

Developmental xenocortication using human-derived organoids in mice

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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)

In this study, Kaganovsky et al generate a mouse model in which the endogenous mouse cortex is largely depleted and replaced by human cortical organoid tissue. The authors characterize the cell populations and behavior of the apallial mice and show that the transplanted organoids populate the cortical region with a variety of glutamatergic cell types including putative L5-ET neurons. The authors measure activity within the transplanted graft and demonstrate robust, synchronized activity using calcium imaging and LFP recordings. Lastly, the authors measure spontaneous behavior using MoSeq and assess motor, sensory, and memory related behaviors across control, apallial and transplanted mice.

The strengths of the manuscript are the establishment of a new xenograft model (this is the first time to my knowledge that hCOs have been transplanted into apallial mice), the state-of-the-art analyses including behavior analysis with MoSeq and in vivo Ca²⁺ imaging and LFPs. The experiments appear to be rigorously done and the data are clearly presented in the figures.

The main weakness is that the new biological conclusions which we can draw from this study are relatively limited. Genetically apallial mice have been generated and well-characterized in several other studies, including in Kweon H, et al Cell Reports, 2021, which the authors should consider citing. hCOs have also been transplanted into rodent hosts in previous studies; therefore this aspect is not particularly novel. The new aspect here is the combination of apallial mice and human hCOs, which, while interesting, seems a somewhat limited advance. The authors suggest that the tAP-hCO model could facilitate disease modeling but it would have been helpful to see an example of how this could be done. It seems that the inherent variability of this system would be challenging to implement in a disease modeling context. I think more could be done in terms of understanding the mechanisms by which the human neurons integrate with and connect to the host cells to define the intrinsic programs that govern this. For example, the authors emphasize that the human cells widely innervate the host tissue but is this connectivity stochastic and random or does it recapitulate the specificity that occurs during typical brain development? Questions like this would be interesting to further explore in this model, beyond the initial characterization that was done here.

Specific points:

- 1) The introduction of multiple organoids into the same host seems like it would increase the potential variability of this model. i.e. it would be challenging to get the same composition of cell types from mouse to mouse as each organoid could be slightly different.
- 2) There are a number of aspects of this graft model that would be important to explore further. For example, do the host GE-derived GABAergic neurons migrate into the host cortical tissue? If yes, when does this occur? If not, do these cells populate other brain regions or do they die off?
- 3) In addition, is there any anatomical structure or layering of the human cortical graft?
- 4) What is the neuron to astrocyte (or other glial cells) ratio within the graft? What is the excitatory neuron to inhibitory neuron ratio? How much infiltration of mouse cells is there (either neurons or glia) into the graft region?

- 5) The authors suggest that the human cortical neurons project to the spinal cord. This is shown with anatomical approaches only and it would be useful to verify this with electrophysiology.
- 6) A key question related to the above point is whether the connectivity of the human neurons is selective or random and stochastic. i.e. Do the human cortical neurons connect to their expected partners? either within the graft or outside of the graft? If the connectivity is imprecise or random, then the model will be less valuable for studying circuit-level questions.
- 7) The authors show that the human graft has robust and synchronous activity. This pattern of activity is very different than the sparse activity that is typically observed in cortical circuits. Does this activity propagate outside of the graft to downstream structures?
- 8) The authors perform a thorough and rigorous investigation of different behaviors in the control, apallial and transplanted mice. However, the overall conclusion that can be drawn from these data is a bit unclear. The authors find some similarities and some differences but what general insight does this provide? For example, how do the authors interpret changes in gait and patterns of paw placement among the different mice?
- 9) The inter-subject variability that the authors report for the behavior analysis could be a concern for using this as a disease model where consistent & reproducible controls are important.
- 10) It looks like only the control and apallial mice were tested in the Von Frey and hot plate tests. It would be interesting to know whether sensory processing is intact in the transplanted mice.

Minor:

- 1) It is unclear what the authors are referring to with "sufficient" in this sentence. pg 5 ...indicating the heterozygosity for Emx1-cre and Esco2 is sufficient"

Referee #2

(Remarks to the Author)

This study by Kaganovsky et al uses mice with an ablated cortex to subsequently transplant hCOs and explore the connections made with the host. While aspects of this paper are done well, I have severe ethical concerns about the study and worry it is an attempt to do something just because we can. I do not believe sufficient efforts to justify the value of this study beyond a Frankensteinian fascination with the ability to do so have been considered, and the ethical considerations are very briefly mentioned in the discussion. In a contentious scientific environment politically, I worry that publication of this study would severely damage the reputation and regard of science in the public sphere, possibly irreparable. For me to feel able to endorse this study, I would want a therapeutic advance so phenomenal that no other system has or could have generated this outcome. As written, the paper definitely makes essentially a human/mouse chimera, ie a mouse with a "humanized brain" raising important ethical questions about sentience and consciousness of the human parts of the brain. If the journal chooses to proceed with publication, I would strongly encourage involving ethicists from the United States and Europe in the review of the manuscript alongside written ethical pieces about the possible considerations of the study. I would also encourage all reviews (including this) to be published alongside the manuscript. Scientifically, I also have some concerns detailed below:

- 1) It is unclear how the biological conclusions differ between this approach and existing in vivo transplantation methods. Although textual explanations for why existing methods are insufficient, a head to head comparison is not provided and should be to identify the advantage of this controversial system.
 - 2) The authors skip directly to behavior but there are many, many steps before that are not addressed and one worries that the behavioral improvements may be an artifact rather than due to the specific nature of the organoid transplant:
 - a. What happens if they put in as a control an organoid from a different brain region – how does it differ then in terms of molecular mechanisms and maturation?
 - b. How does maturation happen? Beautiful and elegant work using iPSC cells from Vanderhaeghe and colleagues has shown that maturation is intrinsic to human neotenic timing – the assays presented seem much too fast for molecular and circuit maturation to be complete in this system.
 - c. Cortical organoids do not have area specificity – do cortical areas emerge in these transplants? By what mechanism? When?
 - d. Although inhibitory neurons are addressed somewhat, it seems unlikely enough would be generated. Are there no seizure like activities? Where do inhibitory neurons migrate?
 - e. Do human-specific progenitor and neuronal populations emerge, or is there a restriction to subtypes shared between mouse and human?
 - f. Many other considerations about mechanisms and evolution that are not addressed or at all considered.
- This is a small number of the scientific considerations missed by this study, and as presented it is a flashy, surface level, ethically challenging paper that I cannot endorse for publication.

Referee #3

(Remarks to the Author)

This study tackles the challenge of limited access to human brain tissue for studying brain development and function in health and disease. Human stem cell-derived models such as organoids and assembloids, especially when transplanted into rodents, have started to address this issue. However, their integration is often constrained by competition with the host brain. To overcome this, the authors developed a new transplantation method using genetically modified mice lacking glutamatergic neurons in the cortex and hippocampus. This provides enough space for the growth of transplanted human cortical organoids (hCO), which integrate robustly. The hCOs expand and provide artificial pallial-like structure and generate

diverse human cell types, including layer 5 extratelencephalic neurons, which are hard to derive in vitro. These neurons project widely through the mouse brain and spinal cord. Functional imaging and electrophysiology reveal organized, cortex-like activity in the grafts. Behavioral analysis shows subtle but distinct effects post-transplantation. Notably, hCOs partially restore motor and working memory deficits caused by cortical depletion. This demonstrates the model's potential to study human neural circuits and therapeutic effects. Despite limitations—such as a lack of cortical layering and proper cerebral cortex morphogenesis—this method significantly advances the field. It may offer insights into circuit-level abnormalities in patient-derived cells. The model will also enable deeper exploration of human neurodevelopment and disease. The manuscript is well-written, scientifically sound, and presents experiments conducted using state-of-the-art, cutting-edge methodologies. Below, I provide suggestions and comments aimed at further strengthening the manuscript.

Major concerns

> Figure 1f-i. The snRNA-seq analysis of adult "apallial" mouse brains reveals a marked reduction in most cortical glutamatergic neurons, as expected. This loss is accompanied by a decrease in GABAergic interneurons, which are typically located within the cortex under normal conditions. In wild-type mice, cortical interneurons originate from the subpallium. In the apallial brains, however, it remains unclear whether these interneurons are lost due to cell death or if they have been spatially redistributed and possibly adopted a new fate. Along this line, it is striking to see that few host GAD65/67 interneurons repopulate the hCO graft at 5 months. This suggests that some host interneurons may have remained localized ventrally but underwent reactivation of their dorsal migration in response to the human graft.

> The manuscript would benefit from a more in-depth immunohistochemical characterization of the cellular composition within the hCO graft. Specifically, are the cell types identified by snRNA-seq in Figures 2g-i also detectable within the graft through immunostaining where are they placed and to which cell partner are they connected with (synapses)? Additionally, is there any evidence of emerging layer-like organization within the graft? Finally, further clarification is needed regarding the presence and distribution of inhibitory and excitatory synapses, particularly between host- and graft-derived interneurons and human projection neurons. Finally, to what extent is the graft undergoing differentiation? What proportion of cycling progenitor cells remains? Additionally, it would be important to assess whether the graft contains any off-target derivatives unrelated to the forebrain lineage.

> Figure 2f. The parasagittal sections illustrate the extensive integration of the graft into the host brain. While graft-derived axonal tracts are visible, there also appears to be graft invasion into the host subpallium. Can this be systematically assessed? If confirmed, what might be the functional implications of such heterotopic localization of graft-derived cells?

> Extended data figure 4. It would be important to clarify whether the hCO graft induces inflammation and/or glial scarring in the host mouse brain. Can this be quantified and reported? Assessing the extent of astrogliosis or microglial activation would be valuable for evaluating the host immune response. This information is particularly relevant from a translational perspective, as it informs the feasibility of using such chimeric models for studying human brain disorders.

> Extended data figure 7h. This figure panel is not referenced in the main text. It is also surprising that the genetically "apallial" mouse does not show deficit in social novelty, as this behavior is largely dependant on CA2 function, and the hippocampus is absent from this model. How can this be explained?

> The authors propose that the hCO-grafted apallial mouse model represents a valuable platform for studying human brain pathology. From a translational standpoint, this approach holds great promise for modeling neurodevelopmental disorders. It would be particularly compelling to investigate whether human cortical organoids (hCOs) derived from patients with genetic conditions, such as those leading for instance to seizures could be used in this chimeric in vivo system. This could allow researchers to assess whether key pathological features—such as hypersynchrony in cortical networks as seen in vivo in epileptic animals but also in cultured human organoids PMID: 34426698—are recapitulated in the grafts. Such findings would spark more interest for this novel mouse model and could provide crucial insights into human disease mechanisms and pave the way for the development of more targeted therapeutic strategies.

Minor concerns

> The references to some Extended Data figures do not follow the chronological order of the main text. These figures should be reorganized to align more closely with the flow of the manuscript.

> The *Emx1-cre+/-;Esco2fl/fl;Prkdcscid/scid* is not completely apallial as the piriform cortex is visible (see extended data figure 1g, figure 1b and PMID 25744204 mentioning some residual cortical neurons in a protruding ridge extending bilaterally from the piriform cortex (also seen in fig 1B)) – the authors should rename the mouse line accordingly.

> Figure 2g. It is interesting to see that the grafted hCOs also generate GABAergic interneurons (shown in green), which may contribute to balancing the activity of the artificial human neural circuits. This important observation should be mentioned in the main text, ideally on page 7, as it highlights a key aspect of functional circuit integration with potential relevance for modeling neurodevelopmental disorders involving excitatory/inhibitory imbalance.

(Remarks to the Author)

I was asked to perform an ethical review of the manuscript. Included in the request I was asked to discuss in my report whether the following aspects of the research comply with the ISSCR guidelines for stem cell research (<https://www.isscr.org/guidelines>) and with local regulations, and whether the authors have provided sufficient details about these aspects in the ethics statement:

- (i) Conditions of procurement/donation of human embryos and tissues;
- (ii) Content and language of the consent forms;
- (iii) Oversight of the research: the name of the committee and, for the most sensitive research, the expertise of its members (researchers, ethicists);
- (iv) Appropriate procedures to ensure privacy of patient data (please confirm that no confidential data are disclosed in the manuscript).

My comments are meant to respond to this request from Nature. I have noted the following Ethics Statement in the manuscript:

"All experiments involving human cells complied with all relevant guidelines and regulations. Human donors in this study consented to the use of their cells to generate hiPS cells and derived cells. The source of the cells and their institutional approvals are listed in the reporting summary. This study also benefitted from a consultation with an external ethical committee. Approval for transplantation of hCO into mice was obtained from the Stanford Laboratory Animal Care (APLAC) Research Compliance Office. No discernible locomotor or memory deficits were detected in transplanted animals and their wellbeing was monitored throughout."

I am encouraged and impressed by the letter from the ad hoc, independent ethics committee formed to provide advice and expert guidance to Dr. Sergiu Pasca and his research team regarding his neural organoid transplantation work. The people on this committee are highly qualified to assess the work, and I was pleased to see that Dr. Pasca engaged this group. I trust these individuals to guide this type of work.

That being said, I have several comments:

- 1) I would like to have seen more information in the letter from the ad hoc committee. What did they assess with respect to this particular study? Their letter states, "Dr. Pasca's work was approved and closely monitored by all the appropriate institutional bodies at Stanford University." However, other than the animal care and use committee, what other committees reviewed and approved the research? No approval documents were provided with the manuscript. While the authors have stated, "This study also benefitted from a consultation with an external ethical committee," it would be helpful to remark on what the committee's comments were with respect to this specific study.
- 2) Although it may seem obvious to the authors that the rodents that incorporate human cells into their brains do not experience consciousness in the human sense, I think it is important under the "ethics statement" to state something to this effect, as this is an ongoing area of concern for many members of the public – scientists, regulators, government officials, and laypersons alike. Two of the members of the special committee were said to have participated in the National Academies committee that authored the 2021 report, "The Emerging Field of Human Neural Organoids, Transplants, and Chimeras." (<https://doi.org/10.17226/26078>.) Some of the questions addressed by that National Academies committee were:
 - a. "How would researchers define or identify enhanced or human awareness in a chimeric animal?" and
 - b. "How large or complex would the ex vivo brain organoids need to be to attain enhanced or human awareness?"
- 3) Thus, could the authors make some comment in the "ethics statement" that addresses these issues and/or that reflects that the external ethical consulting committee reviewed and considered these issues with respect to this research study? Could the authors make some explicit statement in the publication that there was no evidence for "enhanced or human awareness;" the authors could define how they believe this and/or why it would not be possible with the type of transplantation and engraftment that occurred. They could elaborate by stating that the human cells were inadequate to create enhanced or human awareness (assuming this is true).
- 4) Another issue addressed by the National Academies report, "The Emerging Field of Human Neural Organoids, Transplants, and Chimeras" was: "Should patients give explicit consent for their cells to be used to create neural organoids?" While level of granularity may be too specific for most tissue donation consent forms, I would expect consent forms to include iPSC generation and differentiation and transplantation into animals. Indeed, an aspect of my review, as stated above, is to examine the conditions of procurement/donation of human tissues and to view the content and language of the consent forms. However, consent forms were not provided.
- 5) The Reporting Summary states the cell line sources as: "1208-2 UCLA IRB; 8119-1 UCLA IRB; 0524-1 Stanford IRB." However, these appear to be IRB protocol numbers and not cell lines. I have to assume that these IRB protocols were designed to obtain tissue for iPSC generation, but I cannot tell from the information provided. Thus, could the authors provide the titles of these IRB protocols? Could they provide the template consent forms? I would like to see that donors gave informed consent to derivation of iPSC cells and differentiation and even transplantation of the cells/tissue into laboratory animals. Supplementary Table S1 from this article gives a list of cell lines used:
Revah, O., Gore, F., Kelley, K.W. et al. Maturation and circuit integration of transplanted human cortical organoids. *Nature* 610, 319–326 (2022). <https://doi.org/10.1038/s41586-022-05277-w>. Which of these lines were employed in the present study, if any? It would be preferable to state this rather than list IRB approvals.

Version 1:

Reviewer comments:

Referee #2

(Remarks to the Author)

The revision substantially strengthens the manuscript. I appreciate the addition of a disease application, expanded ethical

discussion, clearer description of external ethics oversight, and the new benchmarking against prior transplantation paradigms. The new analyses supporting enrichment of L5-ET/corticospinal-like populations and the hypoxia proof-of-concept address part of my concern that the system's value over existing models was not sufficiently demonstrated.

However, my central concerns are only partially resolved. First, the ethical response remains grounded primarily in institutional oversight and absence of evidence for elevated capacities, rather than a more explicit articulation of the criteria that would justify this model relative to less ethically fraught alternatives. I would ask the authors to sharpen the Discussion to state more clearly: (i) what specific questions can only be answered in this system, (ii) what present limitations prevent over-interpretation of the behavioral findings, and (iii) what future findings would trigger heightened ethical concern or additional safeguards.

Second, while the new comparison to existing transplantation approaches is helpful, some mechanistic specificity remains unresolved. The manuscript would still benefit from a more explicit acknowledgment that the evidence for selective host-graft integration is predominantly anatomical, and that functional validation of long-range connectivity remains a key limitation.

Third, the lack of a region-mismatched graft control still leaves open whether some reported effects depend on cortical identity per se versus general engraftment of a proliferative human neural tissue. The authors claim other regions are not feasible, but there are other neural cells that are sufficiently proliferative that could be used as a control to explore whether this is a cortical identity issue versus a general proliferation question.

Overall, I view the manuscript as considerably improved, but I would recommend further revision focused on narrowing claims, clarifying what is uniquely enabled by this model and testing this with the additional control, and making the ethical justification more explicit and forward-looking.

Referee #3

(Remarks to the Author)

The authors have made a substantial effort in revising and improving their manuscript by incorporating a significant amount of new data and additional experiments. Overall, these additions satisfactorily address the majority of my previous concerns.

With respect to the hypoxia model - the approach taken to demonstrate the translational relevance of this system - a few minor points merit further attention.

First, regarding the figure panels, it would be helpful if the authors could explicitly indicate (e.g., with arrows) the regions of enhanced hypointense vasculature in Figure 5c to improve clarity. In addition, the manuscript would benefit from the inclusion of a triple immunostaining in Extended Data Figure 10a (DAPI, HIF1 α , and HNA) to better support graft localization, in a manner similar to what is presented in Figure 5b.

I am somewhat surprised by the apparent absence of HIF1 α expression in the host brain tissue upon severe hypoxia.

Considering the prolonged hypoxic conditions, one would expect some degree of impact on the surrounding mouse brain tissue, including in control condition, as suggested by the existing literature.

Finally, from a functional standpoint, the hypoxia-exposed tAP-hCO group displays an unexpected behavioral phenotype, with increased three-paw support during Catwalk session, which may indicate altered gait or balance and thus deviates from normal physiological patterns. Could the authors elaborate on how their model and such phenotype are relevant for understanding the disease?

Referee #4

(Remarks to the Author)

I wish to thank the authors for their detailed response to my questions about the ethical aspects and ethics reviews of this protocol. Indeed, I see that the investigators and Stanford University went to great lengths to obtain multiple expert reviews from institutional committees such as the IACUC and SCRO, as well as from a number of bioethicists and neuroscientists. This included Stanford University School of Medicine having convened an external committee composed of neuroscientists, legal scholars, ethicists, and patient advocates who reviewed all aspects of the study multiple times; I see that the review included assessing the rationale, built-in safeguards, monitoring plans, and ethical implications of the study. I am pleased to see the thoroughness with which this research study was reviewed. Thank you for providing the letter signed by the Chair of the ad hoc committee, Dr. Insoo Hyun, on behalf of the Hastings Center for Bioethics, elaborating on the focus of their reviews.

The Ethics statement in the paper is much improved. Besides providing clarification, an added benefit of having a more detailed Ethics statement is that it sets a standard for other researchers in the field. It is also reassuring to regulators and the public alike. Finally, it provides an educational function for students and trainees interested in entering the field.

Thank you for providing the IRB-approved consent forms, which I am glad to see include statements that hiPSCs and further derived cells and products from the participants' specimen donations may be transplanted into animals. In general, these specimen donation consent forms are thorough and fulfill the many requirements of consent forms for pluripotent stem cell work of this nature.

Thank you for revising the Reporting Summary, clarifying which numbers were cell lines and which numbers were IRB protocol numbers (which also corresponded to the consent forms).

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Point-by-Point Responses to Reviewers

We appreciate the reviewers' constructive comments and suggestions to improve our manuscript. We are delighted to see that the reviewers noted "this method significantly advances the field", includes "state-of-the-art analyses", "a thorough and rigorous investigation of different behaviors", is "scientifically sound", and they were "encouraged and impressed by the letter from the ad hoc, independent ethics committee."

To address the concerns raised, we conducted new experiments, provided several new analyses and revised the manuscript, as summarized below and detailed in the point-by-point responses. We believe these revisions have significantly strengthened our work.

In these point-by-point responses, reviewer comments are shown in **black** and our responses are shown in **blue**.

In brief, the major new experiments and analyses are:

1. We developed a disease application using this transplantation model to study hypoxic injury of the developing human cerebral cortex. Critically, we demonstrated the ability of the t^{AP}-hCO system to detect behavioral defects following hypoxic injury.
2. We performed an anatomical characterization of the human graft using both rabies tracing and diffusion weighted imaging to characterize the variability and specificity of connectivity.
3. We mapped the spatial distribution of cell types using immunostaining and MERFISH, revealing local structure and sparse migration of host GABAergic neurons.
4. We further investigated the generation of human L5-ET neurons with new analyses of transcriptomic mapping and direct comparison to other datasets, and leveraging new experiments with age-matched hCO transplantation into the mouse cortex.
5. We increased group size and extensively assessed the variability of t^{AP}-hCO at multiple levels, including in terms of cell diversity, circuitry, and behavior.
6. We elaborated on the ethical consultation process and updated the ethical statement accordingly.

In this revision, we **updated 26** figure panels, added **77 new** figure panels, and revised key sections of the text. We hope these additions and revisions overall address reviewers' concerns and make our manuscript suitable for publication.

Referee #1

In this study, Kaganovsky et al generate a mouse model in which the endogenous mouse cortex is largely depleted and replaced by human cortical organoid tissue. The authors characterize the cell populations and behavior of the apallial mice and show that the transplanted organoids populate the cortical region with a variety of glutamatergic cell types including putative L5-ET neurons. The authors measure activity within the transplanted graft and demonstrate robust, synchronized activity using calcium imaging and LFP recordings. Lastly, the authors measure spontaneous behavior using MoSeq and assess motor, sensory, and memory related behaviors across control, apallial and transplanted mice.

The strengths of the manuscript are the establishment of a new xenograft model (this is the first time to my knowledge that hCOs have been transplanted into apallial mice), the state-of-the-art analyses including behavior analysis with MoSeq and *in vivo* Ca²⁺ imaging and LFPs. The experiments appear to be rigorously done and the data are clearly presented in the figures.

The main weakness is that the new biological conclusions which we can draw from this study are relatively limited. Genetically apallial mice have been generated and well-characterized in several other studies, including in Kweon H, et al Cell Reports, 2021, which the authors should consider citing. hCOs have also been transplanted into rodent hosts in previous studies; therefore this aspect is not particularly novel. The new aspect here is the combination of apallial mice and human hCOs, which, while interesting, seems a somewhat limited advance. The authors suggest that the tAP-hCO model could facilitate disease modeling but it would have been helpful to see an example of how this could be done. It seems that the inherent variability of this system would be challenging to implement in a disease modeling context. I think more could be done in terms of understanding the mechanisms by which the human neurons integrate with and connect to the host cells to define the intrinsic programs that govern this. For example, the authors emphasize that the human cells widely innervate the host tissue but is this connectivity stochastic and random or does it recapitulate the specificity that occurs during typical brain development? Questions like this would be interesting to further explore in this model, beyond the initial characterization that was done here.

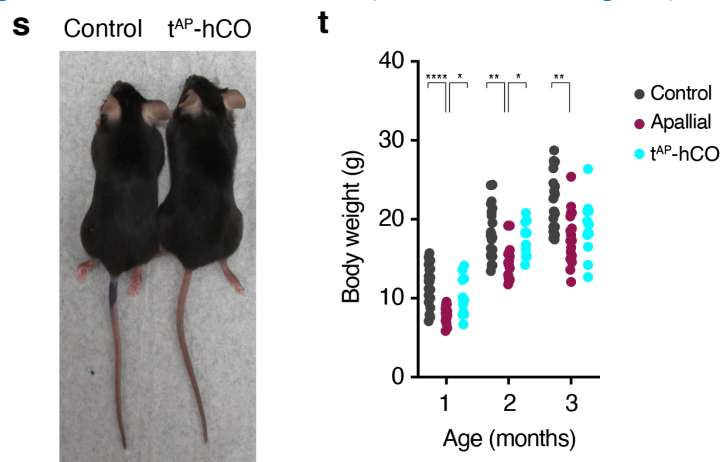
We thank the reviewer for highlighting the potential of this xenograft model. We appreciate the opportunity to further elaborate and contextualize both the apallial mouse model and the hCO transplantation.

