nature portfolio

Peer Review File

Neural xenografts contribute to long-term recovery in stroke via molecular graft-host crosstalk

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this new manuscript by Weber et al., authors demonstrate interesting effects of neural progenitor cell (NPC) transplantation after ischemic stroke in mice. This is an elegant study which contains a wide array of techniques/findings converging towards beneficial effects of NPC transplantation, with a good level of molecular insight into possible mechanisms underlying functional recovery. This is an understudied area of research, hence this paper is important for the field. While very promising, this work will benefit from addressing the following concerns:

Major:

- It is key to test one or two metrics (e.g. CBF, infarct size) when NPCs are implanted earlier (i.e., 2 days after stroke). Seven days post-PT stroke is already far into tissue damage and functional impairments. As recently shown (PMID: 38164600) CBF recovers at one-week post-PT stroke in mice, but is low immediately post-PT and stays low at 48hrs. Hence, the 7-dpi time point chosen by authors probably reduces the sensitivity of their readouts. Authors could also use this citation (PMID: 38164600) to better justify why they have measured CBF 30min post-PT stroke. An alternative to performing new in vivo experiments, if limited by logistics, could be to add NPCs at different time-points to neuronal cultures after oxygen-glucose deprivation, and measure neuronal health.

- Author should incorporate notions from previous work published in the field of neurovascular and gliovascular plasticity after stroke in their introduction, rationale for experiments and discussion (particularly PMIDs 37816732, 33910014, 32873722, and 32848875). It is very important to put their interesting new work in the context of knowledge on endogenous brain repair mechanisms, and how exogenous cells could interfere with plasticity windows and spontaneous biological recovery.

- Biological sex may have impacts on stroke outcomes. In this study, one can find in Methods that both male and female mice were used, which is good. However, no mention is made about biological sex differences statistically. Did author find any sex-related difference? If yes, please report. If not, please mention it was not the focus of the study while acknowledging the existence of such differences.

- How do newly-generated/incorporated cells interact with glial cells and vascular networks? Few co-staining could help add interesting insight into this question.

- I am not really convinced that one can quantify any immunolabeling inside the infarct core, as it is full of dead cells and debris (which infamously alters signal from secondary antibodies). Authors should focus their quantifications on the peri-infract region. I think that, to measure anything related to infarct core, authors should use DAPI or NeuroTrace. But unless authors provide high magnifications of Iba1 (and GFAP) staining in the infract core, images in Fig.2A are not really convincing. I suggest focus on quantifying microglial density/morphology in the peri-infarct region.

- In Fig.3C, while the graph is interesting, one wonders why the core region length (red) would be exactly the same in control and NPC-treated mice. Was this normalized in any way? The stroke core seems a bit larger in control mice (Fig.2A,B; Fig.3B). Authors did quantify infarct lesion size and found no statistical difference (Fig.1F,G), yet is seems the lesion is slightly smaller in NPC-treated mice, which is interesting (also shown by less variability in Fig.1F, right graph). I am

wondering what the p value for this graph is. The lower variability should be discussed. Authors should also indicate on Fig.1 how was infract size measured (e.g., add a staining image).

- It will be important to add p values to all graphs.

Minor:

- Transplantation at 7 dpi: what is the justification for this timing? Is this based on some data or the literature? If the latter, please cite.

- Instead « recovery-associated responses", maybe use "tissue repair responses".

- Introduction, last summary paragraph could be shorter. Instead of "(…) responses five weeks post-stroke. For instance, we observed reduced (…)" authors could focus on some examples: "(…) responses five weeks post-stroke including reduced inflammation,(…)".

- Did authors see decreased cell death in peri-infarct tissues of NPC-transplanted mice?

- Fig.1A: Please increase brightness of DAPI stain for NPCs.

- Fig.1A legend: authors should precise what are Nanog and Nestin: "(…) stained for Nanog and Nestin, markers of… ".

