen the manuscript.

Reviewer#2
Overall, the authors addressed most reviewer's comments. Of improvement, is the addition of the characterization of cell fidelity up to 5 years of age (starting line 248). They are able to align molecular shifts in glia and neurons that occur within this time frame with that of changes that occur in human brain tissue.

We thank the reviewer for appreciating the difficulty of executing this experiment and the importance of the data collected in supporting a central claim of our paper that organoids are able to develop for an unprecedented 5+ years in culture, and that during this time, they continue to mature following molecular and temporal trajectories of endogenous tissue.

The diminished neuron representation at this experiment's endpoint remains a major concern and critical flaw with the dataset given this is a centeral claim. The authors account for this in speculating that neurons are particularly susceptible to dissociation steps in the library preparation for sequencing, but this is not supported with evidence and is not the case for human brain tissue which should be the reference for this study.

The reviewer is correct that the overall proportion of neurons changes. Over time, the cellular composition of the organoids reflects, first the production of all of the cell types, second their maturation, and third, a change in ratio between neurons and glia, with glia constituting a large proportion of the organoid at 5 years. This glial expansion is consistent with our knowledge of endogenous cortical development, and these organoids offer the very first experimental system for the study of human astroglial biology over an unprecedented number of years.

The lowering of neuronal representation over time is also not completely unexpected given that the system lacks processes that promote neuronal maturation and survival in vivo, such as

evoked activity. However, the reduction of neurons we observed in the single-cell RNA sequencing datasets was greater than expected. This is why, given the known fragility of neurons in dissociation, we speculated that the lack of neuronal representation in the older single-cell RNA sequencing datasets may result from differential loss of neurons during dissociation, rather than the absence of neurons in the originating cultures.

We therefore applied other methods to assess neuronal presence that do not require single-cell dissociation of the organoids. Immunohistochemistry data indeed show that neurons are retained throughout the culture period (up to 5.8 years), as demonstrated by NeuN labeling at 2, 3, 4, and 5.8 years and the cell-specific CPN marker SATB2 at 5.8 years, showing the presence of CPN glutamatergic neurons after extended culture (included now in **ED Fig 2j** of the revised manuscript). Similarly, bulk RNA-seq shows expression of markers of both excitatory and inhibitory neurons at 2 and 3 years (included now in **ED Fig 2K** of the revised manuscript). Furthermore, we show that culturing these organoids in activity-permissive media leads to enhanced activity and persistence of neuronal networks for at least 2 years (the latest timepoint for which samples cultured in this media are as yet available; **Figure 4D**), demonstrating that neurons are maintained and remain active within circuitry over multiple years of culture. We have revised the text and figures to clarify all of these points.

Regarding the methods used for profiling endogenous human brain tissue, recent large-scale single-cell profiling of human tissue, in particular postnatal samples, has largely used nuclear profiling rather than whole cells (e.g., *Zhang et al., Nature Neuroscience 2026; Wamsley et al., Science 2026; Wang et al., Nature 2025; Velmeshev et al., Science 2023*). The use of nuclei reduces issues with differential fragility of cell types, allowing better preservation of neuronal representation upon tissue dissociation. We chose to use whole-cell sequencing, in part because retention of endoplasmic transcripts improves sensitivity, and in part to reduce batch effects in comparisons with our existing datasets from younger organoids. Redoing this work by other methods such as nuclear sequencing or spatial transcriptomics would require growing organoids across the entire timeline all the way to 5 years, as we do not currently have any remaining samples for ages older than 1.5 years.

Relatedly, I would like to see a more thorough and clearly articulated discussion of Figure 2b. The authors note that a small population of neurons persists at year 5. But this population is not present at any timepoints prior to 5 years, and they do not address this apparent absence of this "neuron" cluster at earlier time points. This raises questions about how this cluster is defined and why it emerges only at later stages. In addition, while several neuronal subtypes are listed in the cell type breakdown, the identity of this broadly labeled "neuron" cluster is unclear does it correspond to excitatory neurons, or another subtype?

As discussed above, our data shows that neurons are present at all ages along the timeline. We believe the unexpected lack of representation at later ages of the single-cell-sequencing datasets represents differential loss during dissociation; the samples at 5 years happened to retain sufficient neurons to be identifiable upon clustering. Regarding the classification of the 'neuron' cluster observed in the 5-year single-cell RNA sequencing dataset, within this cluster we can identify neurons expressing either excitatory or inhibitory markers (included now in **ED Fig 3b** of the revised manuscript). We decided not to subcluster this cluster further, as the small number of cells in it (101 cells) makes subclustering potentially unreliable, and therefore chose to label this cluster simply as 'neurons' out of an abundance of caution. We also provide a list of all differentially-expressed markers (for all clusters) in **Supplementary Table 5**.