We have a strong interest in developing *in vivo* models of human disease for translational applications, as we have previously done for Timothy syndrome (PMID: 38658687). The need for hCO transplantation into a depleted rodent model emerged as it became more evident that conventional transplantation paradigms involve substantial competition between the rapidly maturing rodent cortex and the neonatal human graft. In such a model, effectively depleting host cortical tissue as well as quantifying the extent of the depletion is critical to disease phenotyping and disentangling contributions of human cells to circuit or behavioral readouts. The novelty of our model lies in enabling a comprehensive, unbiased definition of cortical cell-type depletion, especially as in previous apallial models it remained unclear, beyond anatomical studies, which structures and cell types were affected; in establishing a model with minimal gross locomotor deficits at baseline to avoid confounds in behavioral phenotyping, and in extensively assessing variability at the cellular, circuit, and behavioral levels for disease modeling. In this revision, we now provided more levels of evidence along these lines by providing additional characterization of cell diversity and animal viability in the model (*i*), establishing the specificity and variability of connectivity of human neurons using rabies

virus and diffusion weighted imaging (ii), and introducing a disease application for hypoxic injury to the developing cortex including demonstrating how behavioral defects could be detected (iii).

(i) To quantify the extent of the neocortical depletion, we performed whole brain snRNA-seq (Fig. 1 and Extended Data Figs. 1, 2), and in this revision we add additional analysis of this dataset (Extended Data Fig. 2d,e). Overall, we discovered a depletion of all dorsal pallium-derived glutamatergic cell classes and a preservation of non-neocortical glutamatergic subclasses within amygdala, piriform cortex, and entorhinal areas (Extended Data Figs. 1, 2).

Given that behavioral readouts often define neuropsychiatric conditions, we spend considerable effort optimizing the viability and overall health of the immunocompromised mice. We modified the breeder strategy to avoid germline recombination, supplemented the diet, and we discovered that early “aunting” support allows for the robust generation of apallial mice. As a result, neonatal apallial mice do not have alterations in body weight (Extended Data Fig. 1c), supporting survival after surgery. Similar to previous apallial models (PMIDs: 20399730, 33626347), apallial mice weigh less than control mice at 1, 2, and 3 months of age, but interestingly, data added during revision reveals a normalization of weight in the t^{AP} -hCO condition (Extended Data Fig 7s,t).



Given that altered gross locomotion could confound downstream assays, we measured motor behavior in multiple assays. Contrary to alternative apallial models that either demonstrate increased (PMID: 33626347) or decreased (PMID: 20399730) baseline locomotion, here, we did not observe differences in locomotion across the activity chamber, Y-Maze, and catwalk (Fig. 4i,p; Extended Data Fig. 9a, Supplementary Video 2).

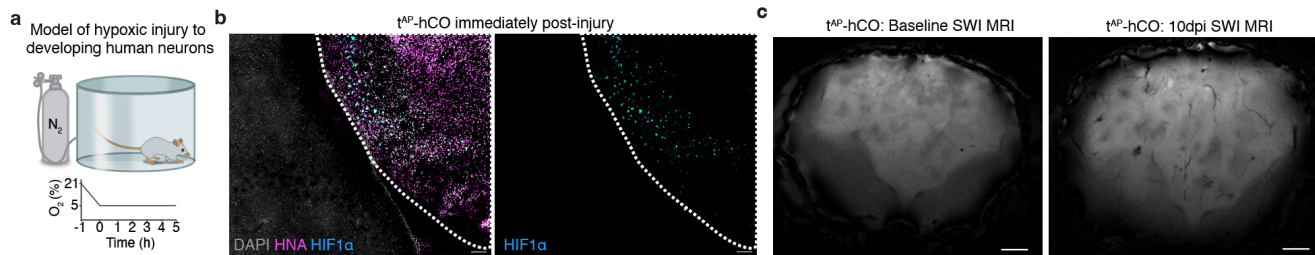
Together, the apallial mouse model presented here allows for robust interpretation of behavioral readouts due to the deeply characterized cortical depletion, minimal gross locomotor deficits, and extensive behavior phenotyping. As suggested, we also include the Kweon et al. reference in the main text below and in discussion:

“Contrary to alternative cortical depletion models, we did not find gross motor changes: control, apallial, and t^{AP} -hCO mice moved at similar rates in the activity chamber, Y-Maze, and catwalk (Fig. 4i,p; Extended Data Fig. 9a, Supplementary Video 2).”

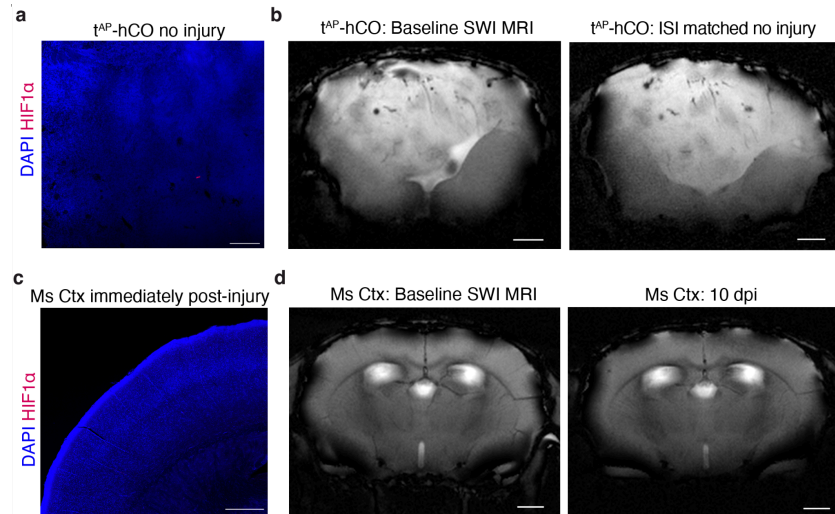
(ii) To address the question of connectivity, we now performed rabies retrograde tracing experiments and MRI-based diffusion-weighted imaging. We provide a detailed response including the new data in the Reviewer point #6 on pages 11-14.

(iii) In this revision, we now include a disease application in the t^{AP} -hCO model for hypoxia-related injury— a common insult associated with behavioral and motor impairments in prenatal and perinatal stages of cortical development. While animal models of hypoxic injury have revealed mechanistic insights, factors such as molecular/cellular differences between species along with changes in the timing of neural development have been limiting. We and others developed human *in vitro* models of hypoxic insult (PMID: 31061540), but *in vitro* systems cannot capture the dynamic organismal-level injury. For example, *in vitro* organoids are not vascularized, are maintained in media containing artificially high glucose, are cultured at static atmospheric oxygen levels with 5% CO₂, and require <1% O₂ for 24–48 hours to induce injury. While previous models of hCO transplantation can integrate organoids *in vivo*, the human graft makes up a relatively small fraction of the total cortical volume, so the effect of injury on the native host cortex and human graft is challenging to disentangle.

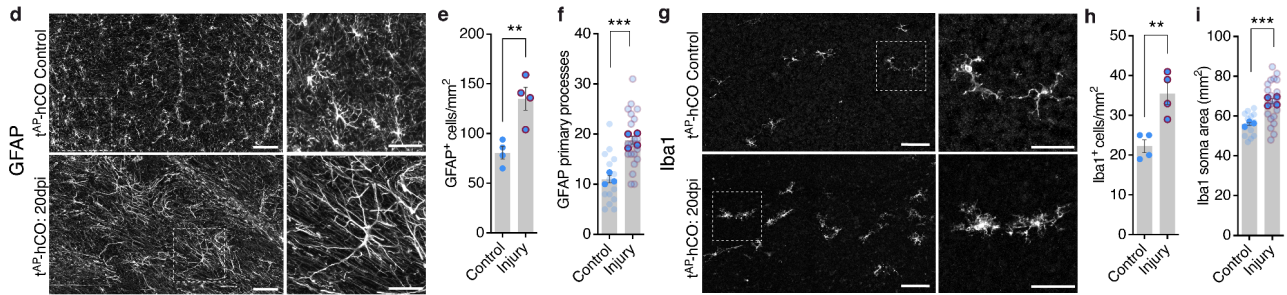
For this new model, we exposed control and t^{AP} -hCO mice to 5% O₂ for 5 hours in a Biospherix chamber (including a graded descent from atmospheric oxygen to 5% O₂, as described in detail in Methods). Immunostaining demonstrated that all t^{AP} -hCO mice exposed to 5% O₂ for 5 hours showed HIF1 α signal in the human graft, but not the mouse paleocortex (Fig. 5a,b). We then performed baseline and post-injury susceptibility weighted imaging (SWI), an MRI method that is highly sensitive to intravascular deoxyhemoglobin and extravascular deposits of blood products (PMID: 17925363). All t^{AP} -hCO mice that underwent 5% O₂ for 5 hours demonstrated more prominent hypointense vasculature 10 days post injury (Fig. 5c). This increased visibility on SWI is consistent with a larger ratio of deoxy- to oxyhemoglobin, and aligned with clinical reports of infants post-hypoxia (PMIDs: 21907881, 24033676).



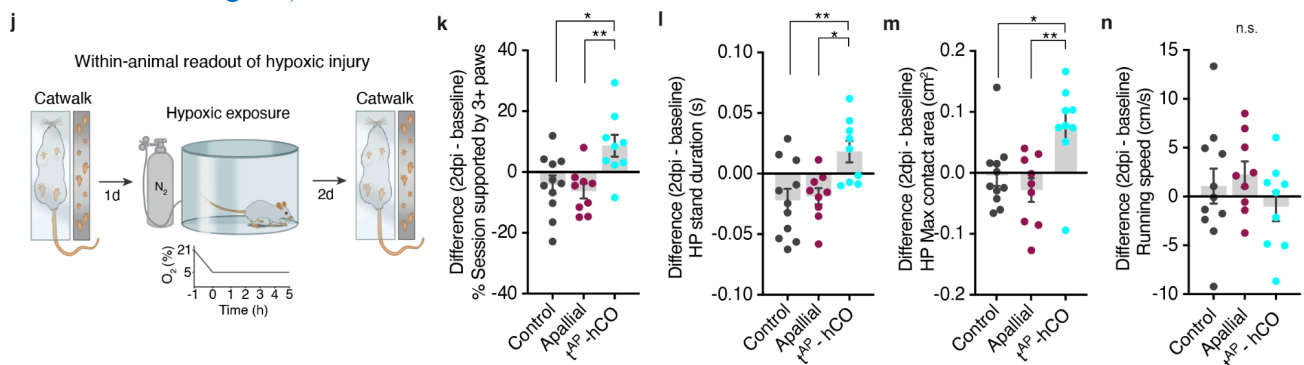
We did not see HIF1 α expression or an increase in SWI hypointensities in t^{AP} -hCO mice exposed to control atmospheric O₂ conditions nor in control mice exposed to 5% O₂ for 5 hours (Extended Data Fig. 10a-d).



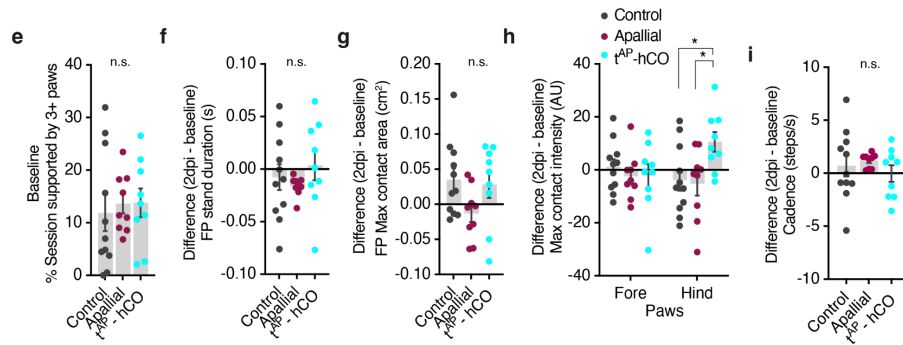
Finally, we found that t^{AP} -hCO mice exposed to 5% O_2 for 5 hours exhibited an increased density and complexity of intragraft Iba1 and GFAP cells (Fig. 5d-i), a shared cellular response to brain insults (PMIDs: 38286816, 40882239, 32770064).



We then tested whether the t^{AP} -hCO platform would allow for functional, behavioral readouts of injury. Because injury to the developing human brain is often associated with motor deficits, we tested mice in the CatWalk before and after injury (Fig. 5j). We found that t^{AP} -hCO mice exposed to 5% O_2 for 5 hours demonstrated an increase in the portion of the session supported by 3–4 paws, a more stable position than the more common 2 paws (Fig. 5k, PMID: 34723800). This was potentially driven by increases in the amount of time the hind paws were in contact with the platform, the contact area of the hind paws, and the maximum intensity of the hind paw print on the glass surface (Figs. 5l,m and Extended Data Fig. 9h).



Critically, these changes were not due to changes in overall running speed or step cadence (Figs. 5n and Extended Data Fig. 10i).



Together, these data demonstrate the feasibility of this approach for modeling aspects of human neurodevelopmental disorders with proof of concept behavioral readouts.

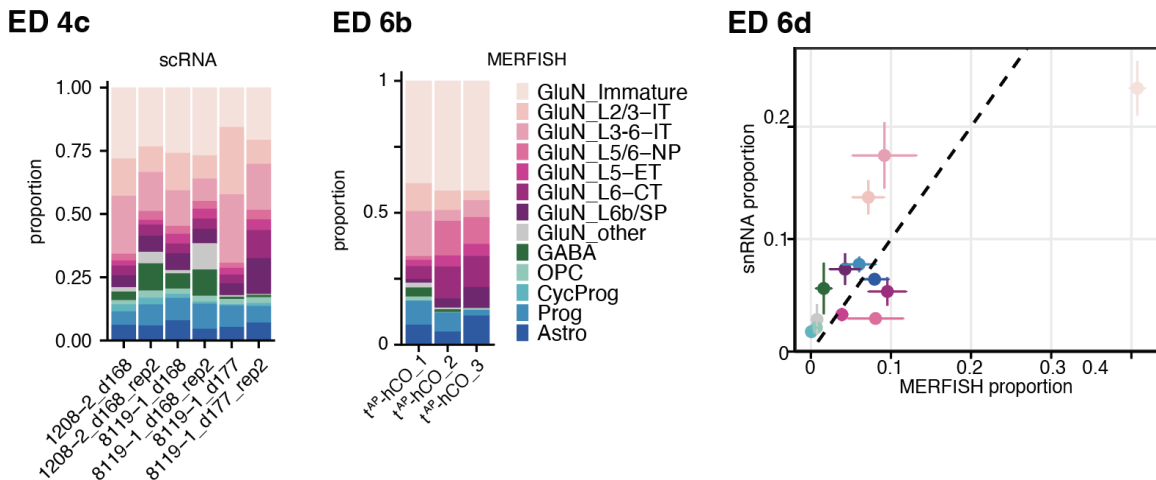
The remaining issues raised by the reviewer are addressed through the specific points below.

Specific points:

1) The introduction of multiple organoids into the same host seems like it would increase the potential variability of this model. i.e. it would be challenging to get the same composition of cell types from mouse to mouse as each organoid could be slightly different.

This is an important point, and as suggested, we further explored variability in this transplantation model in terms of cellular composition, neuronal projections, and behavior.

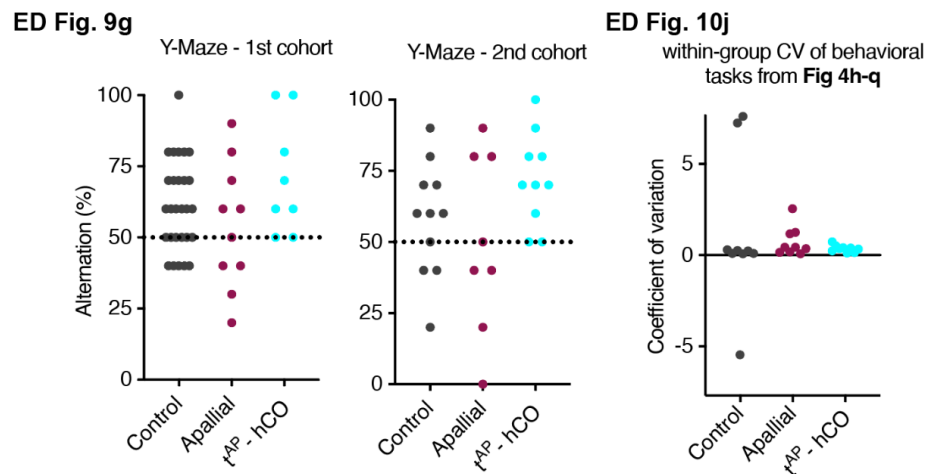
To address the question of variability in the cellular composition, we added analyses and new experiments. We have now performed spatial transcriptomics using MERSCOPE to complement the snRNAseq, and include quantifications of the cell type composition using these modalities. Overall, transcriptional profiling indicates that the percentage of glutamatergic neurons is similar across animals and modalities: $76 \pm 3\%$ by snRNAseq and $84 \pm 3\%$ by MERSCOPE (mean $\pm$ SEM, Extended Data Figs. 4c and 6b). Overall, there is a strong correlation between the two modalities ($r^2=0.53$, p -value = 0.005) (Extended Data Fig. 6d).



With respect to the anatomical variability, we will further elaborate for the response to comment #6 on pages 11-14, but briefly, all t^{AP} -hCO samples demonstrated a very similar pattern of anatomical inputs

to the graft. A cross-correlation between samples revealed a pearson correlation coefficient ranging from 0.92 to 0.99, Extended Data Fig. 3e). Similarly, diffusion weighted imaging (DWI) of tissue microstructure revealed a normalized root mean squared deviation (RMSD) of 0.28 in control and 0.26 in t^{AP} -hCO.

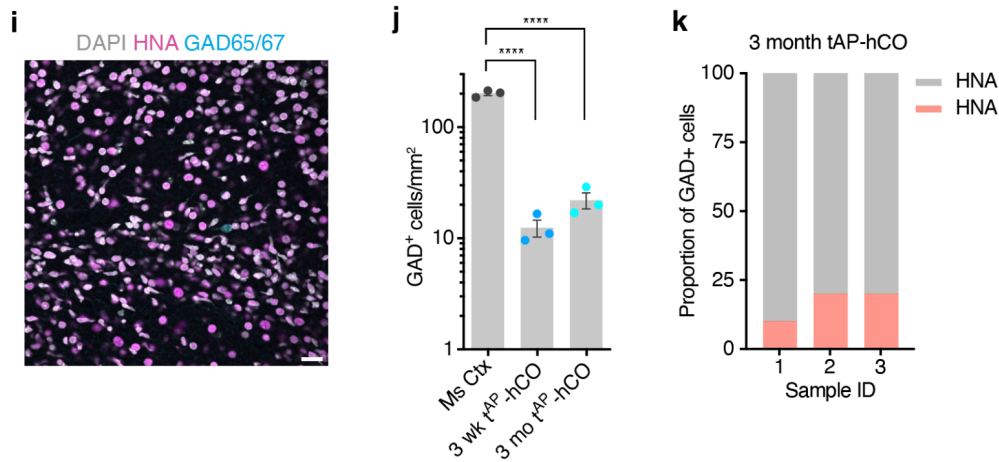
Lastly, we further assessed behavioral variability in the t^{AP} -hCO model, with a detailed response in comment #9 on pages 16-17. Firstly, in this revision, we replicated Y-maze behavioral results using new hCO differentiations, new mouse breeders, and behavioral experiments conducted months apart (Extended Data Fig. 9g), supporting the reliability of behavioral readouts. Secondly, we calculated the coefficient of variation for all the specific behavioral tasks presented in Fig. 4 (Extended Data Fig. 10j). We did not observe a difference between control, apallial, and t^{AP} -hCO conditions, indicating that the t^{AP} -hCO platform generates inter-subject variability in line with control mouse behavior.



Overall, we quantified variability at the cellular, circuit, and behavioral levels (Extended Data Figs. 3e, 3j, 4c, 6b, 6d, 9g, 10j, and 10k), enabling future users of the platform to perform power analyses of the relevant level and determine groups sizes.

2) There are a number of aspects of this graft model that would be important to explore further. For example, do the host GE-derived GABAergic neurons migrate into the host cortical tissue? If yes, when does this occur? If not, do these cells populate other brain regions or do they die off?

This is certainly an important aspect to explore. For this revision, we performed a series of immunostaining analyses. We found GABAergic cells in the graft by 3 weeks post-transplantation (Extended Data Fig. 7i-k). At 3 months post-transplantation, there is no significant increase in the density of GAD⁺ neurons and the majority of the GABAergic (median: 80%) in the graft are host-derived (Extended Data Fig. 7l).



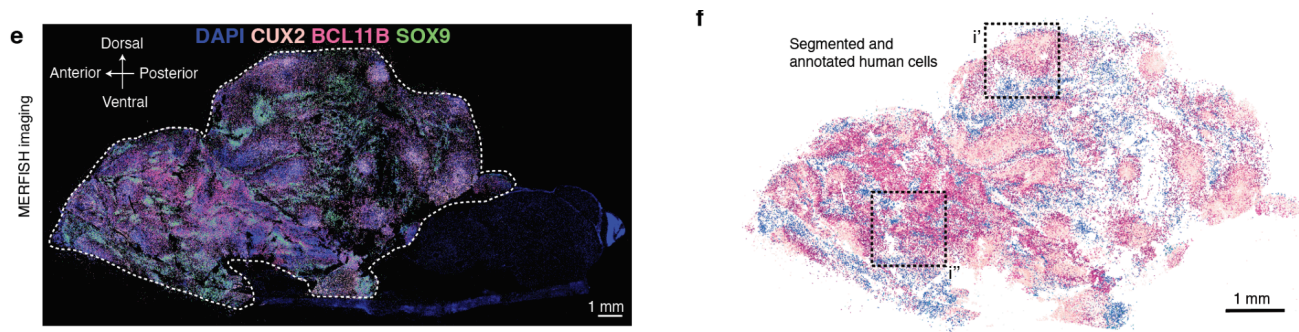
As expected, the transplantation of a primarily glutamatergic hCO resulted in a graft with ~10x fewer GABAergic neurons than control cortex (Extended Data Fig. 7j). Future versions of this model could leverage assembloid approaches to incorporate human GABAergic neurons into the graft. We added the following to the discussion:

“As an initial demonstration of the t^{AP}-hCO model, we transplanted glutamatergic hCO, leading to a low density of GABAergic neurons within the graft. Future versions of this model may use assembloid approaches, particularly for modeling imbalances in excitation and inhibition in neuropsychiatric disorders.”

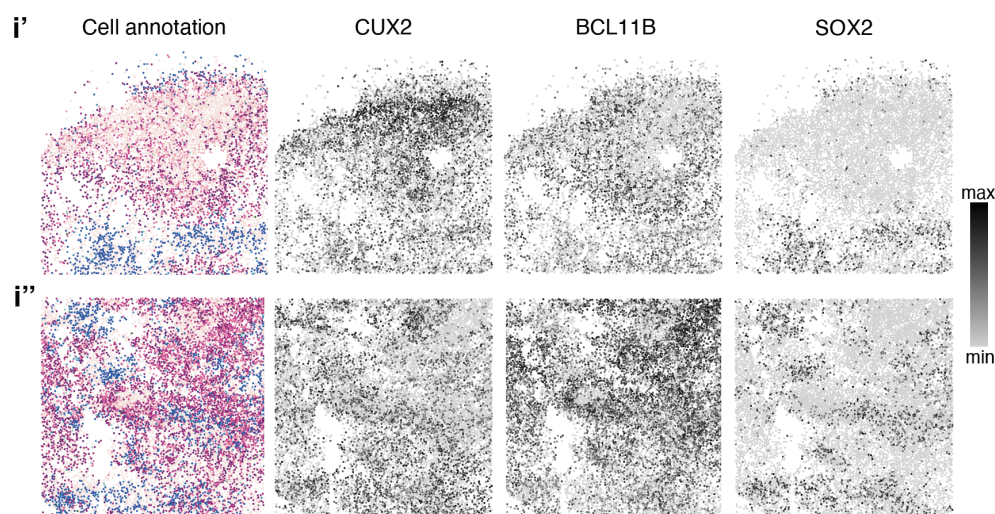
Related to this, we also further explored the fate of cortical GABAergic interneurons in the apallial mouse brain. We performed new analyses of the whole brain snRNA-seq dataset, as detailed in a response to Reviewer #3 below. In brief, new exploratory analyses among cortical GABAergic subclasses, revealed trending reductions across most subclasses, with Sst interneurons appearing the most reduced ($\log_2FC = -2.7$, FDR = 0.11), consistent with their reported dependence on local pyramidal neuron signalling for their survival (PMIDs: 29849154 and 39071350). To further address whether surviving cortical GABAergic neurons have undergone fate alteration, we performed differential expression analysis across all cortical GABAergic subclasses (Extended Data Fig. 2d,e). Most comparisons showed minimal transcriptional changes between control and apallial mice. The Sst subclass showed the most changes (23 up-regulated genes, 38 down-regulated genes; $|\log_2FC| > 1$, FDR < 0.05), and gene ontology (GO) pathway enrichment analysis did not support a change in cell identity programs. Overall, this suggests that surviving Sst interneurons retain their identity but have reductions in transcriptional signatures associated with circuit engagement.

3) In addition, is there any anatomical structure or layering of the human cortical graft?