- Fig.1B panel: for the naïve readership, if might be confusing to read "neural induction" and seeing astrocyte marker S100beta. Maybe rephrase to "neural/glial induction" ?

- Results for Fig.1: This sentence: "(…) one group of mice received a local transplantation of dual-reporter (red firefly luciferase and eGFP (rFluc-eGFP)) expressing iPSC-NPCs, (…)" is confusing. Please rephrase to: "(…) one group of mice received a local transplantation iPSC-NPCs expressing a dual-reporter (red firefly luciferase and eGFP, rFluc-eGFP), (…)".

- Page 4: "Cell survival was confirmed (…)". Please replace by: "Transplanted cell survival was confirmed (…).

- Stars to indicate statistical differences are too small, please increase size.

- Writing "Iba1 expression" is not correct, as authors did not look at gene expression. It would be preferable to use "Iba1 immunoreactivity".

- For vascular staining images (Fig.3B) authors should add lower magnification images so one can better appreciate the vascularization of the ischemic border zone. The images are currently mainly showing the infract core.

- Fig.3D, left graph: please move the stars to top of the graph.

- Fig.4 legend: "Differentiation fate", please remove "Differentiation".

- Fig.5: current panels C and F should be move after current panels D and E (the latter are there to show stroke induces impairments, validating the approach). Validation and metric establishment should come before NPC treatment-related data are shown.

- Fig. 7 title: I would change "Molecular communication" to "Molecular crosstalk".

- Page 17: "we performed single nucleus RNA sequencing (snRNAseq) of the mouse stroke tissue and the (??) containing human cell grafts." I think this sentence misses a word, which makes it confusing.

Reviewer #2

(Remarks to the Author)
The present study performed by Weber and collaborators studies a potential therapy for stroke. Induced pluripotent stem cell (iPSC)-based therapies are emerging as a promising therapeutic approach for stroke recovery. In this study, we demonstrate that local transplantation of research-graded human iPSC-derived neural progenitor cells (NPCs) induces some mechanism of brain repair and reduces neurological deficits after cerebral ischemia in mice. The study has high relevance as currently there are no therapies for stroke. A disease that affects 15 million patients worldwide. Overall, this is a very interesting research project that needs to address some major concerns before publication.

1- Authors indicated "NPC-grafted animals showed increased local axonal sprouting from the IBZ towards the graft, and from the graft towards the IBZ, compared to the vehicle group". BDA labeling does not indicate axon directionality. The data provided does not support this conclusion. It will be very informative to assess if the authors determine if those axons are mouse or human.

2- NeuN/EDU studies. With the data provided authors are not capable of determining if those NeuN/EDU+ cells have migrated from the SVZ. It will be very informative to assess if those NeuN/EDU+ cells are mouse or human.

3- Based on IBA-1 immunostaining quantifications. The authors conclude that they observed an increase in inflammation. Without appropriate inflammation markers, these conclusions can't be supported by the provided data. IBA-1+ cells only represent a higher number of microglial cells.

4- Authors conclude that hiPSC-NPCs transplant improved the blood-brain barrier (BBB). No data in this manuscript supports this conclusion.

5- Authors indicated: "Elevated levels of GFAP expression, indicating glial scar formation, were observed in the stroke core". In stroke, GFAP+ cells are not found in the stroke core, but in the peri-infarct area forming the glial scar.

6- There are no differences in stroke size or blood perfusion between Stroke+Vehicle and Stroke+hiPSC-NPCs (Fig 1E, F, and G). It is unclear how this correlates with the behavioral deficits post-stroke and behavioral recovery observed post-transplant.

7- There are better and more functional metrics to study vascular leakage and integrity. Fibrinogen staining is not sufficient.

8- Co-localization of EDU and vascular makers should be performed to determine if there is a formation of new vasculature.

9- In the Transplanted iPSC-derived NPCs express neuronal markers section co-localization of HuNu/NeuN, HuNu/Glut markers, HuNu/GABAergic markers, and HuNu/OPC markers should be performed.