I am also concerned about the large "unknown" cell population, which appears to comprise approximately 40% of cells at year 5. The manuscript does not provide sufficient interpretation or characterization of this population, making it difficult to assess its biological relevance.

Regarding the classification of the 'unknown' cells observed in the 5-year single-cell RNA sequencing dataset, these cells represent two clusters that emerged from the initial clustering, which collectively make up 33% of the dataset. Cluster 4 (12.6% of the cells at 5yr) expresses markers of truncated radial glia (tRG), however, out of an abundance of caution we initially decided not to classify them as such because they also have high expression of ribosomal genes. It has been reported that tRG have high expression of ribosomal genes, but not to the extent we observe here. The 'Unknown' component of Cluster 0 (20.6% of the cells at 5yr) consists of cells that pass all quality control metrics but show low expression of specific cell type markers; we note that we find small populations of such indistinct cells at other ages as well, although in lower numbers. We and others have also found small unclassifiable populations in single-cell sequencing of endogenous mouse and human tissues. We have been cautious in annotating these cell clusters; however, we provide a list of differentially-expressed markers for all clusters, including these 'unknown' clusters, in Supplementary Table 5.

More broadly, the central claim of the study that extended culture to 5 years yields a well-characterized system that closely tracks human brain development seems well supported up to approximately one year. Beyond this point, however, there appears to be a marked decline in neuronal representation and clarity of cell identity. This raises important concerns about the fidelity and utility of the model at later time points, and it remains unclear what applications these long-term cultures are best suited for in their current form.

We hope that with the clarifications above we have addressed the reviewer's concerns. In this manuscript, we show that human brain development within a reductionist system like an organoid can be extended over unprecedented durations of over 5 years in culture. By enabling such extended timelines, and through multimodal investigation of the organoids, we were able to discover that organoid cells continue to develop and mature along transcriptional and epigenetic programs similar to endogenous human cortex, indicating that temporal trajectories of maturation develop in organoids as self-emergent properties of the system over many years in culture. We show that activity-enhancing conditions allow neuronal survival and maintenance of active neuronal circuitry to be retained beyond previously-known limits. Finally, by mixing cells from young and old organoids within a heterochronic chimeroid system, we show that organoid cells retain an epigenetic memory of developmental time and can recall their current developmental age to direct fate specification of temporally-appropriate progeny.

We agree that this is an *in vitro* system, and as such has limitations. We apologize for not making our reasoning sufficiently clear on some of the important points raised by the reviewer. We hope that we have now clarified these points and the core findings of this study.

Reviewer#3

In our opinion, the authors have provided convincing arguments that address all the points of concern raised during the first round of review.
More, they have remarkably built upon their initial results and therefore significantly strengthened the significance of their work.
In its present form, the submitted work provides unrivalled and remarkable evidence for the ability of organoïds to mimic brain development over extended periods of time.

We thank the reviewer for the thoughtful assessment of our work. We greatly appreciate the recognition that the revised manuscript addresses the concerns raised during the initial review and that the additional analyses and experiments have strengthened the study. We are especially grateful for the reviewer's appreciation of the significance of our findings.

Reviewer#4

Summary: I was asked to specifically review the WGBS-related analyses in the revised manuscript, which were well done. I had a few suggestions around analyses that could provide additional context, as well as a few minor suggestions for the Methods:

Estimating biological age with this additional reference profile – derived from prenatal brain tissue - could perhaps be informative: https://pubmed.ncbi.nlm.nih.gov/34174924/ to contrasts the postnatal cortical clock currently in the manuscript (and pan-tissue Horvath clock)

As suggested by the reviewer, to further contextualize the developmental state of the organoids at the DNA methylation level, we have now applied the fetal brain clock (FBC) developed by Steg et al. (2021), an epigenetic clock specifically trained on human prenatal brain samples spanning gestational weeks (GW) 8-40.

Applying the FBC to our WGBS data revealed that the earliest samples, including NSCs and 1-month organoids, were predicted to correspond to approximately 10-12 gestational weeks, consistent with a first-trimester-like methylation state. Organoids cultured for 3 months or longer were predicted to fall within approximately 18-28 gestational weeks, indicating progressive acquisition of fetal cortical methylation signatures during long-term culture. Across all samples, predicted fetal brain age showed a positive relationship with time in culture (R = 0.54, p = 0.056). This analysis is now included in **ED Fig 2h** of the revised manuscript.