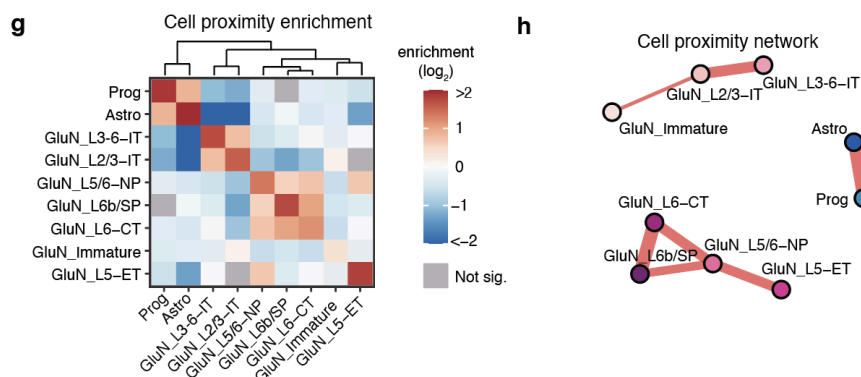
We thank the reviewer for this important question. To comprehensively address the question of cytoarchitectural organization, we performed spatial transcriptomics (MERFISH) of t^{AP}-hCO. As expected based on the presence of multiple ventricular-like zones in organoids without consistent orientation, we did not observe canonical cortical layering throughout the graft (Extended Data Fig. 6e,f).



However, we asked whether local cytoarchitectural organization might emerge within t^{AP} -hCO. We found regions within the graft predominantly occupied by either upper-layer intratelencephalic (IT) or deep-layer non-IT neuron subclasses (Extended Data Fig 6i).

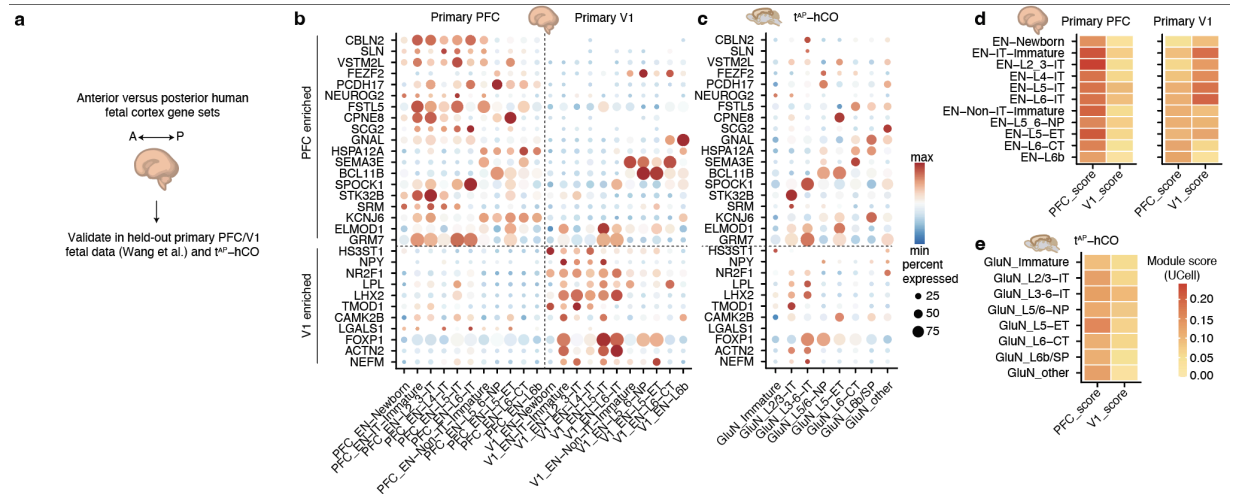


While these regions do not form a continuous laminar structure across the graft, nearest-neighbor analysis reveals that transcriptomically defined subclasses that normally reside in similar cortical layers are found in closer spatial proximity than expected by chance, consistent with local self-organization (Extended Data Fig. 6 g-i).

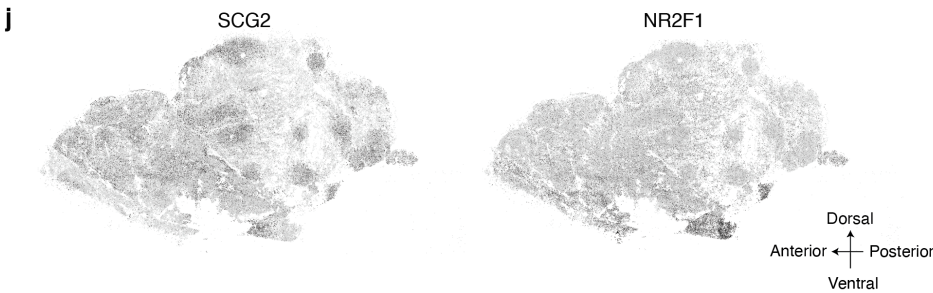


We then investigated whether t^{AP} -hCO exhibit any features of cortical arealization. We reanalyzed our snRNAseq dataset alongside primary fetal PFC and V1 data (PMID: 39779846) for markers of cortical arealization identified from primary fetal human cortical studies (PMIDs: 34616070 and 40369074). We found some evidence of increased expression of PFC-associated markers in t^{AP} -hCO glutamatergic

neurons although this was not as strong as seen in primary human cortical data (Extended Data Fig. 5a-e).



Furthermore, spatial transcriptomics of the t^{AP}-hCO did not reveal a striking gradient along the anterior-posterior axis with available cortical arealization genes present on our MERSCOPE gene panel (Extended Data Fig. 6j). This suggests that the mismatch between the developmental age of the graft and host may not enable robust cortical arealization at this time point. Future work probing the system at later time points may help elucidate whether functional arealization could emerge.



Lastly, we probed the anatomical organization of the graft through diffusion-weighted imaging (DWI). Using advanced tractography methods (Fig. 2g), we estimated fiber orientation distributions (FODs) for each voxel (Fig 2h). While we provide more detail on these results in response to point #6 on pages 11-14, it is worth mentioning here that we observed directionality in the structural organization of t^{AP}-hCO. More specifically, a predominant dorso-ventral orientation (Extended Data Fig. 3j).

Fig. 2g

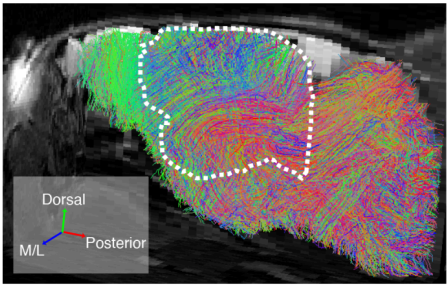
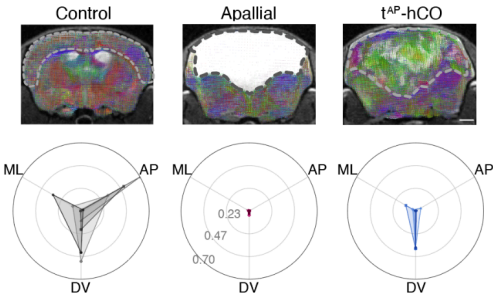
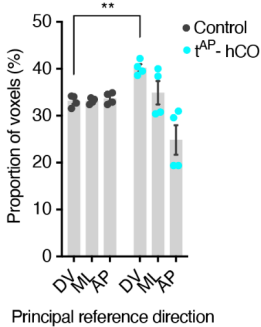


Fig. 2h

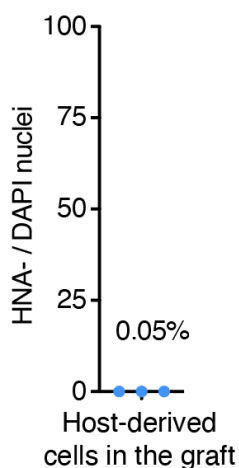


ED Fig. 3j



4) What is the neuron to astrocyte (or other glial cells) ratio within the graft? What is the excitatory neuron to inhibitory neuron ratio? How much infiltration of mouse cells is there (either neurons or glia) into the graft region?

We thank the reviewer for allowing us to elaborate on this question. We added new analyses of the snRNAseq data, we found that the human graft consisted of $81.9 \pm 0.07\%$ human neurons, with $76.3 \pm 3\%$ human glutamatergic neurons per sample, and $5.6 \pm 2.3\%$ human GABAergic neurons per sample. This yields a human neuron to non-neuron ratio of 4.5 ± 0.2 and human glutamatergic to GABAergic ratio of 32 ± 23 across samples. Using immunostaining for DAPI and human nuclear antigen (HNA), we found that 0.051% of the graft nuclei do not co-stain for HNA (Extended Data Fig. 7h).



5) The authors suggest that the human cortical neurons project to the spinal cord. This is shown with anatomical approaches only and it would be useful to verify this with electrophysiology.

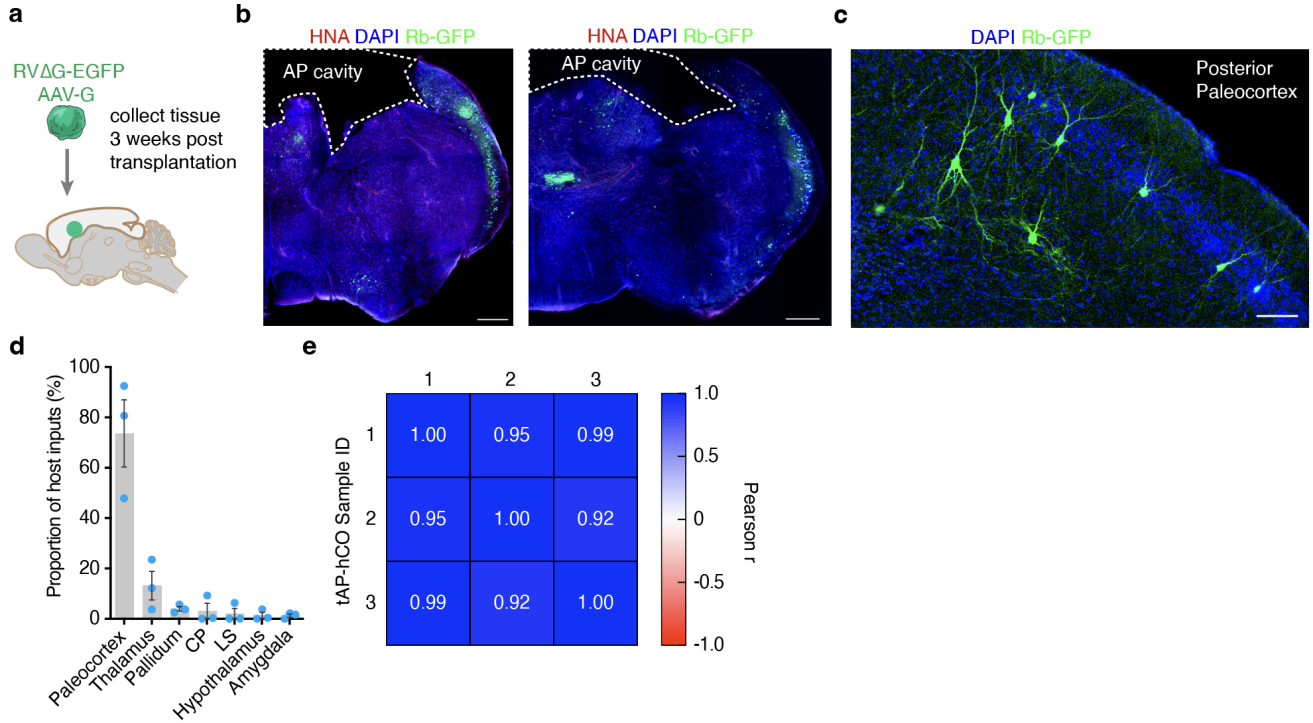
We thank the reviewer for this suggestion. We considered a number of methods to address this point, but direct electrophysiological validation of corticospinal projections in the t^{AP} -hCO model presents a series of technical challenges. The sparsity of human corticospinal projections requires sustained and robust optogenetic activation of the graft coupled with whole-cell electrophysiology recordings in the spinal cord. Elevated spinal cord activity with the expected temporal synaptic delay could demonstrate this connectivity, but t^{AP} -hCO derived neurons may use bulk, and not synaptic, release at this still early developmental time point. We will, however, make it clear in the discussion that this is an important aspect that needs to be pursued:

“Future electrophysiological studies should explore functional connectivity between the graft and host spinal cord and the relative contribution of human neurons.”

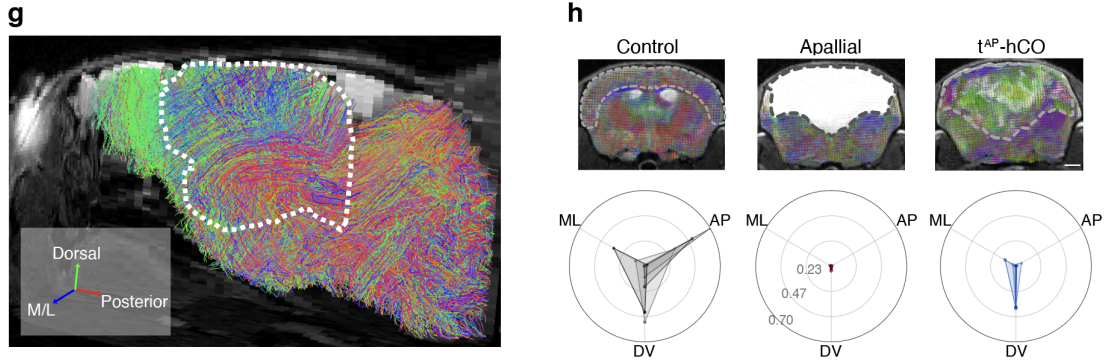
6) A key question related to the above point is whether the connectivity of the human neurons is selective or random and stochastic. i.e. Do the human cortical neurons connect to their expected partners? either within the graft or outside of the graft? If the connectivity is imprecise or random, then the model will be less valuable for studying circuit-level questions.

We agree that this is critical, and we addressed this question through two anatomical approaches: rabies retrograde tracing and MRI-based diffusion-weighted imaging. Broadly, our data support the conclusion that connectivity in t^{AP} -hCO is not random and is reproducible across samples.

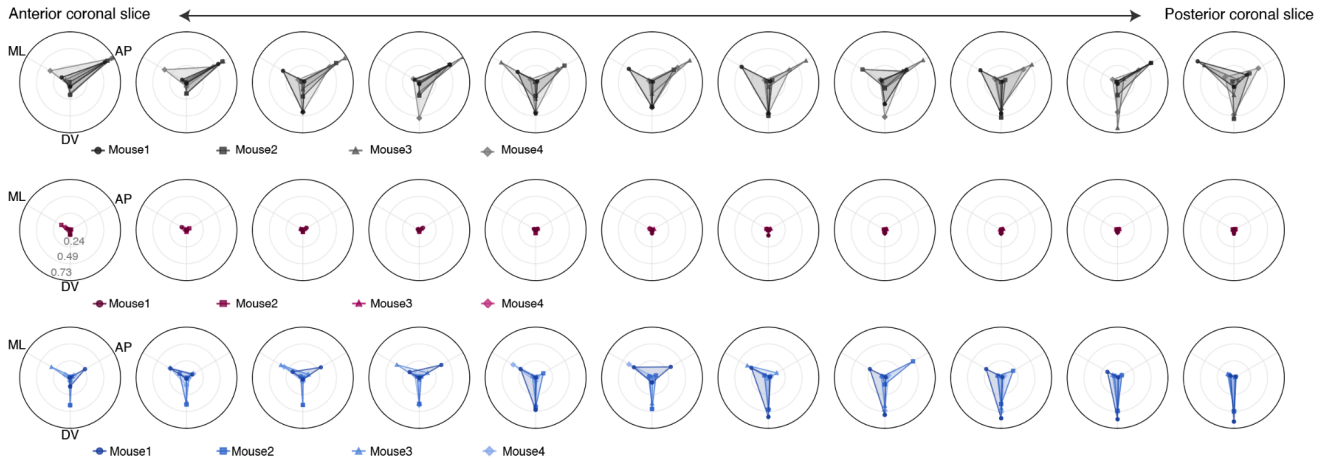
First, we pre-infected hCO with a G-deleted rabies virus encoding GFP and an AAV encoding the helper G protein. The G-deletion of the rabies virus restricts retrograde transport to cells carrying the G protein (i.e. one retrograde step from the organoid). We transplanted the pre-infected hCO into apallial mice and collected tissue 3 weeks later (Extended Data Fig. 3a). We found consistent labeling of neurons in the host paleocortex, thalamus, and pallidum (nucleus of the diagonal band, substantia innominata, globus pallidum external, etc.), but only 1/3 mice exhibited sparse labeling in the caudate putamen (CP, Extended Data Fig. 3b-d). This suggests some degree of specificity of connections: in wild-type mice, CP neurons do not project directly to the cortex, and although the graft borders the CP (Fig 2f, Extended Data Figs. 3f, 3i, 7a), ectopic CP-to-graft projections are rare. Most of the host input neurons resided in the paleocortex, followed by the thalamus and pallidum, with a strong correlation between mice (Extended Data Fig. 3d,e).



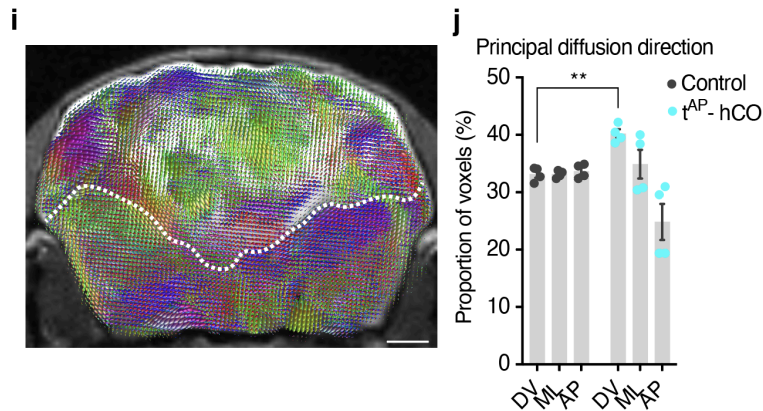
Second, to investigate structural connectivity in t^{AP} -hCO, we acquired diffusion-weighted images (DWI) and applied tractography methods (Fig 2g) to provide a quantitative description of the directionality of local water diffusion, and hence, the organization of tissue microarchitecture. In a representative coronal plane across groups (Fig. 2h), the control group demonstrated a heterogeneous pattern of water diffusion in all directions (AP, ML, DV). In contrast, the water diffusion in the t^{AP} -hCO followed a stereotyped dorso-ventral (DV) trajectory. The lack of an average diffusion direction in the apallial cavity is consistent with the absence of tissue and coherent microstructure.



To comprehensively estimate the structural organization of the graft, we quantified the principal direction of water diffusion across all coronal sections (Extended Data Fig. 3k).



We observe high within-subject consistency across the anterior-posterior axis and high between-subject consistency. Control isocortex demonstrates equal distribution in all directions, while t^{AP} -hCO demonstrates predominantly dorsal-ventral (DV) orientations, with minor components in the medial-lateral (ML) and anterior-posterior (AP) directions. We confirm this observation by quantifying the distribution across all sections of all mice (Extended Data Fig. 3i,j).



To quantify the variability of tissue microarchitecture between grafts, we calculated the root mean squared deviation (RMSD) of the control (0.1445) and t^{AP} -hCO groups (0.0944), and normalized these values to the average magnitude of the vectors (control = 0.5104, t^{AP} -hCO = 0.3663). This yielded a

normalized RMSD of 0.28 (control) and 0.26 (t^{AP} -hCO). Overall, DWI analyses demonstrated a consistent, predominantly dorso-ventral microstructural orientation in the t^{AP} -hCO.

Together, the distribution of host inputs to the graft from rabies tracing along with stereotyped dorso-ventral microstructural organization from DWI support some level of consistency in t^{AP} -hCO connectivity patterns.

7) The authors show that the human graft has robust and synchronous activity. This pattern of activity is very different than the sparse activity that is typically observed in cortical circuits. Does this activity propagate outside of the graft to downstream structures?

We thank the reviewer for this question. While it is currently challenging to directly measure the propagation of this activity to downstream structures, our anatomical data support graft-host integration and the behavioral findings further suggest functional engagement of the graft with the host circuits (including shifts in the behavioral repertoire as captured by MoSeq and movement expression in CatWalk between t^{AP} -hCO and apallial mice). The waves of synchronized, high amplitude graft activity is reminiscent of early network oscillations (ENOs) (as described in animals by the Roska, López-Bendito, Portera-Cailliau, Feller, Ben-Ari, Konnerth, Cossart labs, and others).

We did consider a number of ways to further investigate the spread of activity. Given the spatial and temporal characteristics of the large synchronized events we observed, whole-cortex gCaMP imaging seemed well suited. Unfortunately, assessment of whether this activity spreads into the host would require optical access to the deeper host tissue, and because the graft covers the entire surface, this was not feasible. We then considered targeted recordings of electrical activity with a linear probe spanning the graft-host boundary, but here the probe would need to be inserted in an orientation along the axis of the wave propagation. Therefore, this method is unlikely to conclusively prove or disprove the presence of spatiotemporally-defined events. Whole brain fMRI could provide a global view of brain-wide activity, with the caveat that anesthesia required for rodent fMRI may affect this global activity and its propagation. Regardless, we will seek to pursue this analysis in future studies. Given these considerations, we acknowledged this aspect in the discussion as follows:

“At the electrophysiological level, the waves of synchronized, high amplitude events are reminiscent of an immature form of activity (termed early network oscillations). It remains to be established whether this form of activity propagates outside of the graft.”

8) The authors perform a thorough and rigorous investigation of different behaviors in the control, apallial and transplanted mice. However, the overall conclusion that can be drawn from these data is a bit unclear. The authors find some similarities and some differences but what general insight does this provide? For example, how do the authors interpret changes in gait and patterns of paw placement among the different mice?

We appreciate the reviewer’s recognition of the rigor of the behavioral investigation. In the revised manuscript, we discuss four points related to the interpretation of behavioral outputs of this model. The structured, increasingly complex levels of analysis reveal intact gross locomotor output, subtle changes in limb coordination, altered higher-order organization of behavior, and restricted changes in cognitive function between groups. Given these behavioral results, we were able to design tasks to detect

functional behavioral differences when modeling hypoxic insult of the nervous system. Below, we provide details on our behavioral framework.

First, the three cohorts show largely intact gross locomotor output across assays (activity chamber distance, Y-maze distance, CatWalk speed), indicating that basic locomotor pattern generation and execution remain robust even when pallial structures are depleted.

Second, CatWalk reveals higher-order changes in coordination (phase dispersion, paw synchronization, support patterns, step sequence usage). Specifically, control mice show a highly stereotyped diagonal paw synchronization pattern, whereas apallial and t^{AP} -hCO cohorts deviate from this stereotyped coordination, indicating a shift in how locomotor elements are organized during walking. Importantly, these coordination shifts cannot be reduced to run speed or cadence (steps/s) differences. This pattern is consistent with the idea that cortical circuitry can modulate fine motor parameters via descending pathways, while not being strictly required for locomotor execution.

Third, MoSeq provides an unbiased, data-driven view of spontaneous behavior by decomposing behavior into sub-second modules and their transition structure. In our data, the main MoSeq conclusion is not a single “syllable deficit”, but rather a distributed reweighting of behavioral syllables, with more frequent shifts in their relative usage across time in apallial and t^{AP} -hCO mice relative to controls. In the broader literature, behavior is often conceptualized as hierarchical (goals and state, action selection/sequencing and kinematics), with cortical and corticostriatal loops shaping selection and sequencing, and corticofugal projections modulating kinematic details (e.g. reviewed in PMID: 36030613). Consistent with this, MoSeq-like modular descriptions have been used to argue that basal ganglia/striatal circuits strongly influence the sequencing (“grammar”) of behavioral modules (“syllables”) while leaving execution of individual modules largely intact (PMID: 29779950). In this framework, the combination of preserved gross locomotion, selective kinematic effects, and altered coordination and spontaneous repertoire organization supports a conservative interpretation: the graft can contribute to specific motor control features, but pallial depletion and transplantation together shift higher-order organization of behavior rather than producing a single categorical impairment.

Finally, the targeted behavioral assays provide a positive control for the apallial manipulation and enable interpretation of the t^{AP} -hCO condition. While control mice perform above random chance in a spontaneous alternation Y-maze task, apallial mice performed randomly. This indicates that depletion of neocortical structures results in the expected working memory deficit. Remarkably, t^{AP} -hCO mice performed at above chance levels, suggesting that some forms of working memory may be intact in t^{AP} -hCO mice. In contrast, apallial and t^{AP} -hCO mice showed similar deficits in trace fear conditioning, suggesting the relatively immature, glutamatergic hCO is not sufficient to support longer-term memory. We have revised the text to emphasize these findings as selective functional effects within the context of the predicted developmental stage of t^{AP} -hCO, developmental mismatch between the human graft and mature mouse nervous system, and lack of interneurons. Future work, with proper ethical safeguards, could ascertain whether more mature grafts can generate patterns of neural activity and, therefore, behavioral outputs reminiscent of the postnatal nervous system.

Most importantly, these initial behavioral analyses informed the choice of readouts when modeling aspects of hypoxic injury of the developing nervous system. Specifically, we did not observe major baseline changes in low-level CatWalk measurements, allowing a stable baseline when modeling injury in the three groups.