10- The authors described several times the transplanted cells as "good manufacturing practice (GMP)-compatible iPSC-derived neural progenitor cells (NPCs)". This generates confusion, and it makes the reader think that these cells have been produced under cGMP conditions, which they have not. These are simply research-graded hiPSC-NPCs.

11- Use should properly name their therapeutic product as hiPSC-NPCs. Or at least pick a single way to determine them, throughout the paper they are named NPCS, iPSC-derived NPCs, or iPS-NPCs.

12- The representative image in Figure 3B1 does not show hypovascularization as the authors described in the text.

13- Based on the data provided in Figures 5 D and E it seems like there is a restoration of neurological functions in stroke animals by day 6 post-stroke. While in Figures 5 G, H, and I the Stroke+vehicle group have longer-lasting deficits up to 40 days post-injury. Are stroke and stroke+vehicle cellular and behavioral distinct? And how does affect the comparison with the stroke + hiPSC-NPCs group?

14- The are no data in this manuscript that can sustain the following observation made by the authors: We observed a severe loss of GABAergic neurons in host stroke tissue and preferential differentiation of cell grafts into GABAergic-like phenotype after stroke.

15- There is no analysis or discussion on how the endogenous cells transcriptionally react to the stroke and the cell transplant. A detailed transcriptional analysis during injury and repair should be included.

16- The meaning of Abbreviations like I.e. mGABA, hGlut, etc should be included.

17- The authors indicated that the majority of grafted cells showed hGABA characteristics. But 44% of grafted cells showed hGABA characteristics and 42% of grafted cells showed hGlut characteristics. This is a 50-50 split. It is unclear why authors have decided to solely focused their transcriptional analysis of the GABAergic neurons. Based on the data it seems like the authors should expand their focus.

18- Authors should limit the discussion, to discussion and omit any repetition of results.

19- Methods. The cell differentiation protocol lacks all relevant details. A methods section should be able to be reproduced by other investigators, in this case in impossible.

20- Abbreviations should not be used in the abstract (NRXN, NRG, NCAM, and SLIT).

Reviewer #3

(Remarks to the Author)
Weber et al. have demonstrated the engraftment and therapeutic effects of iPSC-NPCs in MCAO mice. Graft-derived GABAergic neurons communicate with stroke-injured host tissue potentially through neuronal protective signaling pathways including NRXN, NCAM, SLIT and NRG. In general, this study provide some novel insights for cell therapy to treat stroke. However, there are still some major points about the experimental design and data quality that compromised the scientific contributions.
1. The novelty of this work is limited since previous studies have reported the therapeutic effects of iPSC-derived NPCs