DNAm signatures are probably a more stable and predictive substrate for estimating underlying cell composition than RNA levels – it would be interesting to perform analogous transfer learning to pre- and post-natal single cell WGBS datasets (eg https://www.nature.com/articles/s41586-024-08030-7) to complement the existing RNA-based analyses

We agree that leveraging DNA methylation profiles to infer underlying cell composition represents a promising complementary strategy to transcriptome-based approaches, particularly given the relative stability of methylation signatures.

We explored the feasibility of performing analogous transfer-learning analyses using recently published prenatal and postnatal single-cell WGBS reference datasets, including the study suggested by the reviewer (Heffel et al., 2024). However, implementing this analysis would require substantial additional efforts, including acquisition and preprocessing of controlled-access reference datasets, adaptation of transfer-learning pipelines to cross-platform methylome data, and careful benchmarking against our existing RNA-based annotations. We therefore believe that a rigorous implementation and interpretation of such analyses would extend beyond the scope and timeline of the current revision.

Methods:

a) It did not seem obvious why the hg19 reference genome was used for the WGBS alignments, given its successor - hg38 – was released over a decade ago. Some statement/clause in the methods offering some rationale for this choice should be provided.

We agree that hg38 represents the current human genome reference assembly. In this study, hg19 was used primarily to maintain compatibility and coordinate consistency with previously-generated reference datasets, annotation resources, and downstream comparative analyses incorporated into our methylation analysis pipeline, including publicly-available developmental brain methylation datasets and established epigenetic clock resources.

Importantly, all samples and comparative analyses were processed uniformly within the same reference framework. Because our analyses focused primarily on genome-wide and regional DNA methylation patterns rather than fine-resolution structural variation or novel genomic annotations, we do not expect the use of hg19 versus hg38 to materially affect the biological conclusions of the study. We have now clarified this rationale in the Methods section.

b) The targeted sequencing depth/coverage should for the WGBS should also be provided, eg "Sequencing was conducted on an Illumina NovaSeq 6000 platform, generating 150 base paired-end reads[, targeting XX reads (or YYx coverage) per sample]"

We have now updated the Methods section to clarify the targeted sequencing depth and sequencing platforms used for WGBS generation. Specifically, libraries were sequenced using 150 bp paired-end reads, targeting approximately 800–900 million reads per sample.

Reviewer #2:

Overall, the authors addressed most reviewer's comments. Of improvement, is the addition of the characterization of cell fidelity up to 5 years of age (starting line 248). They are able to align molecular shifts in glia and neurons that occur within this time frame with that of changes that occur in human brain tissue.

We thank the reviewer for appreciating the difficulty of executing this experiment and the importance of the data collected in supporting a central claim of our paper that organoids are able to develop for an unprecedented 5+ years in culture, and that during this time, they continue to mature following molecular and temporal trajectories of endogenous tissue.

The diminished neuron representation at this experiment's endpoint remains a major concern and critical flaw with the dataset given this is a centeral claim. The authors account for this in speculating that neurons are particularly susceptible to dissociation steps in the library preparation for sequencing, but this is not supported with evidence and is not the case for human brain tissue which should be the reference for this study.

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We therefore applied other methods to assess neuronal presence that do not require single-cell dissociation of the organoids. Immunohistochemistry data indeed shows that neurons are retained throughout the culture period (up to 5.8 yrs), as demonstrated by NeuN labeling at 2 and 3 years (ED Fig 2i of the revised manuscript; excerpted in Fig A below for ease of reference), and by newly-performed NeuN IHC on 4 and 5.8 year samples (Fig A below, to be included in the ED figures). Similarly, bulk RNA-seq shows expression of markers of both excitatory and inhibitory neurons at 2 and 3 years. Some of these markers were included in ED Fig 2j in the manuscript; we show these markers along with additional markers in Fig B below (to be included in the ED figures). Furthermore, we show that culturing these organoids in activity-permissive media leads to enhanced activity and persistence of neuronal networks for at least 2 years (the latest timepoint for which samples cultured in this media are as yet available; Figure 4D), demonstrating that neurons are maintained and remain active within circuitry over multiple years of culture. We are happy to revise the text and figures to clarify all of these points.