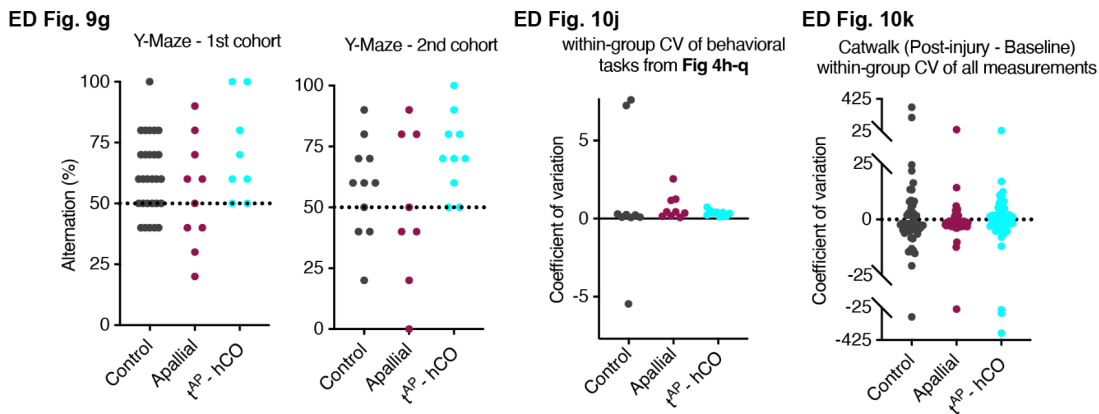
We added the following to the results:

“The behavioral analyses revealed intact gross locomotion, subtle changes in limb coordination, altered higher-order organization of behavior, and restricted changes in cognitive function between groups.”

9) The inter-subject variability that the authors report for the behavior analysis could be a concern for using this as a disease model where consistent & reproducible controls are important.

To explore this aspect, we now provide a quantification of variance in cellular composition, circuit connectivity and behavioral levels. To directly measure the technical variability, we (i) assessed the reliability of the Y-Maze result from the first submission and (ii) measured the level of behavioral variability in the context of the disease model.

(i) We first tested whether a new cohort of t^{AP}-hCO using new hCO differentiations, new mouse breeders, and behavioral experiments conducted months apart could replicate behavioral changes from the first submission, and in particular the performance in the Y-Maze task probing working memory. More specifically, we previously found that control mice performed the Y-Maze at above chance levels while the apallial group performed at chance level, demonstrating the largest data spread of all the groups and a median value of 50%. Intriguingly, the t^{AP}-hCO group performed above chance levels. In this new cohort (total of 27 animals), we found the same results (Extended Data Fig. 9g), supporting the reliability of behavioral testing using the t^{AP}-hCO platform. Next, we calculated the coefficient of variation from all the behavioral assays presented in Fig. 4h-q. The median coefficient of variation within each group was not different between conditions: (Extended Data Fig. 10j, Control: 0.2294; Apallial: 0.3839, tAP-hCO: 0.3496; Kruskal-Wallis test: $H_2 = 1.697$, $P = 0.4281$), indicating no difference in the normalized dispersion between groups.



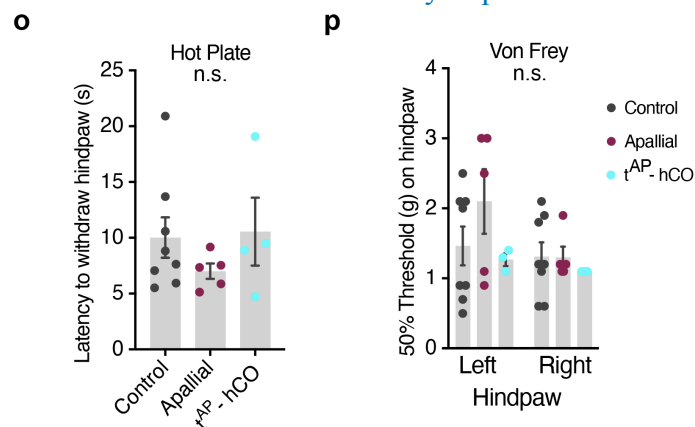
(ii) In this revision, we implemented a hypoxic injury paradigm (5% O₂ for 5 hours) to model a common developmental insult and then asked whether the t^{AP}-hCO platform yields behavioral readouts. We performed CatWalk before and after hypoxia in control, apallial, and t^{AP}-hCO and quantified within-group variability in a way that is directly relevant for detecting injury effects: for each mouse we calculated the pre-to-post change for each of CatWalk behavioral measures and then calculated the within-group coefficient of variation of these changes (Extended Data Fig. 10k). The median coefficient of variation within each group was not different between conditions: (Control: -2.304; Apallial: -1.365, tAP-hCO: 0.8180; Kruskal-Wallis test: H₂ = 3.356, P = 0.1868), indicating normalized variability in the behavioral response to hypoxic injury is not inflated in the transplanted cohort and supports robust behavioral phenotyping.

Lastly, it is also important to mention that in the first version of the manuscript, we used the term “variability” to describe the Kullback–Leibler divergence and coefficients of variation measurements in the MoSeq data, in reference to t^{AP}-hCO mice broadly and dynamically sampling behavioral modules during unconstrained spontaneous behavior in an open field. To avoid confusion, we now use the term dispersion of behavioral repertoire. Therefore, this section was replaced with the following:

“Collectively, our findings show that spontaneous behavioral patterns in apallial and t^{AP}-hCO mice are defined by a concerted re-weighting of multiple behavioral syllables, accompanied by pronounced dispersion of syllable usage within and across animals, yielding a distributed and labile repertoire rather than a discrete, syllable-specific defect.”

10) It looks like only the control and apallial mice were tested in the Ven Frey and hot plate tests. It would be interesting to know whether sensory processing is intact in the transplanted mice.

We agree that this is an interesting question. We have now added new data to present the t^{AP}-hCO condition as well. Across both the Von Frey and Hot plate, we did not observe differences between groups (Extended Data Fig. 9o,p). These data further support unaltered sensory processing and indicate that transplanted mice do not have an increased sensitivity to pain.



Minor:

1) It is unclear what the authors are referring to with "sufficient" in this sentence. pg 5 ...indicating the heterozygosity for *Emx1-cre* and *Esco2* is sufficient"

We thank the reviewer for pointing this out. We added details about the genetic strategy inline for clarity:

“While the survival rate was not statistically different between groups, there was a trend in which both breeders (*Emx1-cre*^{+/-};*Esco2*^{fl/+};*Prkdc*^{scid/scid}) and apallial (*Emx1-cre*^{+/-};*Esco2*^{fl/fl};*Prkdc*^{scid/scid}) mice demonstrated a lower probability of survival at ~2 months of age (Extended Data Fig. 1e). Given that breeders have a grossly intact cortex, cortical depletion does not drive the trend.”

Referee #2

This study by Kaganovsky et al uses mice with an ablated cortex to subsequently transplant hCOs and explore the connections made with the host. While aspects of this paper are done well, I have severe ethical concerns about the study and worry it is an attempt to do something just because we can. I do not believe sufficient efforts to justify the value of this study beyond a Frankensteinian fascination with the ability to do so have been considered, and the ethical considerations are very briefly mentioned in the discussion. In a contentious scientific environment politically, I worry that publication of this study would severely damage the reputation and regard of science in the public sphere, possibly irreparable. For me to feel able to endorse this study, I would want a therapeutic advance so phenomenal that no other system has or could have generated this outcome. As written, the paper definitely makes essentially a human/mouse chimera, ie a mouse with a “humanized brain” raising important ethical questions about sentience and consciousness of the human parts of the brain. If the journal chooses to proceed with publication, I would strongly encourage involving ethicists from the United States and Europe in the review of the manuscript alongside written ethical pieces about the possible considerations of the study. I would also encourage all reviews (including this) to be published alongside the manuscript. Scientifically, I also have some concerns detailed below:

We share the reviewer’s perspective that science unfolds within society and that the ethical implications of research must remain central. As detailed in the response below, we sought extensive ethical review both at the outset of this project and throughout its progression. Our work is motivated by the need to develop improved human models of brain disease and, ultimately, therapeutic approaches, as we have previously done for Timothy syndrome (PMID: 38658687). Existing models, whether animal or human cellular, remain insufficient for capturing key features of many brain disorders, particularly those that depend on the maturation of human neurons within circuits and their consequences at the network and behavioral levels. The rationale for transplanting human cortical organoids into a depleted rodent cortex emerged from the recognition that conventional transplantation paradigms involve substantial competition between the rapidly maturing rodent cortex and the neonatal human graft, thereby limiting the emergence of certain circuit level and behavioral readouts. The present approach is intended to create a more permissive *in vivo* context in which disease relevant properties of human cells can be studied. Importantly, as described in this revision, we provide proof of concept that this model can be used to examine aspects of human disease *in vivo* that would otherwise be difficult to address *in vitro*.

Ethical safeguards

Prior to starting experiments, we sought approval from the Stanford Administrative Panel on Laboratory Animal Care (APLAC). Beyond this conventional review procedure, we requested higher-level consultation from two bioethicists at Stanford who provided an extensive overview of implications. Soon after, we consulted with the Stanford Neuroscience Institute’s Executive Committee that included leading neuroscientists. After the committee consulted with ethicists they extensively discussed the project, and send a report that concluded:

“In summary, you are complying with all pertinent regulations, the experimental path you are following has potential significance for curing human diseases, and we see no credible conceptualization of these experiments as creating organisms with human-like behavioral or cognitive capacities that would lead to elevated ethical concern.”

As we initiated these experiments and established the apallial model in the laboratory, we also sought feedback systematically and confidentially from several neuroscientists around the world. In parallel,

Stanford University School of Medicine convened an external committee composed of neuroscientists, legal scholars, ethicists, and patient advocates to develop a framework for oversight of these studies. All aspects of the experiments were shared transparently and discussed, including the scientific rationale for the work and sequence of studies, laboratory practices and safeguards, ethical dimensions of study design, methods, and data interpretation, and potential social, medical, and ethical implications of the work. We presented this work to the committee on multiple occasions, and their feedback substantially informed our approach.

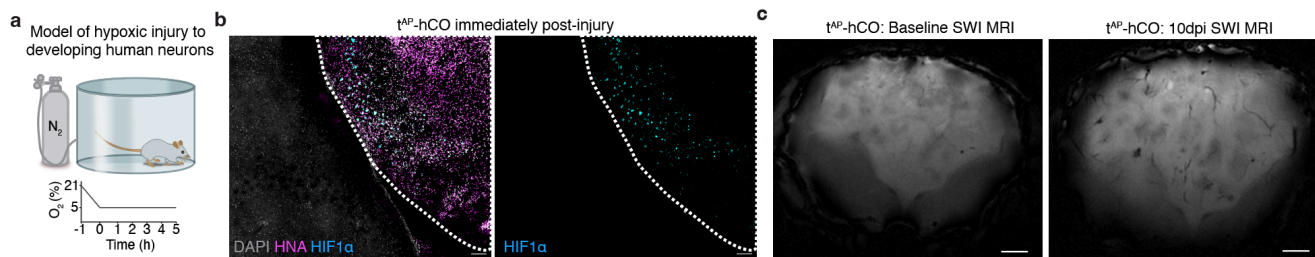
We would also like to direct the reviewer to the committee's letter, included with the original submission of this manuscript. For this revision, we asked the committee to provide additional detail on its process in a second letter, which is also included with this revision. That letter concludes that "all appropriate ethical care was taken by you and your study team."

Finally, reviewer #4 of this manuscript is an ethicist and also provides an independent assessment below.

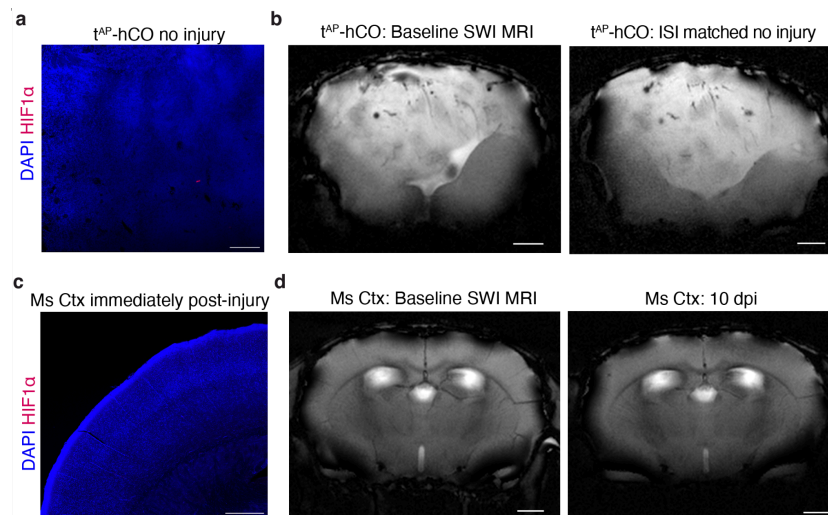
Disease model

We have been actively working to establish a disease application of the t^{AP}-hCO model centered on hypoxia-related injury, and this work has now been finalized for inclusion in the revision. Hypoxia-related injury is associated with behavioral and motor impairments in prenatal and perinatal stages of human cortical development. While animal models of hypoxic injury have revealed mechanistic insights, factors such as molecular/cellular differences between species along with changes in the timing of neural development have been limiting. We and others have developed human *in vitro* models of hypoxic injury (PMID: 31061540), but these systems cannot capture the dynamic, organism-level nature of the insult or its downstream functional consequences, including behavioral outcomes, thereby limiting their clinical relevance. For example, *in vitro* organoids are not vascularized, are maintained in media containing artificially high glucose, are cultured at static atmospheric oxygen levels with 5% CO₂, and require <1% O₂ for 24–48 hours to induce injury. While previous models of hCO transplantation can integrate organoids *in vivo*, the human graft makes up a relatively small fraction of the total cortical volume, so the effect of injury on the native host cortex and human graft is challenging to disentangle.

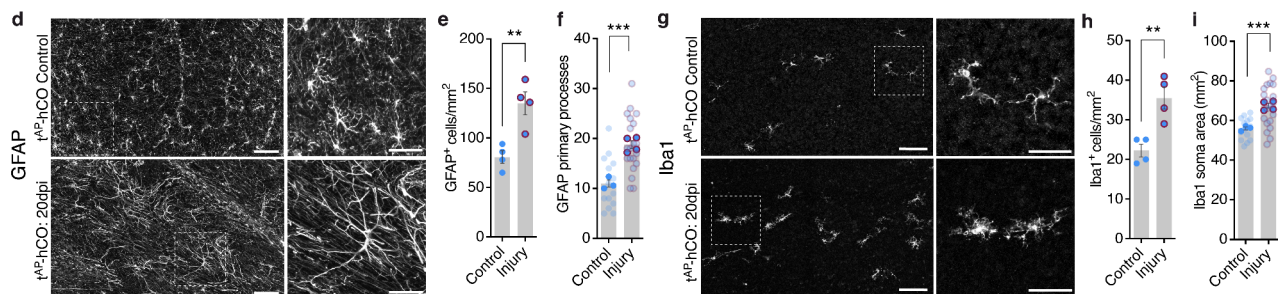
For this new model, we exposed control and t^{AP}-hCO mice to 5% O₂ for 5 hours in a Biospherix chamber (including a graded descent from atmospheric oxygen to 5% O₂, as described in detail in Methods). Immunostaining demonstrated that t^{AP}-hCO mice undergoing 5% O₂ for 5 hours showed HIF1 α signal in the human graft, but not the mouse paleocortex (Fig. 5a,b). We then performed repeated susceptibility weighted imaging (SWI)— an MRI method that is highly sensitive to intravascular deoxyhemoglobin and extravascular deposits of blood products (PMID: 17925363). t^{AP}-hCO mice that underwent 5% O₂ for 5 hours demonstrated more prominent hypointense vasculature 10 days post injury (Fig. 5c). This increased visibility on SWI is consistent with a larger ratio of deoxy- to oxyhemoglobin, and aligned with clinical reports of infants post-hypoxia (PMIDs: 21907881, 24033676).



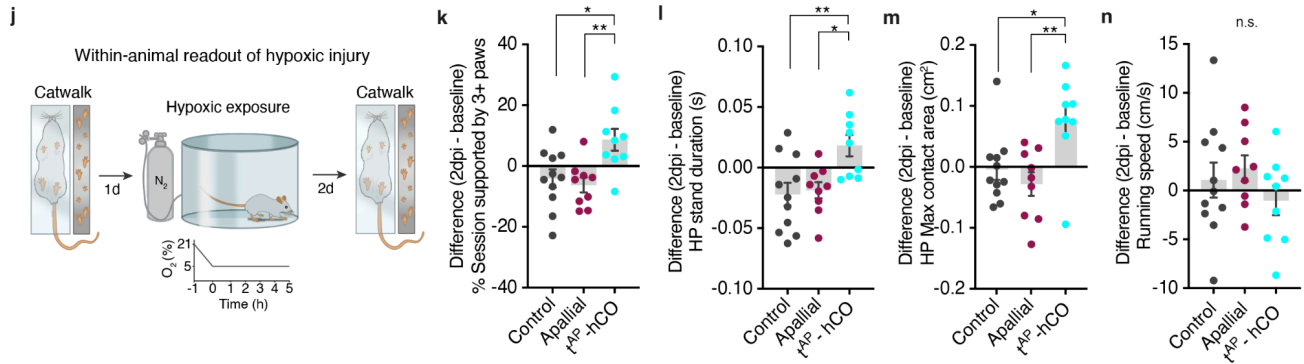
We did not see HIF1 α expression or an increase in SWI hypointensities in t^{AP} -hCO mice exposed to control atmospheric oxygen conditions nor in control mice exposed to 5% O_2 for 5 hours (Extended Data Fig. 10).



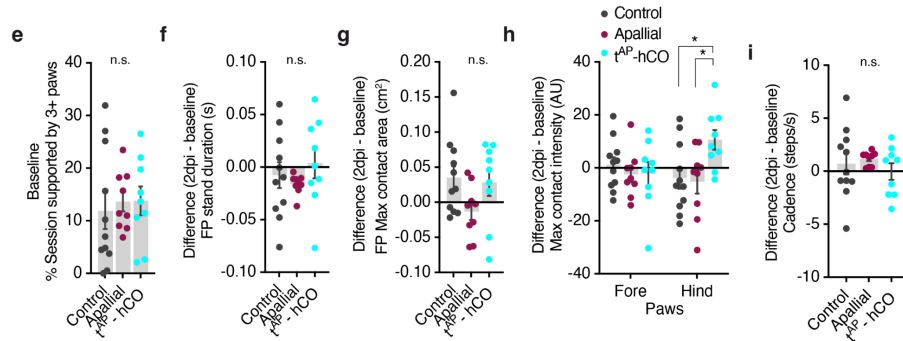
Finally, we found that t^{AP} -hCO mice exposed to 5% O_2 for 5 hours exhibited an increased density and complexity of intragraft Iba1 and GFAP cells (Fig. 5d-i), a shared cellular response to brain insults (PMIDs: 38286816, 40882239, 32770064).



We then tested whether the t^{AP} -hCO platform would allow for functional, behavioral readouts of injury. Because injury to the developing human brain is often associated with motor deficits, we tested mice in the CatWalk before and after injury. We found that 5% O_2 for 5 hours caused the t^{AP} -hCO group to increase the proportion of the session supported by 3–4 paws, a more stable position than the more common 2 paws (Fig. 5i). This was potentially driven by an increase in the amount of time the hind paws were in contact with the platform, the contact area of the hind paws, and the maximum intensity of the hind paw print on the glass surface (Fig. 5j,k and Extended Data Fig. 10g-h).



Critically, these changes were not due to changes in overall running speed or step cadence (Fig. 5l and Extended Data Fig. 10i).



Together, these data establish the feasibility of this approach for modeling selected aspects of human neurodevelopmental disorders in vivo, including proof-of-concept behavioral readouts.

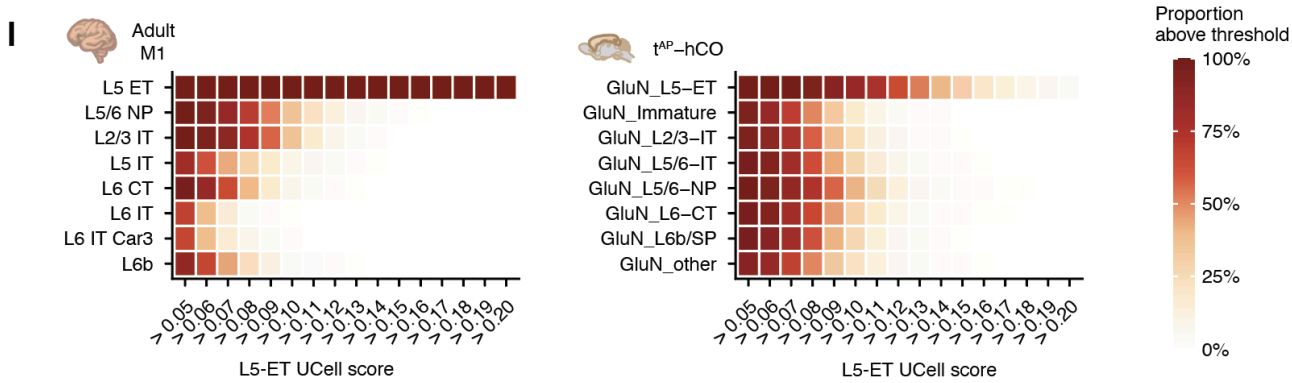
We share the reviewer's aspiration that this platform should enable therapeutic advances. By establishing this proof-of-concept application and defining both its advantages and limitations, we hope the present manuscript provides a foundation for therapeutic progress.

1) It is unclear how the biological conclusions differ between this approach and existing in vivo transplantation methods. Although textual explanations for why existing methods are insufficient, a head to head comparison is not provided and should be to identify the advantage of this controversial system.

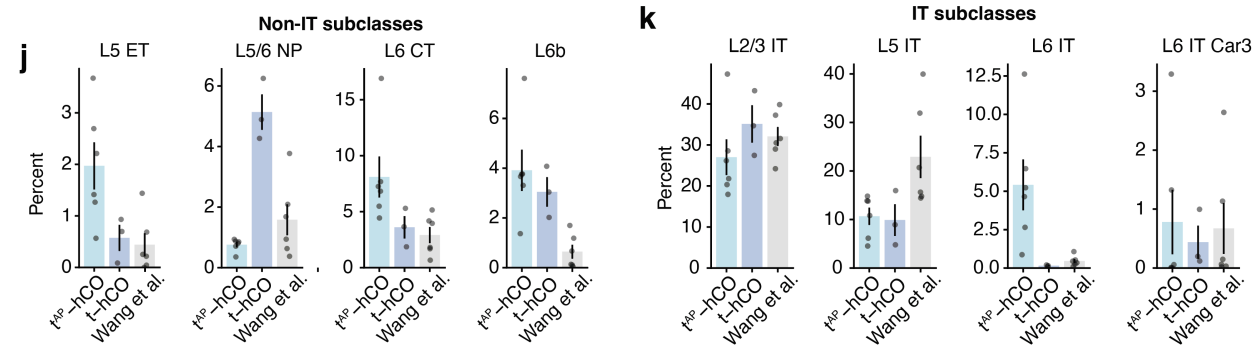
We thank the reviewer for an opportunity to expand on this point. As an example of the unique experimental insights that can arise from this system, we performed new experiments and analyses to highlight an advantage of this platform in the generation of transcriptomically-defined cortical L5-ET (layer 5 - extratelencephalic projecting) neurons, including the generation of a specialized subset of L5-ET neurons— corticospinal projection neurons.

To examine to what extent L5-ET neurons are present in various in vitro cortical organoid models, we performed new analyses of 213,753 dorsal telencephalic glutamatergic neurons from the Human Neural Organoid Cell Atlas (HNOCA), which integrates scRNAseq data across organoid protocols. We scored each neuron for L5-ET identity using UCell with a gene signature derived from the adult human M1 cortex. UCell is a rank-based method whose scores only rely on within-cell gene rankings, making them comparable across datasets. We found that essentially no in vitro organoid neurons showed enrichment for the L5-ET program (3/213,753 neurons had a L5-ET UCell score greater than 0.1).

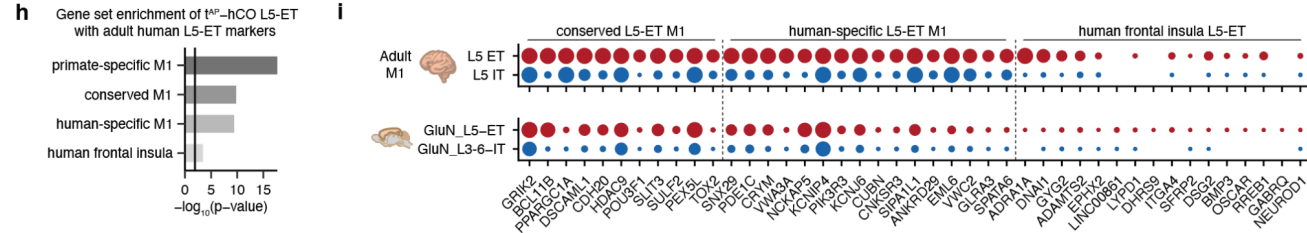
Whereas 85% of t^{AP} -hCO L5-ET neurons and 100% of adult M1 L5-ET neurons exceeded this threshold.