transplantation in stroke animals. In addition, many researchers have devoted considerable efforts to improve the overall viability, promote the differentiation and migration of transplanted cells to augment their efficacy. Transplantation of iPSC-derived neural progenitor cells (NPCs) has been demonstrated to enhance functional recovery in animal models of stroke. What is the significance and innovation of this research?
2. The analysis of the molecular crosstalk between the host and the graft is not comprehensive. Single nucleus profiling of the cell transplants and host stroke tissue was performed in this study and GABAergic cell grafts were primarily identified as involving in graft-host communication and preferentially differentiating towards GABAergic cells. The authors hypothesized that GABAergic grafts interact with damaged host tissue to initiate recovery-associated processes. However, the ligands or receptors mentioned in this study are not exclusively expressed in graft. The NRXN, NRG, NCAM, SLIT signaling pathways are dominantly expressed in mGABA. Given the large number of local host cells compared to the engrafted cells, the communication strength of host between host is much stronger than host between graft. Does secretion from transplanted cells mediate regeneration of the ischemic core? More validation experiments (i.e. loss of function experiments) are necessary to support the purposed molecular mechanisms.
3. Did the transplantation of iPSC-derived NPCs increase the concentration of ligands mentioned in the manuscript, such as slit2 and slit1.
4. Why transplant iPSC-NPCs cells 7 days after stroke? When is the most effective transplantation time point for the treatment of stroke?
5. The detailed protocol to differentiate human iPSCs to NPCs should be presented in the method section.
6. The efficacy of human iPSC-derived NPC transplantation could be evaluated in immunodeficient Rag2-/- mice, which lack functional T cells and B cells, thereby reducing immune rejection of the transplanted cells. However, to fully understand the complex interaction between the host immune system and the transplanted cells. The therapeutic effects and NPCs differentiation towards GABAergic cells should also be determined and validated in normal C57BL/6 mice.
7. The authors only demonstrated that transplanted NPCs could differentiate into neurons and express MAP2, but have not been proven whether the neurons formed by the differentiation of this group of cells exhibit corresponding functions, such as transmitting action potentials.
8. Stroke can lead to sarcopenia and worsening of motor function. Muscle mass should be assessed in the limbs of stroke mice. Does iPSC-derived NPC transplantation improve motor function by preventing sarcopenia.
9. Are experimental behavioral improvements in stroke mice related to changes in the structure or quantity of neuromuscular junctions within the skeletal muscle? If so, are transplanted NPCs involved in the innervation of neuromuscular junctions?
10. Extravasation of tracing dye and quantification of tight junction proteins should be performed to assess the permeability of cerebral blood vessels.
11. In Figure 7F, the authors grouped different signaling pathways via functional and structural similarity. What is the definition of functional and structural similarity? In Figure7I-L, what is the meaning of mediator and influencer. Please describe in detail.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors did an excellent job at addressing all my concerns. The rebuttal letter is extensive and well prepared, making it very easy to see what improvements were made. The revision considerably improves the quality of the manuscript. I would like to congratulate the authors for their excellent work.

Reviewer #2

(Remarks to the Author)
Weber et al. have made a commendable effort in addressing the reviewers' concerns. They have incorporated several new experiments and analyses, added new figures, and expanded the discussion with important new insights. Additionally, they have appropriately downplayed points that are no longer strongly supported by the new data. Overall, these revisions have improved the manuscript. However, several points still remain to be addressed.

Based on the new results, NPC transplantation does not affect BBB integrity. The abstract must be revised accordingly. NPCs do not primarily differentiate into GABAergic interneurons. Instead, 44% differentiate into GABAergic interneurons, while 42% become glutamatergic neurons. The abstract must be revised accordingly. A more accurate statement would be that the graft preferentially differentiates into neurons.

Two out of three reviewers requested a justification for the selected transplant day. The authors cited their own BioRxiv manuscript, which supports 7-day over 24-hour transplantation, but did not acknowledge the potential for chronic-phase transplantation—a stage where most stroke patients reside and where intervention may be more clinically relevant. There is extensive literature, including clinical trials, on therapeutic windows for stem cell-based therapies in stroke and other types of brain/spinal cord injury models, and failing to discuss this represents a missed opportunity to address a critical issue in the field.

What markers were used to assess stroke size? In the rebuttal letter (Reviewer 1, Question 6), the authors imply that HuNu

was used, but GFAP, IBA-1, and NeuN would be more appropriate for this purpose. Clarification is necessary.

At what time point was the caspase-3 experiment performed? Clarification is needed.

The authors suggest that NPC therapy does not affect apoptosis, but they did not perform colocalization with caspase-3. Was caspase-3 expressed in transplanted NPCs? If so, in what proportion? In which host cell types was caspase-3 expressed? Are different cell populations undergoing apoptosis in stroke alone versus stroke + NPCs?

What is the cellular composition of the final therapeutic product? A substantial proportion of S100β+ cells appears to be present. How reproducible is this differentiation protocol across batches? Are the authors consistently transplanting the same proportions of cell types? This is a crucial consideration for potential clinical translation.