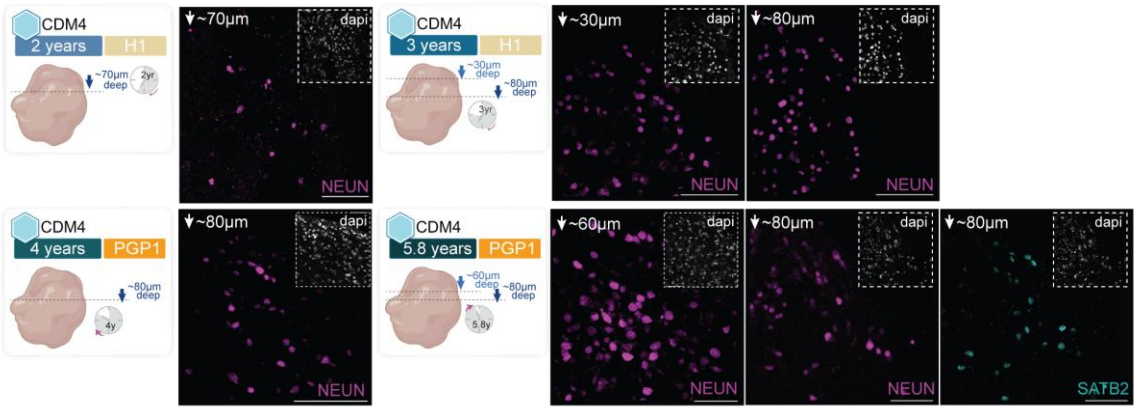

Fig A. Expression of NeuN in CDM4 organoids by IHC at 2 years and 3 years (top, excerpted from ED Fig2i for ease of reference), and at 4 and 5.8-years (bottom, new data), demonstrating the presence of neurons throughout the culture period.

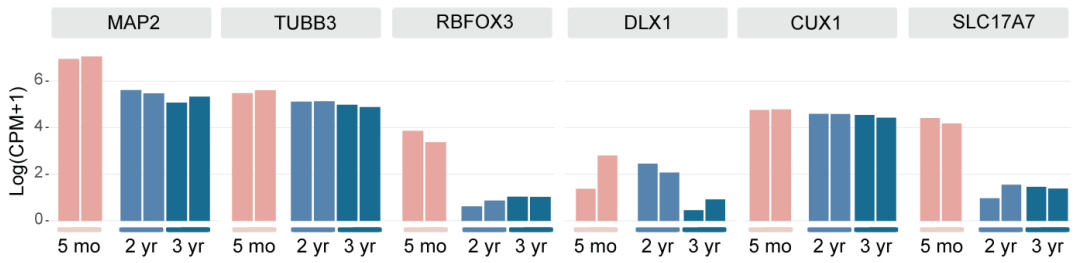

Fig B. Expression of excitatory and inhibitory neuronal markers at 5 months, 2 years, and 3 years by bulk RNA sequencing.

Regarding the methods used for profiling endogenous human brain tissue, recent large-scale single-cell profiling of human tissue, in particular postnatal samples, has largely used nuclear profiling rather than whole cells (e.g., Zhang et al., Nature Neuroscience 2026; Wamsley et al., Science 2026; Wang et al., Nature 2025; Velmeshev et al., Science 2023). The use of nuclei reduces issues with differential fragility of cell types, allowing better preservation of neuronal representation upon tissue dissociation. We chose to use whole-cell sequencing, in part because retention of endoplasmic transcripts improves sensitivity, and in part to reduce batch effects in comparisons with our existing datasets from younger organoids. Redoing this work by other methods such as nuclear sequencing or spatial transcriptomics would require growing organoids across the entire timeline all the way to 5 years, as we do not currently have any remaining samples for ages older than 1.5 years.

Relatedly, I would like to see a more thorough and clearly articulated discussion of Figure 2b. The authors note that a small population of neurons persists at year 5. But this population is not present at any timepoints prior to 5 years, and they do not address this apparent absence of this “neuron” cluster at earlier time points. This raises questions about how this cluster is defined and why it emerges only at later

stages. In addition, while several neuronal subtypes are listed in the cell type breakdown, the identity of this broadly labeled “neuron” cluster is unclear does it correspond to excitatory neurons, or another subtype?

As discussed above, our data shows that neurons are present at all ages along the timeline. We believe the unexpected lack of representation at later ages of the single-cell-sequencing datasets represents differential loss during dissociation; the samples at 5 years happened to retain sufficient neurons to be identifiable upon clustering. Regarding the classification of the ‘neuron’ cluster observed in the 5 year single-cell RNA sequencing dataset, within this cluster we can identify neurons expressing either excitatory or inhibitory markers (Fig D below). We decided not to subcluster this cluster further, as the small number of cells in it (101 cells) makes subclustering potentially unreliable, and therefore chose to label this cluster simply as ‘neurons’ out of an abundance of caution. This said, we are happy to include a discussion of the markers expressed by this cluster in the manuscript. We also provide a list of all differentially-expressed markers (for all clusters) in Supplementary Table 5.