We next directly benchmarked cortical organoid transplantation procedures with respect to L5-ET neuron generation. We analyzed all of the available snRNA-seq datasets from transplanted cortical organoids, including our previous neonatal rat somatosensory cortex transplantation approach (PMID: 36224417, “ t -hCO”) and an adult mouse cortical cavity transplantation approach (PMID: 38418648 “Wang et al.”). Applying the same transcriptomic mapping procedure to each dataset, we found that the t^{AP} -hCO platform produced over three-fold greater proportion of L5-ET neurons compared to prior approaches (Extended Data Fig. 5j).

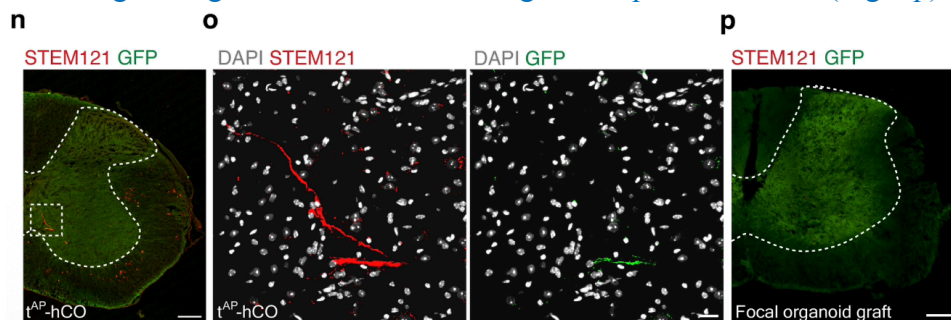


Beyond generating a greater proportion of L5-ET neurons, we leveraged the t^{AP} -hCO platform to characterize the molecular identity of these cells in greater detail. Gene set enrichment analysis of t^{AP} -hCO L5-ET neurons revealed enrichment for human-specific L5-ET genes identified in primary motor cortex, as well as genes characteristic of L5-ET neurons in human frontoinsular cortex, which contains von Economo neurons, a specialized population implicated in social cognition and neuropsychiatric disease (Extended Data Fig. 5h,i).



Second, because corticospinal neurons are an anatomically defined subset of L5-ET, and we observed graft-derived GFP and STEM121 in the spinal cord of t^{AP} -hCO mice (Fig. 2n,o), we tested whether transplantation of age-matched hCO into control, immunocompromised age-matched mice with an intact cortex (which we refer to here as focal organoid graft for contrast with grafts into apallial mice)

would similarly support corticospinal projections. We generated focal organoid grafts using hCO pre-infected with a lentivirus expressing either GFP or gCaMP and performed immunostaining and imaging of GFP and STEM121 in the cervical spinal cord. We could not detect human graft projections in mice with a focal organoid graft in contrast to hCO grafted apallial animals (Fig. 2p).



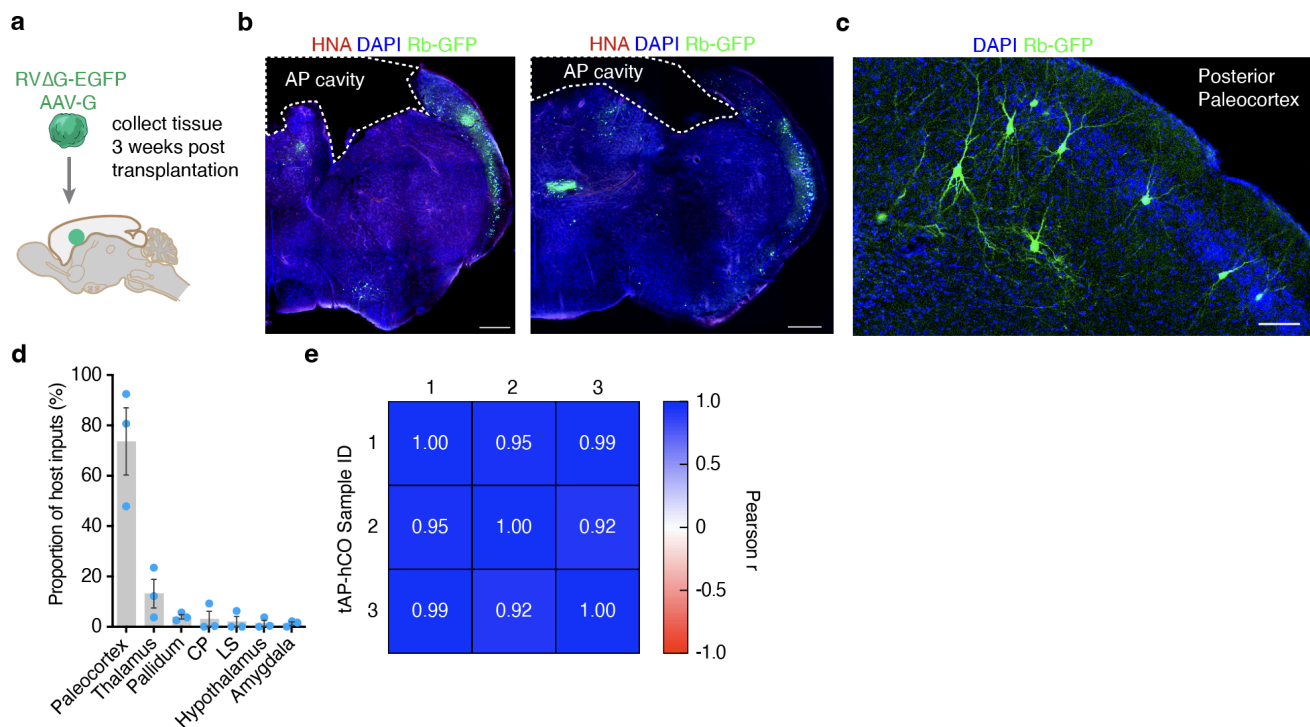
Together, these data imply that the genetic depletion of the host cortex may enable the enrichment of L5-ET neurons, including corticospinal neurons. This will be particularly useful for modeling disorders that affect this vulnerable population (PMID: 32127543, PMID: 38907103).

2) The authors skip directly to behavior but there are many, many steps before that are not addressed and one worries that the behavioral improvements may be an artifact rather than due to the specific nature of the organoid transplant:

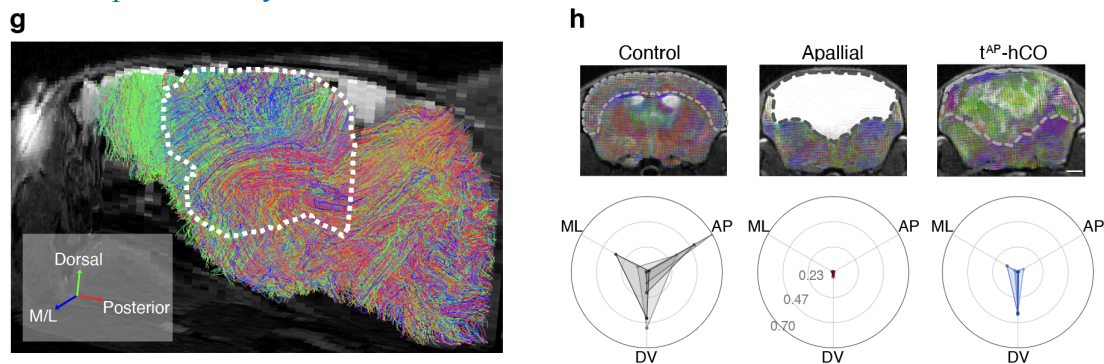
a. What happens if they put in as a control an organoid from a different brain region – how does it differ then in terms of molecular mechanisms and maturation?

We thank the reviewer for this suggestion. We did consider transplanting a control region, such as the subpallium, for this purpose. However, in separate work we found that subpallial grafts do not expand to the same extent as transplanted hCO, likely because of more limited proliferative capacity of other non-dorsal forebrain progenitors. This suggests that robust engraftment into the space created by depletion may be achievable primarily with highly proliferative populations, such as cortical radial glia. Due to these constraints, we focused on verifying the specificity of the connectivity t^{AP}-hCO using two anatomical methods: rabies retrograde tracing and MRI-based diffusion-weighted imaging. Broadly, our data support the conclusion that connectivity in t^{AP}-hCO is not random and is reproducible across samples.

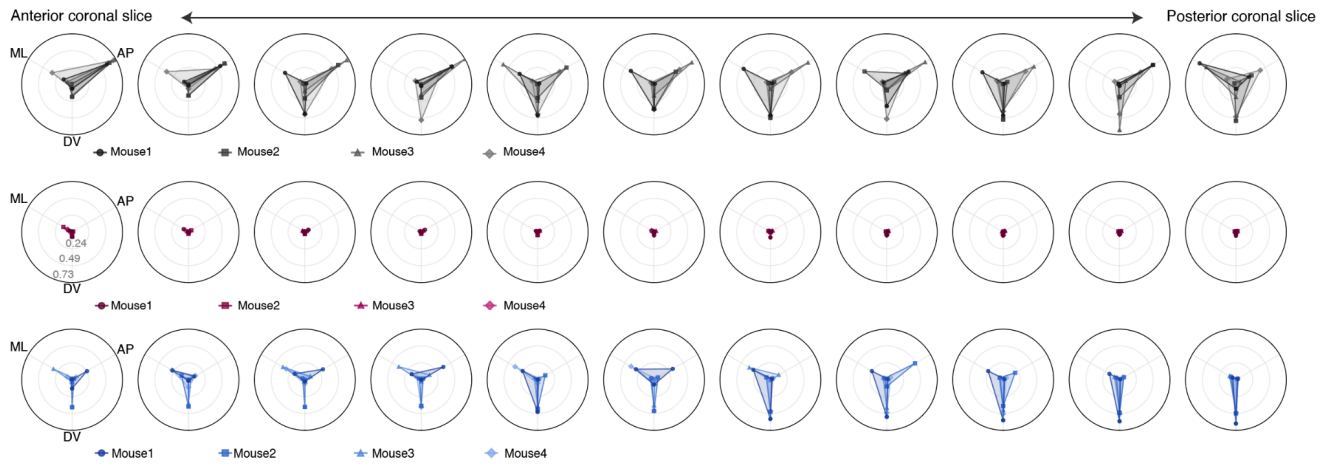
First, we pre-infected hCO with a G-deleted rabies virus encoding GFP and an AAV encoding the helper G protein. The G-deletion of the rabies virus restricts retrograde transport to cells carrying the G protein (i.e. one retrograde step from the organoid). We transplanted the pre-infected hCO into apallial mice and collected tissue 3 weeks later (Extended Data Fig. 3a). We found consistent labeling of neurons in the host paleocortex, thalamus, and pallidum (nucleus of the diagonal band, substantia innominata, globus pallidum external, etc.), but only 1/3 mice exhibited sparse labeling in the caudate putamen (CP, Extended Data Fig. 3b-d). This suggests some degree of specificity of connections: in wild-type mice, CP neurons do not project directly to the cortex, and although the graft borders the CP (Fig 2f, Extended Data Figs. 3f, 3i, 7a), ectopic CP-to-graft projections are rare. Most of the host input neurons resided in the paleocortex, followed by the thalamus and pallidum, with a strong correlation between mice (Extended Data Fig. 3d,e).



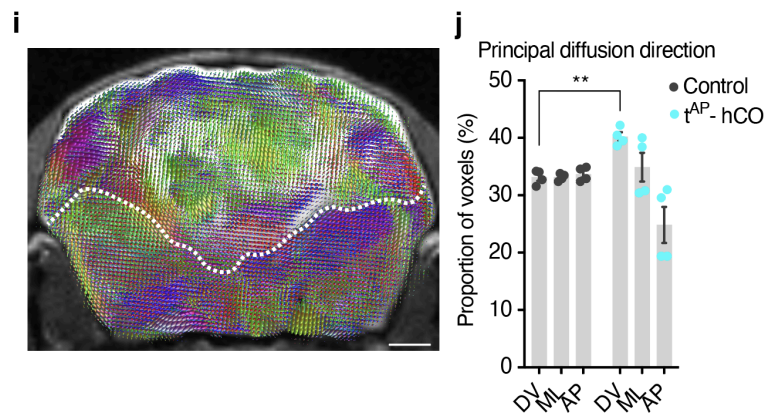
Second, to investigate structural connectivity in t^{AP} -hCO, we acquired diffusion-weighted images (DWI) and applied tractography methods (Fig 2g) to provide a quantitative description of the directionality of local water diffusion, and hence, the organization of tissue microarchitecture. In a representative coronal plane across groups (Fig. 2h), the control group demonstrated a heterogeneous pattern of water diffusion in all directions (AP, ML, DV). In contrast, the water diffusion in the t^{AP} -hCO followed a stereotyped dorso-ventral (DV) trajectory. The lack of an average diffusion direction in the apallial cavity is consistent with the absence of tissue and coherent microstructure.



To comprehensively estimate the structural organization of the graft, we quantified the principal direction of water diffusion across all coronal sections (Extended Data Fig. 3k).



We observe high within-subject consistency across the anterior-posterior axis and high between-subject consistency. Control isocortex demonstrates equal distribution in all directions, while t^{AP} -hCO demonstrates predominantly dorsal-ventral (DV) orientations, with minor components in the medial-lateral (ML) and anterior-posterior (AP) directions. We confirm this observation by quantifying the distribution across all sections of all mice (Extended Data Fig. 3i,j).



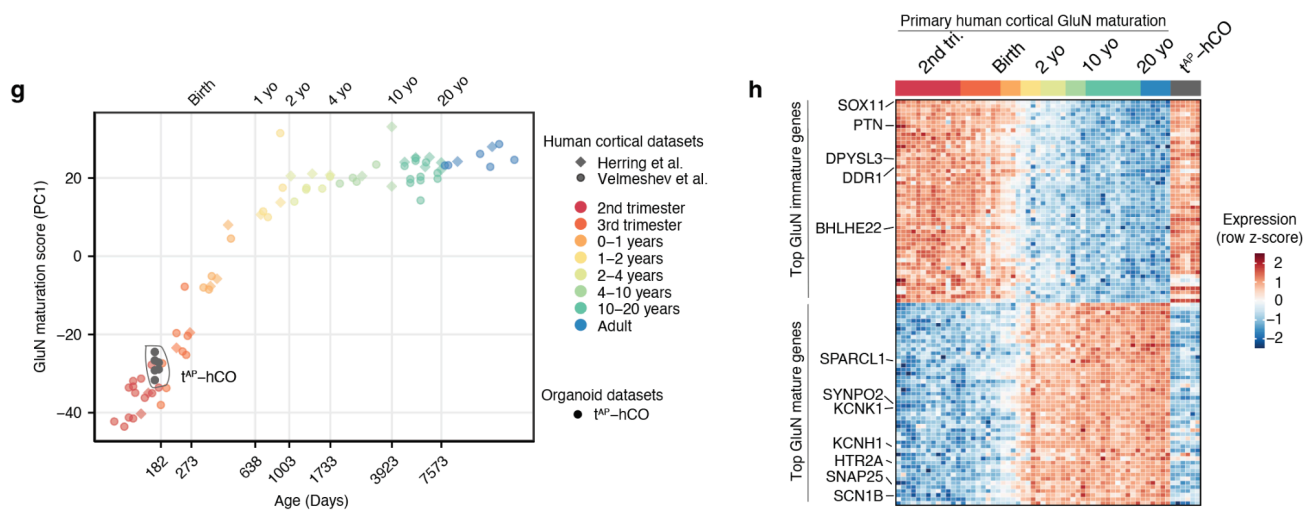
To quantify the variability of tissue microarchitecture between grafts, we calculated the root mean squared deviation (RMSD) of the control (0.1445) and t^{AP} -hCO groups (0.0944), and normalized these values to the average magnitude of the vectors (control=0.5104, t^{AP} -hCO=0.3663). This yielded a normalized RMSD of 0.28 (control) and 0.26 (t^{AP} -hCO). Overall, DWI analyses demonstrated a consistent, predominantly dorso-ventral microstructural orientation in the t^{AP} -hCO.

Together, the distribution of host inputs to the graft from rabies tracing along with stereotyped dorso-ventral microstructural organization from DWI support some level of consistency in t^{AP} -hCO connectivity patterns.

b. How does maturation happen? Beautiful and elegant work using iPSC cells from Vanderhaegen and colleagues has shown that maturation is intrinsic to human neonatal timing – the assays presented seem much too fast for molecular and circuit maturation to be complete in this system.

We thank the reviewer for this insightful comment. Confirming Vanderhaegen's pioneering work on integrating human neurons into the rodent cortex, data from our lab showed that hCO transplanted into

the developing rat cortex largely maintains its intrinsic timing across molecular, morphological, and physiological assays. To more precisely estimate the maturation stage of t^{AP} -hCO, we performed new analyses benchmarking glutamatergic neuron maturation against primary developing human cortical snRNA-seq datasets (PMIDs: 37824647 and 36318921) (Extended Data Fig. 4g,h). Briefly, we generated pseudobulk expression profiles from t^{AP} -hCO glutamatergic neurons and performed PCA alongside pseudobulk profiles spanning primary human cortical development of these two reference datasets. We then constructed a linear model relating PC1 scores to gestational age from the Velmeshev et al. (PMID: 37824647) samples and validated it on held-out Herring et al. (PMID: 36318921) data, restricting to samples younger than 4 years postnatal age where model performance was strongest. This analysis estimated that t^{AP} -hCO samples 24 weeks post-differentiation map onto the primary dataset at about 24 gestational weeks.

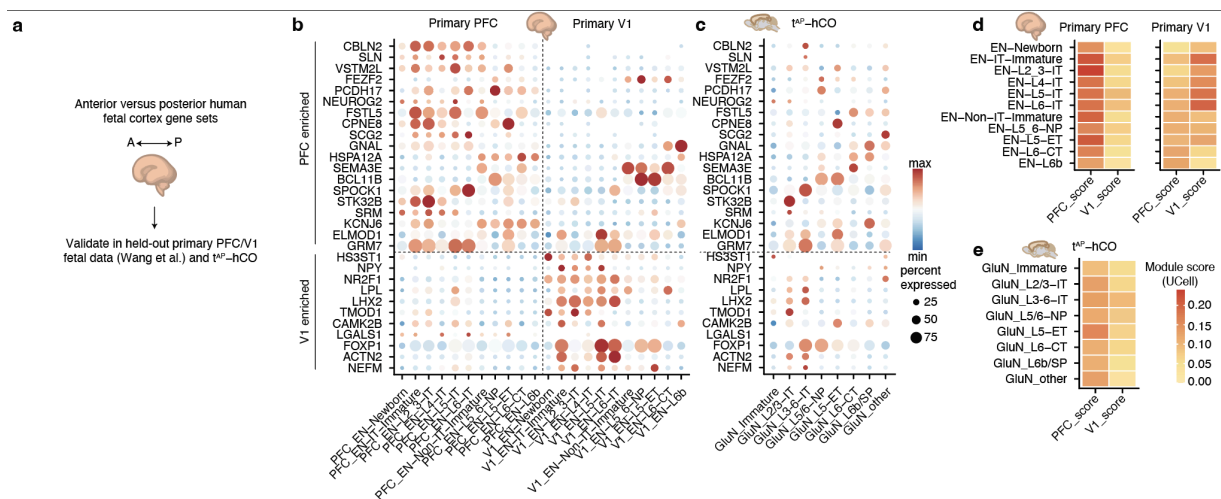


Putting these data together with the presence of synchronous network activity detected in the whole cortex gCaMP imaging, data in t^{AP} -hCO presented in this manuscript reflect a human mid-corticogenesis stage. We added the following to the results to clarify this issue:

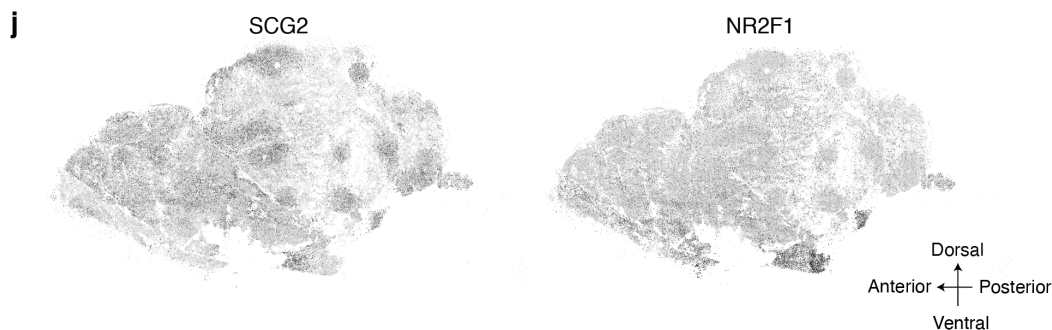
“We benchmarked t^{AP} -hCO glutamatergic neuron maturation against primary developing human cortical snRNA-seq datasets (PMIDs: 37824647 and 36318921) (Extended Data Fig. 4g,h). Consistent with prior work (PMIDs: 23395372, 31761708, 33619405, 36224417), the transcriptomic maturation state of t^{AP} -hCO largely followed in vivo developmental timing: t^{AP} -hCO samples 24 weeks post-differentiation map onto the primary dataset at 24 gestational weeks.”

c. Cortical organoids do not have area specificity – do cortical areas emerge in these transplants? By what mechanism? When?

We thank the reviewer for this question. We reanalyzed our snRNAseq dataset alongside primary fetal PFC and V1 data (PMID: 39779846) for markers of cortical arealization identified from primary fetal human cortical studies (PMIDs: 34616070 and 40369074). We found some evidence of increased expression of PFC-associated markers in t^{AP} -hCO glutamatergic neurons although this was not as strong as seen in primary human cortical data (Extended Data Fig. 5a-e).

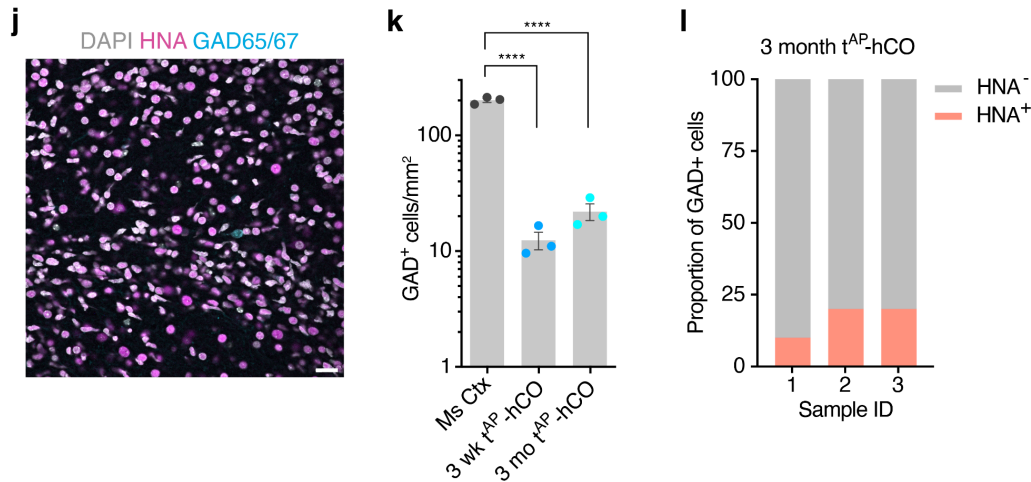


Furthermore, spatial transcriptomics of the t^{AP}-hCO did not reveal a striking gradient along the anterior-posterior axis with available cortical arealization genes present on our MERSCOPE gene panel (Extended Data Fig. 6j). This suggests that the mismatch between the developmental age of the graft and host may not enable robust cortical arealization at this time point. Future work probing the system at later time points will help elucidate whether functional arealization will emerge.



d. Although inhibitory neurons are addressed somewhat, it seems unlikely enough would be generated. Are there no seizure like activities? Where do inhibitory neurons migrate?

This is an important issue to investigate. We first performed a series of immunostaining analyses of GABAergic cells. We found interneurons in the graft by 3 weeks post-transplantation (Extended Data Fig. 7i,j). At 3 months post-transplantation, there was no significant increase in the density of GAD⁺ neurons and the majority of the interneurons (median: 80%) in the graft are host-derived (Extended Data Fig. 7k).



As expected, the transplantation of a primarily glutamatergic hCO resulted in a graft with ~10x fewer GABAergic neurons than wild-type cortex (Extended Data Fig. 7i,j). Future versions of this model could leverage assembloid approaches to incorporate human GABAergic neurons into the graft. We added the following to the discussion:

“As an initial demonstration of the t^{AP}-hCO model, we transplanted glutamatergic hCO, leading to a low density of GABAergic neurons within the graft. Future versions of this model may use assembloid approaches, particularly for modeling imbalances in excitation and inhibition in neuropsychiatric disorders.”