The authors state that “the majority of ligands were upregulated in the mouse stroke tissue” and cite their own BioRxiv manuscript (Weber et al., 2024) to describe transcriptional changes during stroke repair. Since most of these upregulated ligands are in host cells, how do the current findings relate to the stroke-induced transcriptional shifts in the previous study? A direct transcriptional comparison between stroke and stroke + NPCs appears necessary.

Reviewer 3 raised concerns about the study's novelty, and the authors do not provide compelling evidence to counter this critique. i.e., NPC transplantation has been extensively studied in permanent stroke rodent models.

It is unclear why SLIT2 was chosen as a proof-of-concept ligand for the in vitro assay. SLIT signaling is primarily associated with neuroinflammation rather than migration, and the transplanted cells exhibited minimal migration to the peri-infarct area.

In their rebuttal letter, the authors acknowledge that BDA tracing alone does not confirm axonal projection directionality. The main manuscript must be revised accordingly, particularly in the second paragraph of the “Increased neurite outgrowth and endogenous neurogenesis in NPC-receiving animals” section.

While graft-derived GABAergic neurons communicate with stroke-injured host cells, it is well established that stroke disproportionately affects GABAergic interneurons compared to glutamatergic neurons. Given that an equal proportion of NPCs differentiate into glutamatergic and GABAergic neurons, it is important to determine how graft-derived glutamatergic neurons interact with host cells and which pathways they influence.

Methods sections should be reviewed. As an example, DAB experiments are not included. Make sure all new and old methodologies are included in this section.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
All my concerns have been addressed.

Congratulations to the authors.

Reviewer #4

(Remarks to the Author)
The study by Weber et al presents a detailed investigation into the effects of transplanted neural progenitor cells (NPCs) on stroke pathophysiology. One major component of this study is to explore graft-host interactions using snRNA-seq. While this approach is innovative and potentially informative, several concerns arise, such as the lack of methodological details and validation, which may undermine robustness of the conclusions.

1. It is unclear how the host and transplanted cells were separately isolated for sequencing. Cell sorting?
2. The Methods section indicates that non-stroke mouse cortices were collected and sequenced. However, such data do not appear to be presented or used as a reference. Instead, the authors relied on a previously published dataset for reference, which may not be optimal due to potential technical variations. This needs to be clarified.
3. The authors should provide unbiased clustering data files, containing top differentially expressed genes (DEGs) for each cluster. Such data are important for interpreting their analyses.
4. Suppl. Fig. 17. It is unclear how reliability and prediction analyses were performed.
5. Only 1149 nuclei from transplanted cells were sequenced. Were these cells pooled from 5 mice as well? The cell number seems relatively low.
6. For Supplemental Table 2, how was this DEG analysis performed? It seems that a public reference dataset was used as a reference. If so, again, this comparison is not convincing.
7. Fig. 7 F. Given the emphasis on GABAergic neurons, it would be important to include common markers such as GAD65

(GAD2) and GAD67 (GAD1) for immunostaining.
8. Another major concern is related to their data regarding graft-host interactions. The authors conducted an extensive analysis of cell-cell communication using several bioinformatics tools. While this can be valuable, such analysis can sometimes lead to overinterpretation, particularly when not supported by repeated sequencing or validation experiments. Here, it appears that only a single pooled snRNA-seq sample was analyzed for each group. Although an in vitro study exploring Slit2 signaling was included as a proof-of-concept, its relevance is questionable because the snRNAs-seq study used cells one month after transplant, when most transplanted NPCs showed mature properties. An in vivo validation would provide stronger support for their conclusions.

Version 3:

Reviewer comments:

Reviewer #4

(Remarks to the Author)
The detailed response is greatly appreciated. However, given the focus on GABAergic neurons, staining with a pan-GABAergic marker still seems necessary. The authors note that “VIP, SST, and PV are important GABAergic subtypes that account for the majority (with some studies suggesting nearly 90%) of GABAergic neurons in the cortex”; however, these three subtypes together represent only ~15% of all $HuNu^+$ cells in their study (Fig. 7F,G), which is substantially lower than the ~44% revealed by the snRNA-seq analysis. This discrepancy needs to be clarified.