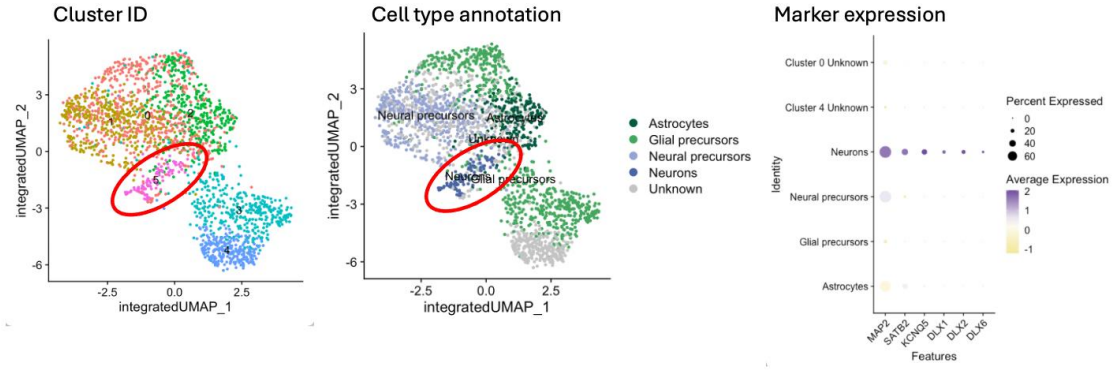

Fig D. Expression of neuronal markers within the ‘neuron’ cluster in the 5 year single-cell RNA sequencing dataset

I am also concerned about the large “unknown” cell population, which appears to comprise approximately 40% of cells at year 5. The manuscript does not provide sufficient interpretation or characterization of this population, making it difficult to assess its biological relevance.

Regarding the classification of the ‘unknown’ cells observed in the 5 year single-cell RNA sequencing dataset, these cells represent two clusters that emerged from the initial clustering, which collectively make up 33% of the dataset. Cluster 4 (12.6% of the cells at 5yr) expresses markers of truncated radial glia (tRG), however, out of an abundance of caution we initially decided not to classify them as such because they also have high expression of ribosomal genes. It has been reported that tRG have high expression of ribosomal genes, but not to the extent we observe here. A possible alternative approach would be to label this cluster as high-Ribosomic tRG and include this caveat in the text. The ‘Unknown’ component of Cluster 0 (20.6% of the cells at 5yr) consists of cells that pass all quality control metrics but show low expression of specific cell type markers; we note that we find small populations of such indistinct cells at other ages as well, although in lower numbers. We and others

have also found small unclassifiable populations in single-cell sequencing of endogenous mouse and human tissues. We have been cautious in annotating these cell clusters; however, we provide a list of differentially-expressed markers for all clusters, including these ‘unknown’ clusters, in Supplementary Table 5. We can also add some explanation of this in the text, of course.

More broadly, the central claim of the study that extended culture to 5 years yields a well-characterized system that closely tracks human brain development seems well supported up to approximately one year. Beyond this point, however, there appears to be a marked decline in neuronal representation and clarity of cell identity. This raises important concerns about the fidelity and utility of the model at later time points, and it remains unclear what applications these long-term cultures are best suited for in their current form.

We hope that with the clarifications above we have addressed the reviewer’s concerns. In this manuscript, we show that human brain development within a reductionist system like an organoid can be extended over unprecedented durations of over 5 years in culture. By enabling such extended timelines, and through multimodal investigation of the organoids, we were able to discover that organoid cells continue to develop and mature along transcriptional and epigenetic programs similar to endogenous human cortex, indicating that temporal trajectories of maturation develop in organoids as self-emergent properties of the system over many years in culture. We show that activity-enhancing conditions allow neuronal survival and maintenance of active neuronal circuitry to be retained beyond previously-known limits. Finally, by mixing cells from young and old organoids within a heterochronic chimeroid system, we show that organoid cells retain an epigenetic memory of developmental time and can recall their current developmental age to direct fate specification of temporally-appropriate progeny.

We agree that this is an in vitro system, and as such has limitations. We apologize for not making our reasoning sufficiently clear on some of the important points raised by the reviewer. We hope that we have now clarified these points and the core findings of this study.