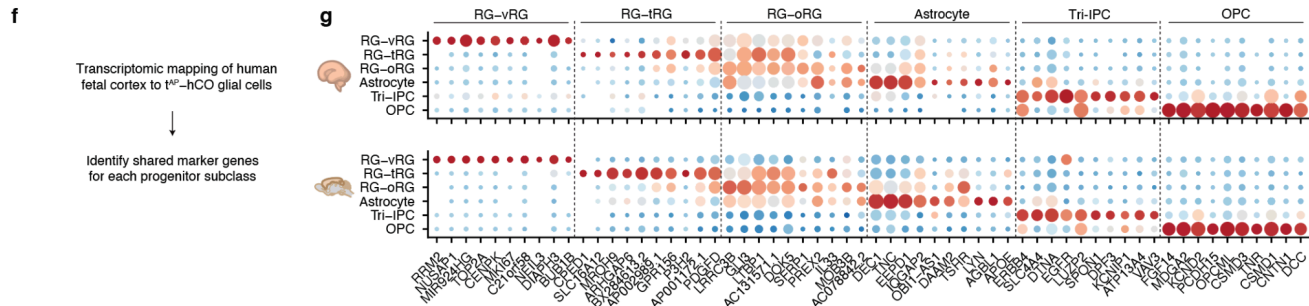
As the reviewer pointed out, many models of adult epilepsy would predict that a feed-forward excitatory network, similar to the t^{AP}-hCO or previous rat t-hCO model, should become epileptic foci. However, we have not found evidence for this. This may reflect the fact that t^{AP}-hCO generates activity reminiscent of developmental stages and/or the engagement of host homeostatic mechanisms, particularly given that transplantation is performed early in development. Indeed, characterization of t^{AP}-hCO activity with gCaMP imaging showed synchronized, high amplitude events, similar to early network oscillations (ENOs) in development (which was previously described by Roska, López-Bendito, Portera-Cailliau, Feller, Ben-Ari, Konnerth, Cossart labs and others). Critically, GABA is required for high frequency activity in the gamma range, and work from Ben-Ari’s group and others demonstrate that GABA and high frequency activity is required for epileptogenesis in developing networks. As a proof of principle, our previous characterization of the t-hCO transplantation model, in which hCO is transplanted into the neonatal rat cortex, demonstrated normal, non-epileptic EEG activity (PMID: 36224417). We added a sentence to the discussion contextualizing these results:

“At the electrophysiological level, the waves of synchronized, high amplitude events are reminiscent of an immature form of activity (termed early network oscillations). It remains to be established whether this form of activity propagates outside of the graft.”

e. Do human-specific progenitor and neuronal populations emerge, or is there a restriction to subtypes shared between mouse and human?

To address whether human-enriched progenitor populations emerge in the t^{AP}-hCO system, we transcriptomically mapped our snRNA-seq data to a recently published, comprehensively annotated

atlas of human neocortical development (PMID: 39779846). Using label transfer to second trimester data, we annotated our non-neuronal cells and identified signatures consistent with the major human cortical progenitor subtypes, including outer radial glia (oRG), a population expanded in primates; truncated radial glia (tRG), a later stage progenitor enriched in gyrencephalic mammals and absent in mice; ventricular radial glia (vRG); and tripotential intermediate progenitor cells (Tri-IPCs), a recently described population proposed to be capable of generating GABAergic neurons, oligodendrocyte precursor cells, and astrocytes. The local production of GABAergic neurons from dorsal cortical progenitors is a process that is minimal or absent in rodents. We confirmed these annotations by demonstrating high overlap between marker genes of t^{AP} -hCO progenitor subclasses and their primary tissue counterparts (Extended Data Fig. 5f,g).



Regarding neuronal populations, as described above, t^{AP} -hCO generate transcriptomically defined L5-ET neurons enriched for human-specific M1 L5-ET genes and genes expressed in von Economo neurons. Consistent with transcriptomic enrichment, we further identified cells with the characteristic bipolar morphology and large soma diameter of von Economo neurons in all t^{AP} -hCO examined (Figure 2l,m and Extended Data Fig. 7p-r). Together, these findings demonstrate that the t^{AP} -hCO platform recapitulates human-enriched features of cortical development at both the progenitor and neuronal level, including cell types that are not present in mice.

f. Many other considerations about mechanisms and evolution that are not addressed or at all considered.

This is a small number of the scientific considerations missed by this study, and as presented it is a flashy, surface level, ethically challenging paper that I cannot endorse for publication.

We understand the position, and we have taken extensive steps to address concerns. Based on reviewer comments, we have strengthened the manuscript through the addition of a disease model, quantification of anatomical specificity, an in-depth characterization of the spatial distribution of cell types within t^{AP} -hCO, a direct comparison of this model to other organoid transplantation methods, replication of behavioral findings, and clarifying comments on the scope and limitations of the model.

Referee #3

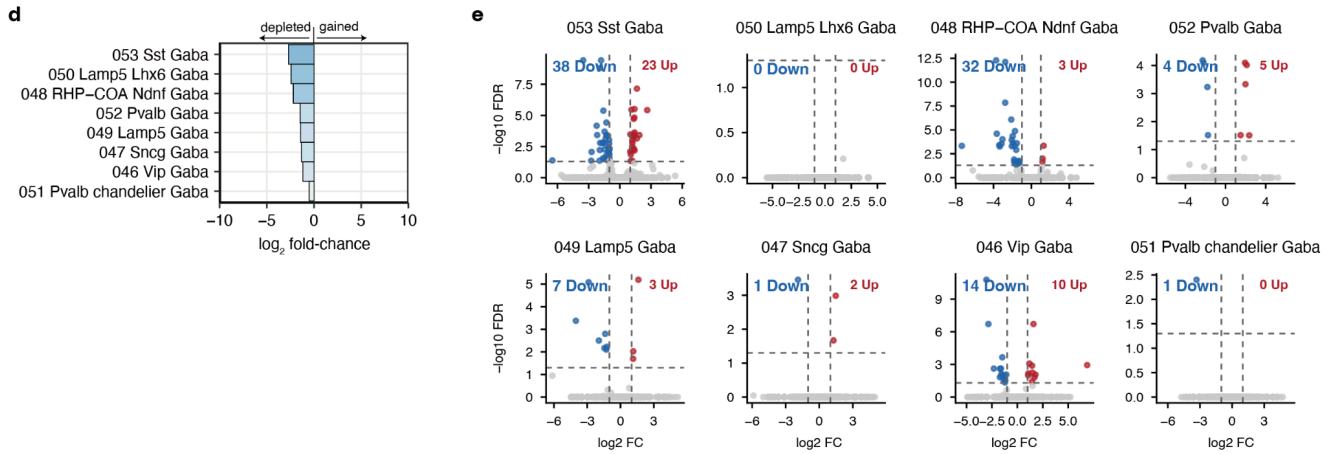
This study tackles the challenge of limited access to human brain tissue for studying brain development and function in health and disease. Human stem cell–derived models such as organoids and assembloids, especially when transplanted into rodents, have started to address this issue. However, their integration is often constrained by competition with the host brain. To overcome this, the authors developed a new transplantation method using genetically modified mice lacking glutamatergic neurons in the cortex and hippocampus. This provides enough space for the growth of transplanted human cortical organoids (hCO), which integrate robustly. The hCOs expand and provide artificial pallial-like structure and generate diverse human cell types, including layer 5 extratelencephalic neurons, which are hard to derive *in vitro*. These neurons project widely through the mouse brain and spinal cord. Functional imaging and electrophysiology reveal organized, cortex-like activity in the grafts. Behavioral analysis shows subtle but distinct effects post-transplantation. Notably, hCOs partially restore motor and working memory deficits caused by cortical depletion. This demonstrates the model's potential to study human neural circuits and therapeutic effects. Despite limitations—such as a lack of cortical layering and proper cerebral cortex morphogenesis—this method significantly advances the field. It may offer insights into circuit-level abnormalities in patient-derived cells. The model will also enable deeper exploration of human neurodevelopment and disease. The manuscript is well-written, scientifically sound, and presents experiments conducted using state-of-the-art, cutting-edge methodologies. Below, I provide suggestions and comments aimed at further strengthening the manuscript.

Major concerns

> Figure 1f-i. The snRNA-seq analysis of adult "apallial" mouse brains reveals a marked reduction in most cortical glutamatergic neurons, as expected. This loss is accompanied by a decrease in GABAergic interneurons, which are typically located within the cortex under normal conditions. In wild-type mice, cortical interneurons originate from the subpallium. In the apallial brains, however, it remains unclear whether these interneurons are lost due to cell death or if they have been spatially redistributed and possibly adopted a new fate. Along this line, it is striking to see that few host GAD65/67 interneurons repopulate the hCO graft at 5 months. This suggests that some host interneurons may have remained localized ventrally but underwent reactivation of their dorsal migration in response to the human graft.

We agree that the fate of cortical GABAergic interneurons in the apallial mouse brain is an interesting developmental question. We note that our original differential abundance analysis did not reveal a statistically significant reduction in cortical MGE or CGE derived GABAergic neuron classes, although there was a trend in reduction, especially for MGE interneurons ($\log_2\text{FC} = -2.2$, nominal $p\text{-value} = 0.02$, $\text{FDR} = 0.21$). To further address this question, we performed new analyses among cortical GABAergic subclasses, revealing trending reductions across most subclasses, with SST interneurons appearing the most reduced ($\log_2\text{FC} = -2.7$, $\text{FDR} = 0.11$), consistent with their reported dependence on local pyramidal neuron signalling for their survival (PMIDs: 29849154 and 39071350). These reductions did not reach statistical significance, potentially reflecting both low statistical power ($n = 2$ per group) and preserved pyramidal neurons within ventrolateral pallial structures, such as the piriform cortex, in our model.

To further address whether surviving cortical GABAergic neurons have undergone fate alteration, we performed differential expression analysis across all cortical GABAergic subclasses (Extended Data Fig. 2d,e).

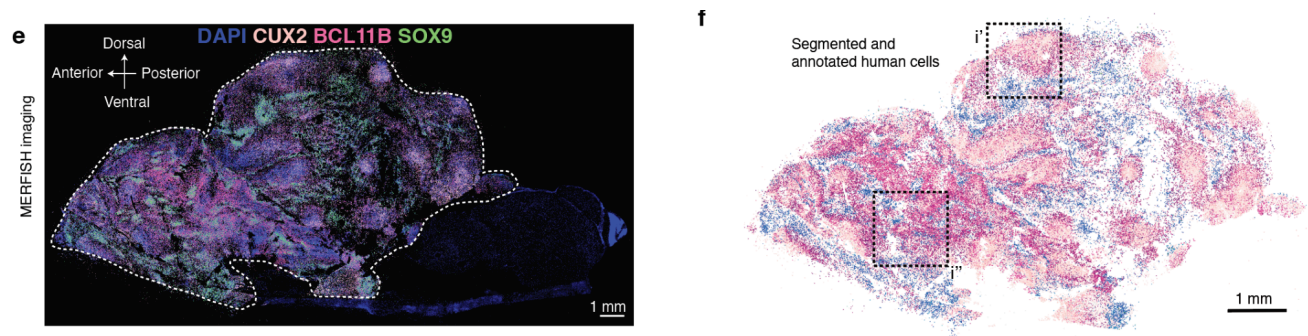


Most comparisons showed minimal transcriptional changes between control and apallial mice. The Sst subclass showed the most changes (23 up-regulated genes, 38 down-regulated genes; $|\log_2 FC| > 1$, $FDR < 0.05$), and gene ontology pathway enrichment analysis did not support a change in cell identity programs. Rather, significant downregulated pathways included regulation of synaptic plasticity, driven by trending reductions in NMDA receptor subunits and activity-dependent genes; upregulated pathways converged on ERK/MAPK signaling. While most individual genes within these pathway enrichments do not meet multiple testing correction, the overall picture suggests surviving Sst interneurons retain their identity but have reductions in transcriptional signatures associated with circuit engagement. Lastly, we did not observe a statistically significant increase in other forebrain GABAergic classes in the apallial mice, arguing against large-scale spatial redistribution.

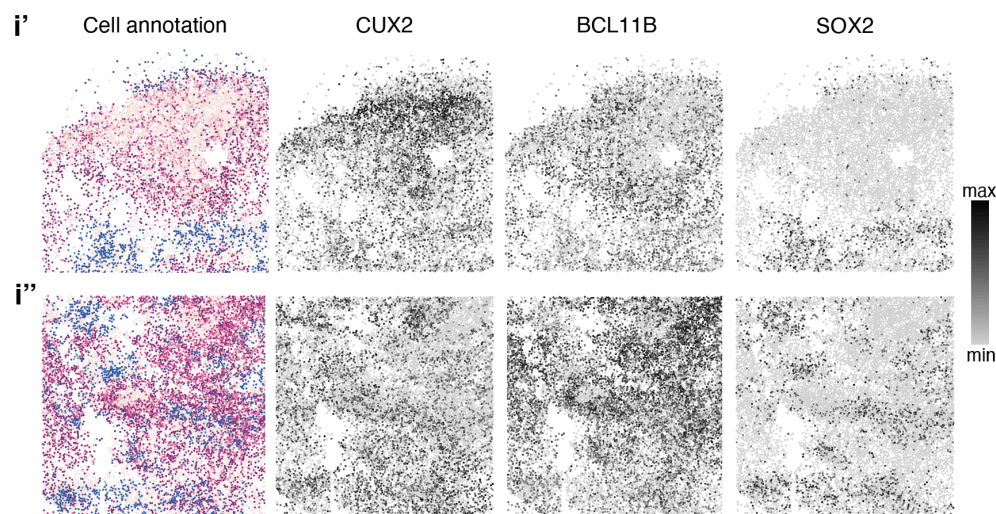
> The manuscript would benefit from a more in-depth immunohistochemical characterization of the cellular composition within the hCO graft. Specifically, are the cell types identified by snRNA-seq in Figures 2g–i also detectable within the graft through immunostaining where are they placed and to which cell partner are they connected with (synapses)? Additionally, is there any evidence of emerging layer-like organization within the graft? Finally, further clarification is needed regarding the presence and distribution of inhibitory and excitatory synapses, particularly between host- and graft-derived interneurons and human projection neurons. Finally, to what extent is the graft undergoing differentiation? What proportion of cycling progenitor cells remains? Additionally, it would be important to assess whether the graft contains any off-target derivatives unrelated to the forebrain lineage.

We thank the reviewer for these suggestions. To address these points, we have performed new analyses and experiments, including spatial transcriptomics, rabies retrograde tracing, diffusion weighted imaging, and benchmarking against primary human fetal datasets.

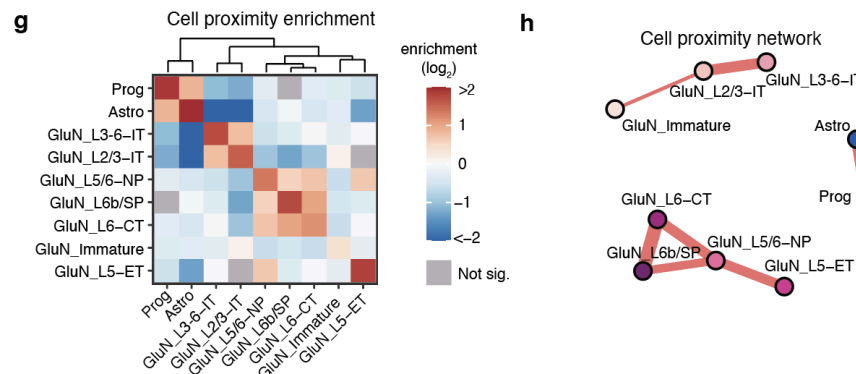
To comprehensively address the question of cytoarchitectural organization, we performed spatial transcriptomics (MERFISH) of t^{AP} -hCO. As expected based on the presence of multiple ventricular-like zones in organoids without consistent orientation, we did not observe canonical cortical layering throughout the graft (Extended Data Fig. 6e,f).



However, we asked whether local cytoarchitectural organization might emerge within t^{AP} -hCO. We found regions within the graft predominantly occupied by either upper-layer intratelencephalic (IT) or deep-layer non-IT neuron subclasses (Extended Data Fig 6i).

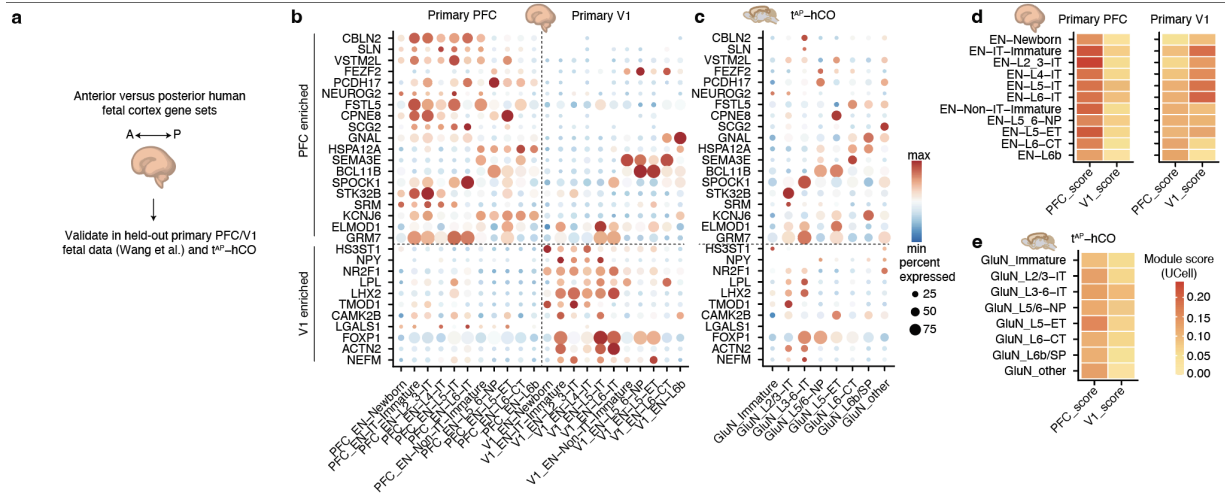


While these regions do not form a continuous laminar structure across the graft, nearest-neighbor analysis reveals that transcriptomically defined subclasses that normally reside in similar cortical layers are found in closer spatial proximity than expected by chance, consistent with local self-organization (Extended Data Fig. 6 g-i).

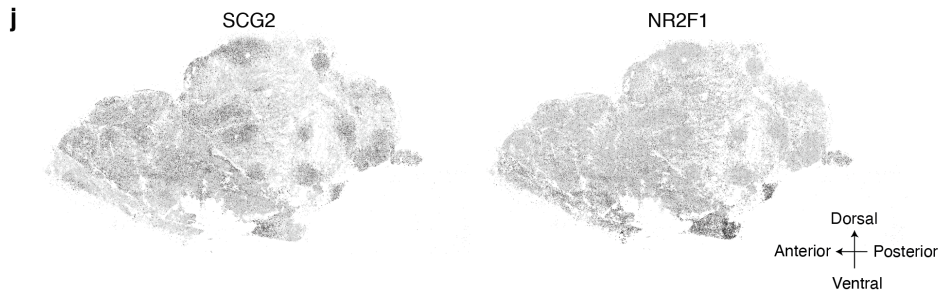


We then investigated whether t^{AP} -hCO exhibit any features of cortical arealization. We reanalyzed our snRNAseq dataset alongside primary fetal PFC and V1 data (PMID: 39779846) for markers of cortical arealization identified from primary fetal human cortical studies (PMIDs: 34616070 and 40369074). We found some evidence of increased expression of PFC-associated markers in t^{AP} -hCO glutamatergic

neurons although this was not as strong as seen in primary human cortical data (Extended Data Fig. 5a-e).

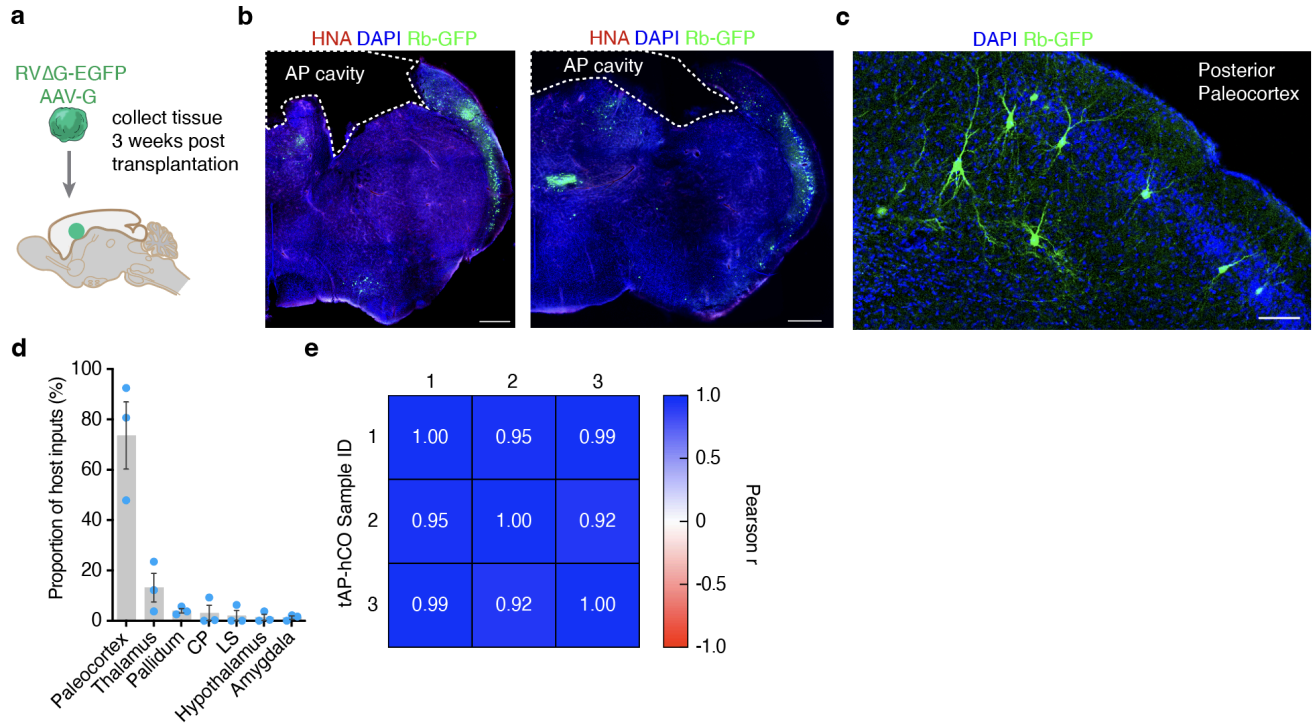


Furthermore, spatial transcriptomics of the t^{AP} -hCO did not reveal a striking gradient along the anterior-posterior axis with available cortical arealization genes present on our MERSCOPE gene panel (Extended Data Fig. 6j). This suggests that the mismatch between the developmental age of the graft and host may not enable robust cortical arealization at this time point. Future work probing the system at later time points will help elucidate whether functional arealization will emerge.

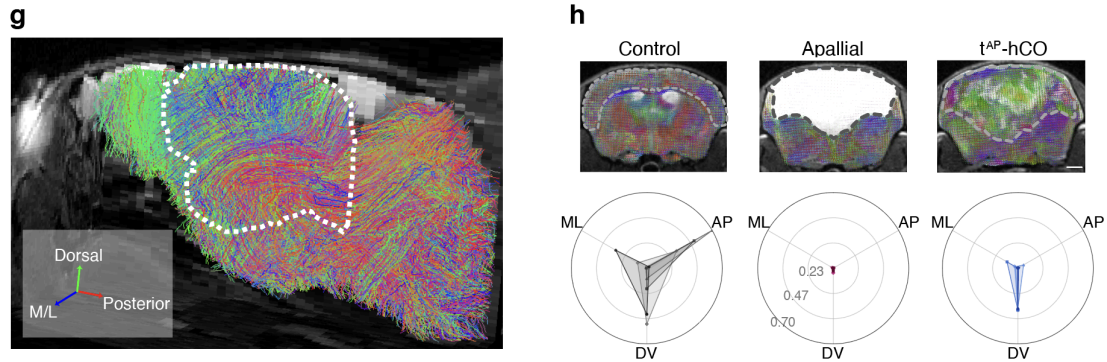


To systematically probe the question of long-range connectivity, we used two anatomical approaches: rabies retrograde tracing and MRI-based diffusion-weighted imaging. Broadly, our data support the conclusion that connectivity in t^{AP} -hCO is not random and is reproducible across samples.