Response to Reviewers

We would like to thank the reviewers for their thoughtful comments on our work. We appreciate the reviewers' overall support of our study, which explores the potential of induced pluripotent stem cell (iPSC)-derived neural progenitor cells (NPCs) as a novel therapy for ischemic stroke. All three reviewers acknowledge the relevance of our work, given the urgent need for effective interventions in a disease affecting millions of patients around the globe. The reviewers recognize that our approach (ranging from histological assays to behavioral assessments) provide important insights into how cell transplantation may lead to long term post-stroke recovery.

Nevertheless, the reviewers have raised several major points that require either additional experiments, additional analysis or additional clarification. Among these points are questions regarding the timing of cell transplantation (why 7 days post-stroke rather than an earlier time point), the specificity and interpretation of certain immunohistochemical markers (e.g., Iba1 for inflammation, BDA labeling for axonal sprouting), and the strength of our conclusions regarding e.g. the blood–brain barrier integrity or the molecular interaction between graft and host.

Additionally, the reviewers asked to improve our methodological transparency, for example, by detailing our differentiation protocol more comprehensively, or by ensuring that claims of cell type specificity (e.g., mouse vs. human axons, or GABAergic differentiation) are well substantiated, and they asked to incorporate additional measures/parameters (e.g., extravasation assays, transcriptional analyses) to strengthen our discussion of underlying mechanisms.

In revising this manuscript, we have performed numerous experiments to address the reviewers' suggestions and concerns. We have performed additional experiments to examine early transplantation time points (24h versus 7d post injury). We have also added a more functional metric to study vascular leakage: In the revised manuscript, we use Evans Blue as a leakage marker to further assess the impact of NPC therapy on blood brain barrier integrity.

In collaboration with Prof. Markus Rüegg (Biozentrum, Universität Basel), we imaged neuromuscular junctions (NMJs) of two different muscle types (*extensor digitorum longus* (fast-twitch muscle) and *soleus* (slow-twitch muscle)) in the hindlimbs of NPC- and vehicle-treated animals, to evaluate whether the behavioral improvements in cell-therapy treated mice are related to changes in the structure or quantity of NMJs.

We further expanded our analysis of microglial activation by quantifying morphological parameters of Iba1$^+$ cells, such as branching complexity and ramification indices. This provides a more reliable indication of microglial activation and was requested by all three reviewers.

Below is a point-by-point response to the reviewers' comments including a description of the changes made to the figures and manuscript. We highlighted the changes in the manuscript yellow. We appreciate the time and effort of the reviewers, and we hope that these revisions effectively demonstrate the robustness and clinical relevance of our findings.

Point-by-point responses:

Reviewer #1

In this new manuscript by Weber et al., authors demonstrate interesting effects of neural progenitor cell (NPC) transplantation after ischemic stroke in mice. This is an elegant study which contains a wide array of techniques/findings converging towards beneficial effects of NPC transplantation, with a good level of molecular insight into possible mechanisms

underlying functional recovery. This is an understudied area of research; hence this paper is important for the field.