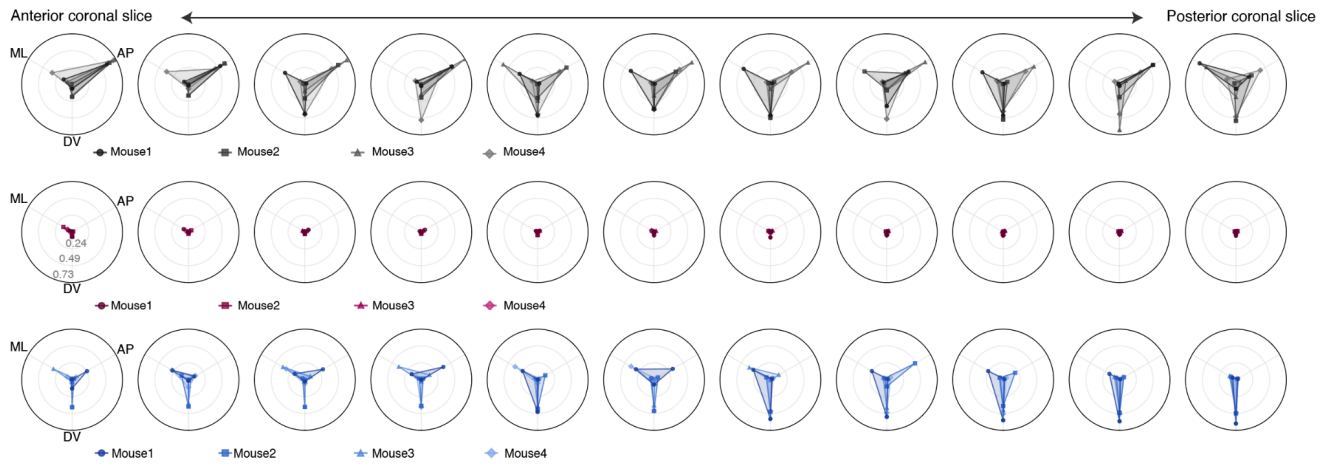
First, we pre-infected hCO with a G-deleted rabies virus encoding GFP and an AAV encoding the helper G protein. The G-deletion of the rabies virus restricts retrograde transport to cells carrying the G protein (i.e. one retrograde step from the organoid). We transplanted the pre-infected hCO into apallial mice and collected tissue 3 weeks later (Extended Data Fig. 3a). We found consistent labeling of neurons in the host paleocortex, thalamus, and pallidum (nucleus of the diagonal band, substantia innominata, globus pallidum external, etc.), but only 1/3 mice exhibited sparse labeling in the caudate putamen (CP, Extended Data Fig. 3b-d). This suggests some degree of specificity of connections: in wild-type mice, CP neurons do not project directly to the cortex, and although the graft borders the CP (Fig 2f, Extended Data Figs. 3f, 3i, 7a), ectopic CP-to-graft projections are rare. Most of the host input neurons resided in the paleocortex, followed by the thalamus and pallidum, with a strong correlation between mice (Extended Data Fig. 3d,e).



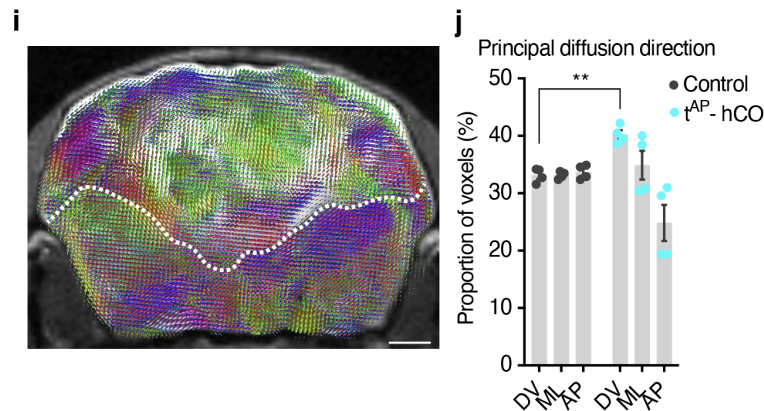
Second, to investigate structural connectivity in t^{AP} -hCO, we acquired diffusion-weighted images (DWI) and applied tractography methods (Fig 2g) to provide a quantitative description of the directionality of local water diffusion, and hence, the organization of tissue microarchitecture. In a representative coronal plane across groups (Fig. 2h), the control group demonstrated a heterogeneous pattern of water diffusion in all directions (AP, ML, DV). In contrast, the water diffusion in the t^{AP} -hCO followed a stereotyped dorso-ventral (DV) trajectory. The lack of an average diffusion direction in the apallial cavity is consistent with the absence of tissue and coherent microstructure.



To comprehensively estimate the structural organization of the graft, we quantified the principal direction of water diffusion across all coronal sections (Extended Data Fig. 3k).



We observe high within-subject consistency across the anterior-posterior axis and high between-subject consistency. Control isocortex demonstrates equal distribution in all directions, while t^{AP} -hCO demonstrates predominantly dorsal-ventral (DV) orientations, with minor components in the medial-lateral (ML) and anterior-posterior (AP) directions. We confirm this observation by quantifying the distribution across all sections of all mice (Extended Data Fig. 3i,j).

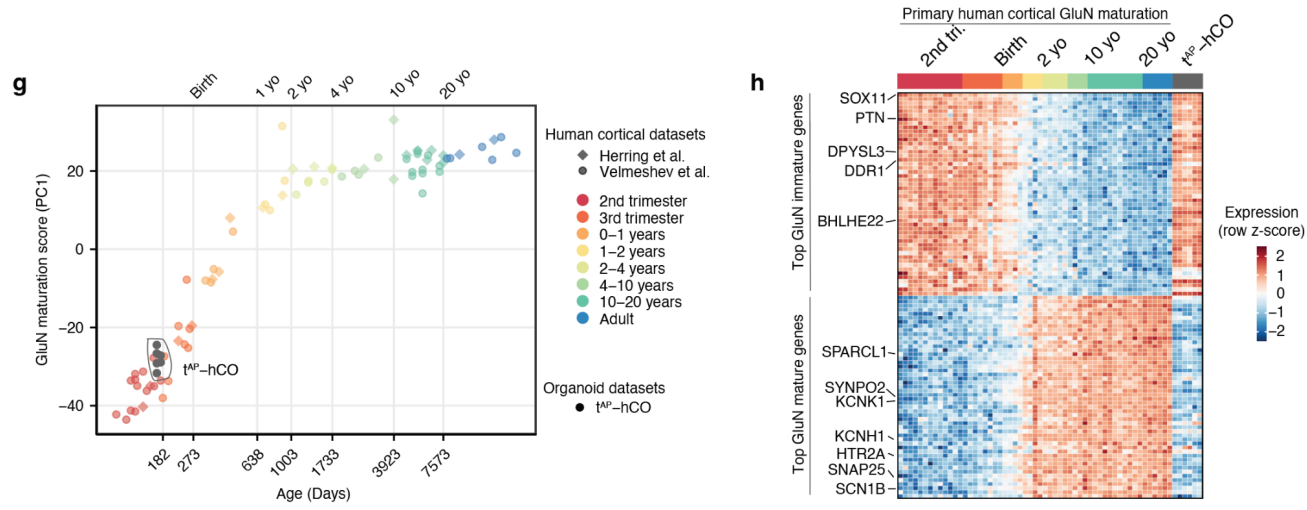


To quantify the variability of tissue microarchitecture between grafts, we calculated the root mean squared deviation (RMSD) of the control (0.1445) and t^{AP} -hCO groups (0.0944), and normalized these values to the average magnitude of the vectors (control=0.5104, t^{AP} -hCO=0.3663). This yielded a normalized RMSD of 0.28 (control) and 0.26 (t^{AP} -hCO). Overall, DWI analyses demonstrated a consistent, predominantly dorso-ventral microstructural orientation in the t^{AP} -hCO.

Together, the distribution of host inputs to the graft from rabies tracing along with stereotyped dorso-ventral microstructural organization from DWI support some level of consistency in t^{AP} -hCO connectivity patterns.

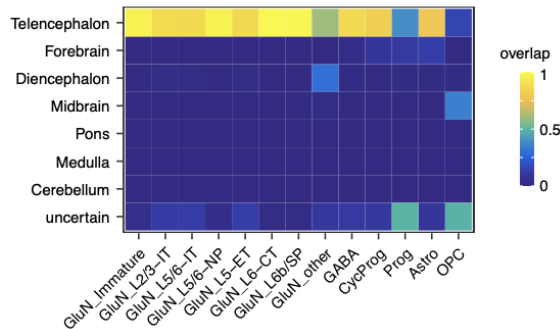
To address the question of differentiation, we performed new analyses benchmarking glutamatergic neuron maturation against primary developing human cortical snRNA-seq datasets (PMIDs: 37824647 and 36318921) (Extended Data Fig. 4g,h). Briefly, we generated pseudobulk expression profiles from t^{AP} -hCO glutamatergic neurons and performed PCA alongside pseudobulk profiles spanning primary human cortical development of these two reference datasets. We then constructed a linear model relating PC1 scores to gestational age from the Velmeshev et al. (PMID: 37824647) samples and

validated it on held-out Herring et al. (PMID: 36318921) data, restricting to samples younger than 4 years postnatal age where model performance was strongest. This analysis estimated that t^{AP} -hCO samples 24 weeks post-differentiation map onto the primary dataset at about 24 gestational weeks.



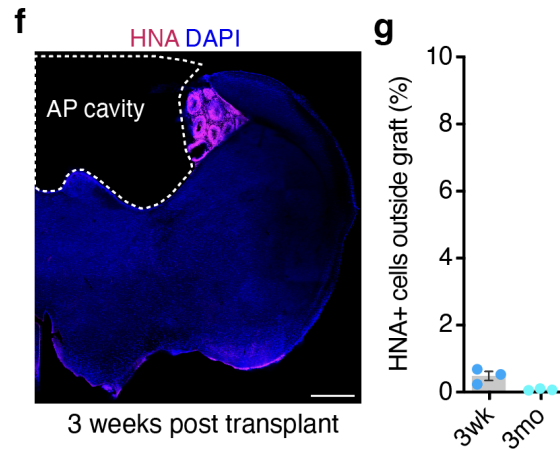
Regarding cycling progenitors, we further analyzed the snRNAseq data of the graft and observed $1.8 \pm 0.6\%$ of the cells were cycling, which is comparable to second trimester human cortical data ($1.3 \pm 0.2\%$ cycling progenitors; PMID: 39779846).

Lastly, to address the question of off-target derivatives unrelated to the forebrain lineage, we performed new transcriptomic mapping analyses to a human fetal whole-brain scRNAseq atlas (PMID: 37824650) to address the regional identity of t^{AP} -hCO cells. Among cells with confident predictions, the vast majority (>95%) mapped to fetal telencephalon (ED Fig. 4d).



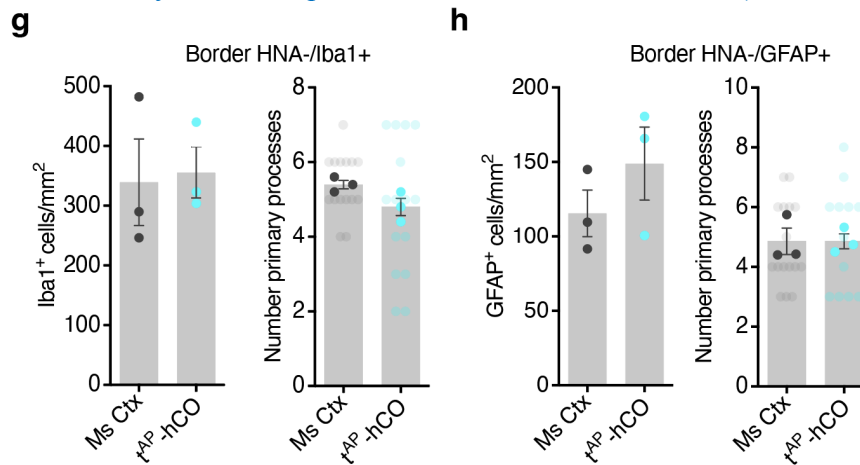
>Figure 2f. The parasagittal sections illustrate the extensive integration of the graft into the host brain. While graft-derived axonal tracts are visible, there also appears to be graft invasion into the host subpallium. Can this be systematically assessed? If confirmed, what might be the functional implications of such heterotopic localization of graft-derived cells?

We performed a new experiment staining for human nuclei at 3 weeks and at 3 months post-transplantation. We found that 0.49% (3 weeks) and 0.08% (3 months) of the human nuclei in a coronal section exist outside the graft (Extended Data Fig. 3f,g), indicating that extra-graft human nuclei are rare.



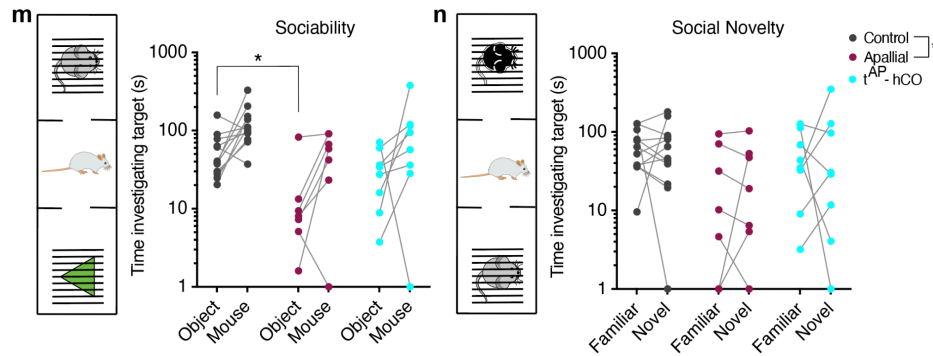
>Extended data figure 4. It would be important to clarify whether the hCO graft induces inflammation and/or glial scarring in the host mouse brain. Can this be quantified and reported? Assessing the extent of astrogliosis or microglial activation would be valuable for evaluating the host immune response. This information is particularly relevant from a translational perspective, as it informs the feasibility of using such chimeric models for studying human brain disorders.

We thank the reviewer for the suggestion. Guided by the reviewer's question of glial scarring in the host mouse brain, we performed new immunostaining experiments characterizing host-derived GFAP and Iba1 at the border between the graft and host. We found that both the density and morphology of mouse microglia and astrocytes were equivalent to control mouse cortex (*Esco2*^{fl/fl}; *Prkdc*^{scid/scid}).



>Extended data figure 7h. This figure panel is not referenced in the main text. It is also surprising that the genetically “apallial” mouse does not show deficit in social novelty, as this behavior is largely dependant on CA2 function, and the hippocampus is absent from this model. How can this be explained?

We thank the reviewer for raising this issue. To probe this question further, we increased the sample size for the three-chamber sociability assay (Extended Data Fig. 9m,n; control n = 12, apallial n = 7, tAP-hCO n = 8).



A repeated measure ANOVA confirmed effects of group and target (object vs mouse), but post-hoc tests comparing the within-group differences in time spent with object and mouse are not significant (Extended Data Fig. 9m). While a trend hinting at social preference remains, this is confounded by a significant post-hoc test demonstrating that apallial mice spent less time investigating the object than control mice. This may indicate a deficit in object exploration as opposed to social preference. The non-specific effect of pallial depletion on social memory is further supported by the social novelty task (Extended Data Fig. 9n). Here, none of the groups show a preference for a novel mouse over a familiar mouse, but there is a main effect of the group. Together with posthoc tests, this indicates that the apallial condition spends less time investigating both the novel and familiar mice, as compared to the control group.

Taken in the context of reduced sociability of *Prkdc^{scid}* mice (PMID: 27409813), these data are consistent with previous studies on the role of hippocampal subregion CA2 (PMID: 24572357), which is required for social novelty (akin to the task in Extended Data Fig. 9n) but not for baseline sociability (akin to the task in Extended Data Fig. 9m).

We thank the reviewer for pointing out a missed figure citation and added a reference to these analyses in the main text:

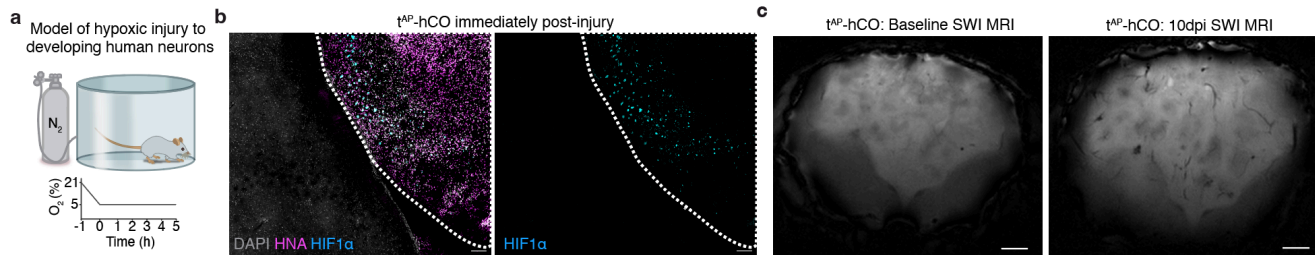
“Using a three-chamber sociability assay, in which mice were given the choice to interact with a novel object or a novel social partner, we found that none of the groups demonstrated a preference for social interaction over novel object interaction (Extended Data Fig. 9m,n). This replicated previous findings demonstrating no preference for social interaction over a novel object in control mice on a *Prkdc^{scid}* background (PMID: 27409813).”

> The authors propose that the hCO-grafted apallial mouse model represents a valuable platform for studying human brain pathology. From a translational standpoint, this approach holds great promise for modeling neurodevelopmental disorders. It would be particularly compelling to investigate whether human cortical organoids (hCOs) derived from patients with genetic conditions, such as those leading for instance to seizures could be used in this chimeric in vivo system. This could allow researchers to assess whether key pathological features—such as hypersynchrony in cortical networks as seen in vivo in epileptic animals but also in cultured human organoids PMID: 34426698—are recapitulated in the grafts. Such findings would spark more interest for this novel mouse model and could provide crucial insights into human disease mechanisms and pave the way for the development of more targeted therapeutic strategies.

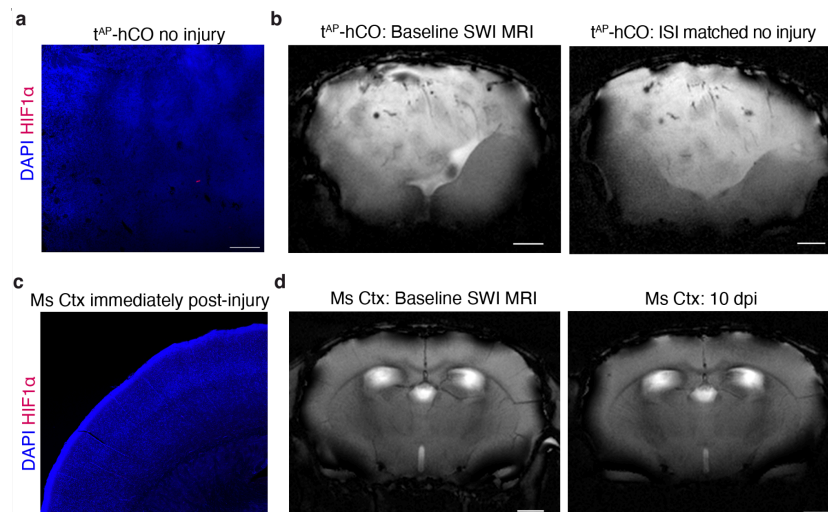
We thank the reviewer for sharing our enthusiasm for the potential of this model with respect to neurodevelopmental disorders. In this revision, we include a disease application in the t^{AP}-hCO model

that we have been developing over the past period. This is related to hypoxia-related injury— a common insult associated with behavioral and motor impairments in prenatal and perinatal stages of cortical development. While animal models of hypoxic injury have revealed mechanistic insights, factors such as molecular/cellular differences between species along with changes in the timing of neural development have been limiting. We and others developed human *in vitro* models of hypoxic insult (PMID: 31061540), but *in vitro* systems cannot capture the dynamic organismal-level injury. For example, *in vitro* organoids are not vascularized, are maintained in media containing artificially high glucose, are cultured at static atmospheric oxygen levels with 5% CO₂, and require <1% O₂ for 24–48 hours to induce injury. While previous models of hCO transplantation can integrate organoids *in vivo*, the human graft makes up a relatively small fraction of the total cortical volume, so the effect of injury on the native host cortex and human graft is challenging to disentangle.

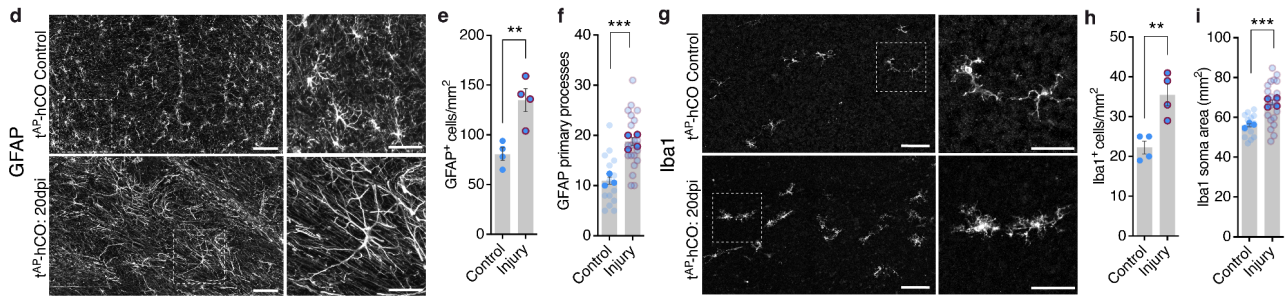
For this new model, we exposed control and t^{AP}-hCO mice to 5% O₂ for 5 hours in a Biospherix chamber (including a graded descent from atmospheric oxygen to 5% O₂, as described in detail in Methods). Immunostaining demonstrated that all t^{AP}-hCO mice exposed to 5% O₂ for 5 hours showed HIF1α signal in the human graft, but not the mouse paleocortex (Fig. 5a,b). We then performed baseline and post-injury susceptibility weighted imaging (SWI), an MRI method that is highly sensitive to intravascular deoxyhemoglobin and extravascular deposits of blood products (PMID: 17925363). All t^{AP}-hCO mice that underwent 5% O₂ for 5 hours demonstrated more prominent hypointense vasculature 10 days post injury (Fig. 5c). This increased visibility on SWI is consistent with a larger ratio of deoxy- to oxyhemoglobin, and aligned with clinical reports of infants post-hypoxia (PMIDs: 21907881, 24033676).



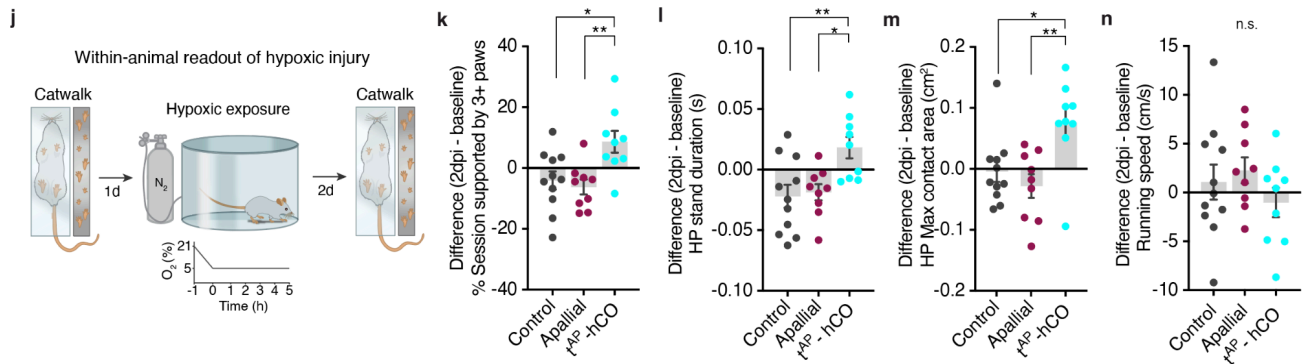
We did not see HIF1α expression or an increase in SWI hypointensities in t^{AP}-hCO mice exposed to control atmospheric oxygen conditions nor in control mice exposed to 5% O₂ for 5 hours (Extended Data Fig. 10a-d).



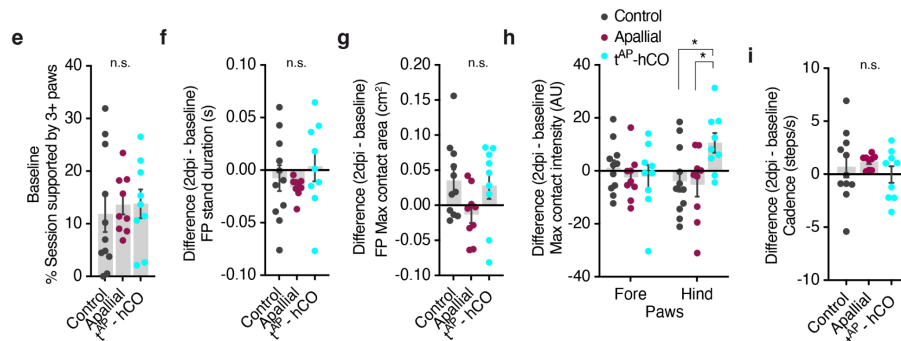
Finally, we found that t^{AP} -hCO mice exposed to 5% O₂ for 5 hours exhibited an increased density and complexity of intragraft Iba1 and GFAP cells (Fig. 5d-i), a shared cellular response to brain insults (PMIDs: 38286816, 40882239, 32770064).



We then tested whether the t^{AP} -hCO platform would allow for functional, behavioral readouts of injury. Because injury to the developing human brain is often associated with motor deficits, we tested mice in the CatWalk before and after injury (Fig. 5j). We found that t^{AP} -hCO mice exposed to 5% O₂ for 5 hours demonstrated an increase in the portion of the session supported by 3-4 paws, a more stable position than the more common 2 paws (Fig. 5k, PMID: 34723800). This was potentially driven by an increases in: the amount of time the hind paws were in contact with the platform, the contact area of the hind paws, and the maximum intensity of the hind paw print on the glass surface (Figs. 5l,m and Extended Data Fig. 9h).



Critically, these changes were not due to changes in overall running speed or step cadence (Figs. 5n and Extended Data Fig. 10i).



Together, these data demonstrate the feasibility of this approach for modeling aspects of human neurodevelopmental disorders with proof of concept behavioral readouts.

Minor concerns

>The references to some Extended Data figures do not follow the chronological order of the main text. These figures should be reorganized to align more closely with the flow of the manuscript.

We appreciate the suggestion to improve the readability of the manuscript. We have reorganized the order of Extended Data figures in the main text to follow the manuscript flow and updated all in-text citations, captions, and the figure legends accordingly.

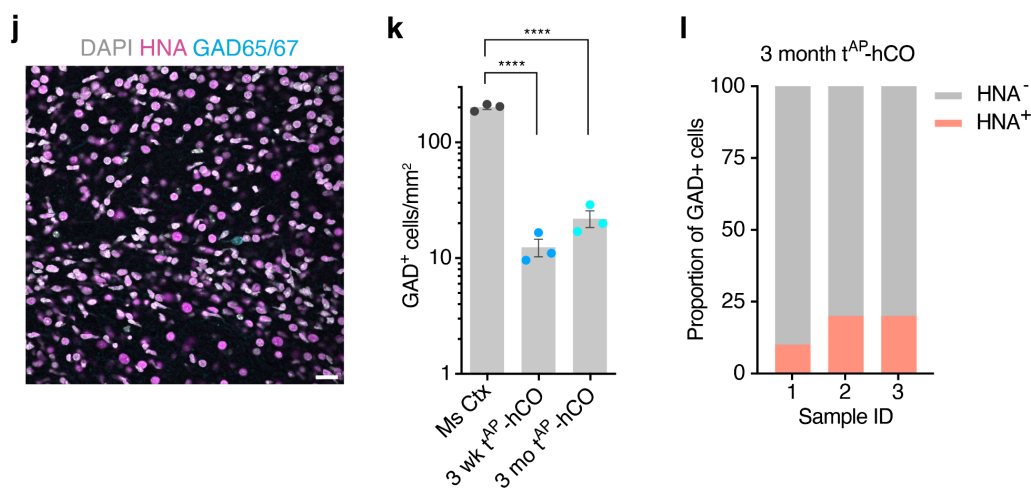
> The *Emx1-cre^{+/-};Esco2^{fl/fl};Prkdcsid/scid* is not completely apallial as a the piriform cortex is visble (see extended data figure 1g, figure 1b and PMID 25744204 mentionning some residual cortical neurons in a protruding ridge extending bilateraly from the piriform cortex (also seen in fig 1B)) – the authors should rename the mouse line accordingly.

We fully agree that *Emx1-cre^{+/-};Esco2^{fl/fl}* mice retain the piriform cortex. We have now explicitly acknowledged this point in the main text.

“Consistent with previous reports^{5,6}, ventral/lateral glutamatergic pallial neuronal subclasses were preserved (i.e. paleocortex), including those from amygdala and piriform cortex (Fig. 1h,i and Extended Data Fig. 2c). We nonetheless retain the term “apallial” as an operational designation for this model, recognizing that it does not denote complete elimination of all pallial derivatives.”

> Figure 2g. It is interesting to see that the grafted hCOs also generate GABAergic interneurons (shown in green), which may contribute to balancing the activity of the artificial human neural circuits. This important observation should be mentioned in the main text, ideally on page 7, as it highlights a key aspect of functional circuit integration with potential relevance for modeling neurodevelopmental disorders involving excitatory/inhibitory imbalance.

We appreciate the opportunity to expand on this. To quantify the proportions of host- and graft-derived GABAergic interneurons, we performed immunostaining analysis with GAD65/67 and HNA antibodies. We found the large majority of GAD65/67⁺ interneurons within the graft to be host-derived (HNA⁻), with approximately 16.7% being graft-derived (HNA⁺) (Extended Data Fig. 7k-l).



We have added a sentence describing this in the main text and updated Extended Data Fig. 7 to reflect the quantification. We also consider the potential implications for excitatory/inhibitory balance and modeling of neurodevelopmental disorders in the Discussion.

“As an initial demonstration of the t^{AP} -hCO model, we transplanted glutamatergic hCO, leading to a low density of GABAergic neurons within the graft. Future versions of this model may use assembloid approaches, particularly for modeling imbalances in excitation and inhibition in neuropsychiatric disorders.”

Referee #4:

I was asked to perform an ethical review of the manuscript. Included in the request I was asked to discuss in my report whether the following aspects of the research comply with the ISSCR guidelines for stem cell research (<https://www.isscr.org/guidelines>) and with local regulations, and whether the authors have provided sufficient details about these aspects in the ethics statement:

- (i) Conditions of procurement/donation of human embryos and tissues;
- (ii) Content and language of the consent forms;
- (iii) Oversight of the research: the name of the committee and, for the most sensitive research, the expertise of its members (researchers, ethicists);
- (iv) Appropriate procedures to ensure privacy of patient data (please confirm that no confidential data are disclosed in the manuscript).

My comments are meant to respond to this request from Nature. I have noted the following Ethics Statement in the manuscript:

“All experiments involving human cells complied with all relevant guidelines and regulations. Human donors in this study consented to the use of their cells to generate hiPS cells and derived cells. The source of the cells and their institutional approvals are listed in the reporting summary. This study also benefitted from a consultation with an external ethical committee. Approval for transplantation of hCO into mice was obtained from the Stanford Laboratory Animal Care (APLAC) Research Compliance Office. No discernible locomotor or memory deficits were detected in transplanted animals and their wellbeing was monitored throughout.”

I am encouraged and impressed by the letter from the ad hoc, independent ethics committee formed to provide advice and expert guidance to Dr. Sergiu Pasca and his research team regarding his neural organoid transplantation work. The people on this committee are highly qualified to assess the work, and I was pleased to see that Dr. Pasca engaged this group. I trust these individuals to guide this type of work.

That being said, I have several comments:

1) I would like to have seen more information in the letter from the ad hoc committee. What did they assess with respect to this particular study? Their letter states, “Dr. Pasca’s work was approved and closely monitored by all the appropriate institutional bodies at Stanford University.” However, other than the animal care and use committee, what other committees reviewed and approved the research? No approval documents were provided with the manuscript. While the authors have stated, “This study also benefitted from a consultation with an external ethical committee,” it would be helpful to remark on what the committee’s comments were with respect to this specific study.

We thank the reviewer for this comment. Prior to starting experiments, we sought approval from the Stanford Administrative Panel on Laboratory Animal Care (APLAC). Beyond this conventional review procedure, we requested consultation from two bioethicists at Stanford who provided an extensive overview of implications. Soon after, we consulted with the Stanford Neuroscience Institute's Executive Committee that included leading neuroscientists. As we initiated these experiments and established the apallial model in the laboratory, we also sought feedback systematically and confidentially from several neuroscientists around the world. In parallel, Stanford University School of Medicine convened an external committee composed of neuroscientists, legal scholars, ethicists, and patient advocates to develop a framework for oversight of these studies. All aspects of the experiments were shared transparently and discussed, including the scientific rationale for the work and sequence of studies, laboratory practices and safeguards, ethical dimensions of study design, methods, and data interpretation, and potential social, medical, and ethical implications of the work. We presented this work to the committee on multiple occasions, and their feedback substantially informed our approach.

We asked the external committee to elaborate on their process, and we are now uploading a letter that describes what they have evaluated. As per this letter, this included: "monitoring plan for animal welfare, including any evidence of unique gain-of-function outcomes not typically assessed or deliberated upon by an IACUC". They "concluded that all appropriate ethical care was taken".

2) Although it may seem obvious to the authors that the rodents that incorporate human cells into their brains do not experience consciousness in the human sense, I think it is important under the "ethics statement" to state something to this effect, as this is an ongoing area of concern for many members of the public – scientists, regulators, government officials, and laypersons alike. Two of the members of the special committee were said to have participated in the National Academies committee that authored the 2021 report, "The Emerging Field of Human Neural Organoids, Transplants, and Chimeras." (<https://doi.org/10.17226/26078>.) Some of the questions addressed by that National Academies committee were:

- a. "How would researchers define or identify enhanced or human awareness in a chimeric animal?" and
- b. "How large or complex would the ex vivo brain organoids need to be to attain enhanced or human awareness?"

3) Thus, could the authors make some comment in the "ethics statement" that addresses these issues and/or that reflects that the external ethical consulting committee reviewed and considered these issues with respect to this research study? Could the authors make some explicit statement in the publication that there was no evidence for "enhanced or human awareness;" the authors could define how they believe this and/or why it would not be possible with the type of transplantation and engraftment that occurred. They could elaborate by stating that the human cells were inadequate to create enhanced or human awareness (assuming this is true).

As we designed and conducted these experiments, and through discussions with external advisors, we considered whether transplantation into apallial mice could give rise to features warranting heightened ethical concern. At the cellular and cytoarchitectural levels, we did not observe canonical cortical layering or gyrification, and the proportion of GABAergic cells remained low, consistent with transplantation of dorsal forebrain tissue only. The human cortical grafts exhibited synchronous waves of activity reminiscent of early stages of cortical development. At the behavioral level, we performed extensive phenotyping spanning unconstrained spontaneous behavior, including MoSeq-quantification, as well as motor, sensory, and multiple task-structured assays probing specific cognitive readouts. As described in Figure 4 of the article, across these behavioral assays we identified differences in behavioral states that were relatively subtle, defects in some tasks (e.g., fear conditioning) and improved performance in transplanted vs apallial animals in some other tasks (e.g., spontaneous alternation Y-maze).

These experiments were also reviewed in consultation with the external committees. As stated in the letter, “The Ethics Committee examined your monitoring plan for animal welfare, including any evidence of unique gain-of-function outcomes not typically assessed or deliberated upon by an IACUC.” In addition, the Stanford Neuroscience Institute’s Executive Committee concluded that “we see no credible conceptualization of these experiments as creating organisms with human-like behavioral or cognitive capacities that would lead to elevated ethical concern.”

The Ethical statement has been updated to read:

“All experiments involving human cells were predicated on ethical guidelines and safeguards and were adherent to all relevant guidelines and regulations. Human donors consented to the use of their cells to generate hiPS cells, derived cells, and their in vivo transplantation. The source of the cells and their institutional approvals are listed in the reporting summary. This study benefited from prospective and ongoing consultation with bioethicists at Stanford University, review by the Stanford Neuroscience Institute Executive Committee, and evaluation and guidance provided by an external independent ad hoc ethics committee. Experiments were shared transparently and discussed, including the scientific rationale for the work and sequence of studies, laboratory practices and safeguards, ethical dimensions of study design, methods, and data interpretation, the possibility of altered or improved capacities in the experimental mice, and potential social, medical, and ethical implications of the work. Approval for transplantation of human cortical organoids into mice was obtained from the Stanford’s Stem Cell Research Oversight (SCRO) committee and Institutional Animal Care and Use Committee (IACUC). To assess altered or improved capacities in the experimental mice, systematic and comprehensive locomotor and cognitive testing was performed, and their wellbeing was carefully monitored throughout. In addition to fully adhering to all relevant guidelines and regulations, we recommend that all future experiments with this model should be performed in close consultation with ethicists in designing, conducting, interpreting, and communicating this work.”

4) Another issue addressed by the National Academies report, “The Emerging Field of Human Neural Organoids, Transplants, and Chimeras” was: “Should patients give explicit consent for their cells to be used to create neural organoids?” While level of granularity may be too specific for most tissue donation consent forms, I would expect consent forms to include iPSC generation and differentiation and transplantation into animals. Indeed, an aspect of my review, as stated above, is to examine the conditions of procurement/donation of human tissues and to view the content and language of the consent forms. However, consent forms were not provided.

We now included for the referees’ review the blank consent forms, which include a statement on the use of their cells for in vivo applications. We also mention in the statement:

“Human donors consented to the use of their cells to generate hiPS cells, derived cells, and their in vivo transplantation.”

5) The Reporting Summary states the cell line sources as: “1208-2 UCLA IRB; 8119-1 UCLA IRB; 0524-1 Stanford IRB.” However, these appear to be IRB protocol numbers and not cell lines. I have to assume that these IRB protocols were designed to obtain tissue for iPSC generation, but I cannot tell from the information provided. Thus, could the authors provide the titles of these IRB protocols? Could they provide the template consent forms? I would like to see that donors gave informed consent to derivation of iPS cells and differentiation and even transplantation of the cells/tissue into laboratory animals. Supplementary Table S1 from this article gives a list of cell lines used:

Revah, O., Gore, F., Kelley, K.W. et al. Maturation and circuit integration of transplanted human cortical organoids. *Nature* 610, 319–326 (2022). <https://doi.org/10.1038/s41586-022-05277-w>. Which of these lines were employed in the present study, if any? It would be preferable to state this rather than list IRB approvals.

Thank you for your attention to this important point. We now updated the information on the cell lines and IRB in the reporting summary as follows:

“Cell lines are presented followed by the IRB in parentheses: 1208-2 (UCLA IRB#13-000296); 8119-1 (UCLA IRB#13-000296); 0524-1 (Stanford IRB-27795).”

Response to Reviewers

Referee #2

The revision substantially strengthens the manuscript. I appreciate the addition of a disease application, expanded ethical discussion, clearer description of external ethics oversight, and the new benchmarking against prior transplantation paradigms. The new analyses supporting enrichment of L5-ET/corticospinal-like populations and the hypoxia proof-of-concept address part of my concern that the system's value over existing models was not sufficiently demonstrated.

We thank the reviewer for their thoughtful assessment and appreciate that the additions to the manuscript have addressed a number of their concerns. We have carefully considered the remaining points raised below and revised the manuscript accordingly. Please note that “t^{AP}-hCO” is now referred to as “xenocortex” (XCX).

However, my central concerns are only partially resolved. First, the ethical response remains grounded primarily in institutional oversight and absence of evidence for elevated capacities, rather than a more explicit articulation of the criteria that would justify this model relative to less ethically fraught alternatives. I would ask the authors to sharpen the Discussion to state more clearly: (i) what specific questions can only be answered in this system, (ii) what present limitations prevent over-interpretation of the behavioral findings, and (iii) what future findings would trigger heightened ethical concern or additional safeguards.

We appreciate the reviewer's specific suggestions, and we have restructured the Discussion to address the three related points:

(i) We modified the Discussion to describe the features of XCX mice that may be relevant to specific applications in studying human brain development and disease:

This neural transplantation paradigm has several advantages in comparison to previous in vitro stem cell-based or in vivo transplantation models. First, XCX mice capture cellular and circuit phenotypes induced by genetic and environmental perturbations in engrafted human neural cells, together with associated behavioral outcomes, with less interference from the host rodent cortex than in other transplantation models. Second, the presence of L5-ET neurons enables functional studies of this population, including specialized subtypes such as VENs, whose restriction to a few large-brained species has limited investigation of their disease susceptibility. More broadly, XCX mice could enable new insights into the biology and evolution of human cortical cells. Third, the system enables therapeutic testing across a large, integrated volume of developing human cortical tissue, together with assessment of effects on broader circuit activity. Fourth, it could provide a foundation for the preclinical development and evaluation of cell therapies for microcephaly or severe ischemic injury during early development.

(ii) We agree that the behavioral results from this system should be interpreted in the context of several limitations. We added the following considerations in the Discussion:

The observed behavioral outputs likely reflect interactions between the human graft and preserved mouse subcortical circuits, but whether graft-derived neurons are necessary or sufficient for specific behaviors remains unknown. Although the graft forms anatomical connections with host tissue in the brain and spinal cord, pathway-specific recordings and manipulations will be needed to determine the extent of functional integration. The present experiments used organoids up to 177 days post-differentiation, an early developmental stage marked by synchronized, high-amplitude calcium waves. In a previous rat t-hCO study, sensory-evoked graft responses emerged at later stages. Although graft activity was strongly correlated with orofacial movement in XCX mice, whether these early network oscillations propagate beyond the graft remains unknown. At the time points examined, the xenocortex also lacked several features of mature cortical circuitry, including complete areal patterning, a well-defined laminar cortical plate, full representation of GABAergic interneurons, and a mature transcriptional profile. Ultimately, the mismatch between the rapid maturation of the rodent nervous system and the prolonged developmental trajectory of the human graft may limit integration.

(iii) We share the reviewer's concern regarding appropriate safeguards for transplantation experiments. We therefore revised the Discussion to provide examples of features that, if pursued in future studies, may warrant early input from ethicists and other relevant stakeholders:

Generating XCX mice with more mature and canonical cortical organization will require early and proactive engagement to establish ethical guidance. Relevant considerations may include studies at later stages of maturation, cortical layering or gyrification, physiological representation of excitatory and inhibitory neurons, and mature network properties, such as sparse activity with experience-dependent adaptation. These considerations may become especially important if the developmental mismatch between the graft and host is minimized, for example by transplanting organoids into cortically depleted embryonic rodent hosts or non-human primates. Close collaboration between ethicists, researchers, patient representatives and regulatory bodies and will be essential for the responsible advancement of transplantation models and support their application to investigating human biology and developing therapeutics.

Second, while the new comparison to existing transplantation approaches is helpful, some mechanistic specificity remains unresolved. The manuscript would still benefit from a more explicit acknowledgment that the evidence for selective host-graft integration is predominantly anatomical, and that functional validation of long-range connectivity remains a key limitation.

We have now revised the Discussion section to state this limitation more clearly:

Although the graft forms anatomical connections with host tissue in the brain and spinal cord, pathway-specific recordings and manipulations will be needed to determine the extent of functional integration.

Third, the lack of a region-mismatched graft control still leaves open whether some reported effects depend on cortical identity per se versus general engraftment of a proliferative human neural tissue. The authors claim other regions are not feasible, but there are other neural cells that are sufficiently proliferative that could be used as a control to explore whether this is a cortical identity issue versus a general proliferation question.

We agree that this is an interesting mechanistic point and have updated the Discussion accordingly:

Although the present study focused on hCOs, transplanting non-cortical organoids into the apallial cavity will help distinguish cortical lineage-specific effects from general effects of engraftment.

Overall, I view the manuscript as considerably improved, but I would recommend further revision focused on narrowing claims, clarifying what is uniquely enabled by this model and testing this with the additional control, and making the ethical justification more explicit and forward-looking.

We believe the reviewer's comments have strengthened the manuscript and appreciate the thoughtful questions.

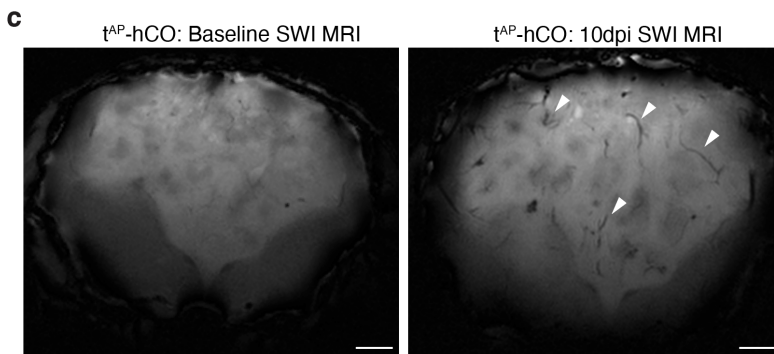
Referee #3

The authors have made a substantial effort in revising and improving their manuscript by incorporating a significant amount of new data and additional experiments. Overall, these additions satisfactorily address the majority of my previous concerns.

With respect to the hypoxia model - the approach taken to demonstrate the translational relevance of this system - a few minor points merit further attention.

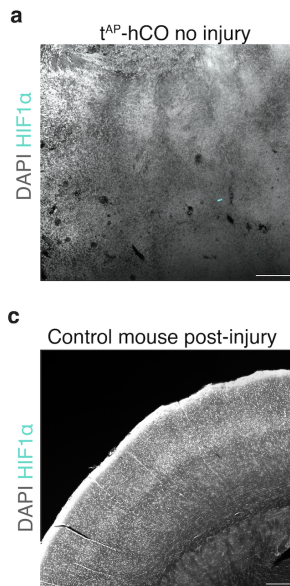
First, regarding the figure panels, it would be helpful if the authors could explicitly indicate (e.g., with arrows) the regions of enhanced hypointense vasculature in Figure 5c to improve clarity. In addition, the manuscript would benefit from the inclusion of a triple immunostaining in Extended Data Figure 10a (DAPI, HIF1 α , and HNA) to better support graft localization, in a manner similar to what is presented in Figure 5b.

We appreciate the reviewer's suggestions. For Fig 5c, we added white arrows indicating selected examples of tortuous hypointensities that were new or increased post-hypoxia. Please note that "t^{AP}-hCO" is now referred to as "xenocortex" (XCX).



In Extended Data Figure 10a, we did not display the HNA channel because the field of view is located entirely within the graft, and displaying overlapping fluorescence made the absence of HIF1 α signal more difficult to visualize. We have updated the figure legend to explicitly state that the field of view is within the graft, as indicated by the organoid-like cytoarchitecture. Finally, we revised the color scheme to better represent the lack of HIF1 α in the absence of a hypoxic insult and to maintain consistency with the immunostaining presented in the original Fig. 5b.

Extended Data Fig. 10



I am somewhat surprised by the apparent absence of HIF1α expression in the host brain tissue upon severe hypoxia. Considering the prolonged hypoxic conditions, one would expect some degree of impact on the surrounding mouse brain tissue, including in control condition, as suggested by the existing literature.

We appreciate the reviewer allowing us to clarify this point. While our data reveal HIF1α localized to the human graft, we now include text clarifying that this finding does not imply the absence of hypoxia in mouse tissue (PMIDs: 35279653, 36313199). Our goal was to determine whether HIF1α could be detected in human host cells, which required immunohistochemistry to co-localize human nuclear antigen (HNA) and HIF1α. We expanded upon this in the Results section:

To assess the hypoxic response with cellular and species specificity, we used immunohistochemistry to evaluate HIF1α together with human nuclear antigen (HNA). In 3/3 tAP-hCO mice exposed to 5% O₂ for 5 hours, HIF1α immunoreactivity was detected in the human graft, whereas HIF1α signal was not appreciable in the adjacent mouse paleocortex (Fig. 5a,b) or in control mice exposed to the same hypoxia protocol (Extended Data Fig. 10c). Thus, our analysis localizes the most prominent HIF1α immunoreactivity to the human graft. Prior bulk protein immunoblotting studies provide complementary evidence that HIF1α can also be elevated in the adult mouse brain following a milder hypoxia protocol (PMID: 11919510).

Finally, from a functional standpoint, the hypoxia-exposed tAP-hCO group displays an unexpected behavioral phenotype, with increased three-paw support during Catwalk session, which may indicate altered gait or balance and thus deviates from normal physiological

patterns. Could the authors elaborate on how their model and such phenotype are relevant for understanding the disease?

We thank the reviewer for allowing us to expand on this point. Clinically, perinatal hypoxia is associated with cerebral palsy (PMID: 32765409), a group of movement and posture disorders that frequently affect gait. In the present manuscript, the t^{AP} -hCO group demonstrated the largest hypoxia-related changes in paw support strategies and measures of hind-paw weight bearing, which is consistent with compensatory postural shifts reported across rodent models of movement disorders (PMIDs: 34723800, 23151254, 26695533). Together these findings suggested that t^{AP} -hCO could be used to model aspects of human neurodevelopmental disorders. To integrate the reviewer's suggestion, we added the following text to the Discussion:

For example, t^{AP} -hCO captures gait deficits in response to hypoxic injury highlighting the potential for modeling perinatal hypoxia and associated cerebral palsy⁴⁷, as well as human neurodevelopmental disorders more broadly.

Referee #4

I wish to thank the authors for their detailed response to my questions about the ethical aspects and ethics reviews of this protocol. Indeed, I see that the investigators and Stanford University went to great lengths to obtain multiple expert reviews from institutional committees such as the IACUC and SCRO, as well as from a number of bioethicists and neuroscientists. This included Stanford University School of Medicine having convened an external committee composed of neuroscientists, legal scholars, ethicists, and patient advocates who reviewed all aspects of the study multiple times; I see that the review included assessing the rationale, built-in safeguards, monitoring plans, and ethical implications of the study. I am pleased to see the thoroughness with which this research study was reviewed. Thank you for providing the letter signed by the Chair of the ad hoc committee, Dr. Insoo Hyun, on behalf of the Hastings Center for Bioethics, elaborating on the focus of their reviews.

The Ethics statement in the paper is much improved. Besides providing clarification, an added benefit of having a more detailed Ethics statement is that it sets a standard for other researchers in the field. It is also reassuring to regulators and the public alike. Finally, it provides an educational function for students and trainees interested in entering the field.

Thank you for providing the IRB-approved consent forms, which I am glad to see include statements that hiPSCs and further derived cells and products from the participants' specimen donations may be transplanted into animals. In general, these specimen donation consent forms are thorough and fulfill the many requirements of consent forms for pluripotent stem cell work of this nature.

Thank you for revising the Reporting Summary, clarifying which numbers were cell lines and which numbers were IRB protocol numbers (which also corresponded to the consent forms).

We are grateful to the reviewer for their thorough examination of the ethical aspects of this work and for their recognition of the oversight processes. Please note that "t^{AP}-hCO" is now referred to as "xenocortex" (XCX).