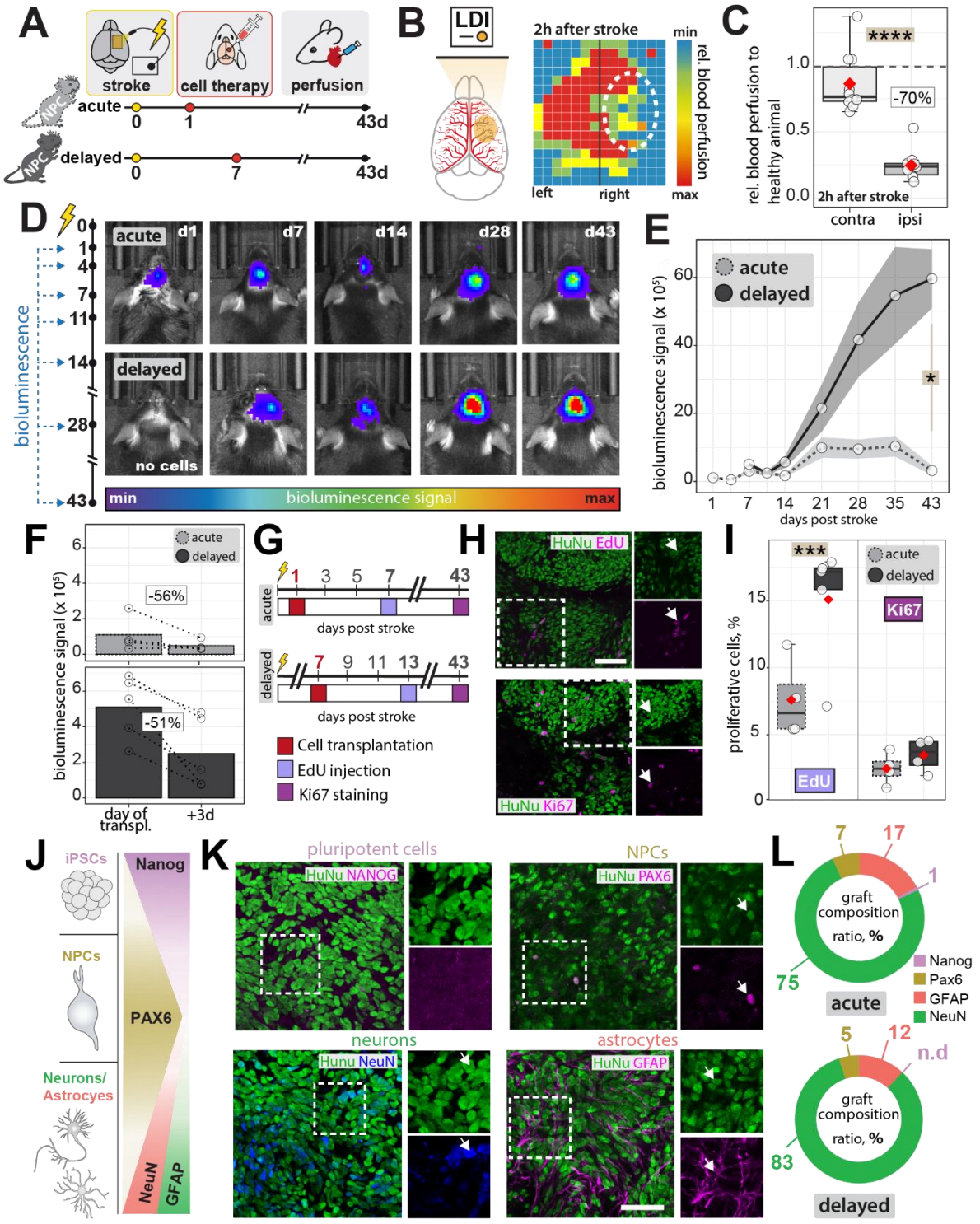
Thank you for this thoughtful summary of our work.

While very promising, this work will benefit from addressing the following concerns:

1. It is key to test one or two metrics (e.g. CBF, infarct size) when NPCs are implanted earlier (i.e., 2 days after stroke). Seven days post-PT stroke is already far into tissue damage and functional impairments. As recently shown (PMID: 38164600) CBF recovers at one-week post-PT stroke in mice but is low immediately post-PT and stays low at 48hrs. Hence, the 7-dpi time point chosen by authors probably reduces the sensitivity of their readouts. Authors could also use this citation (PMID: 38164600) to better justify why they have measured CBF 30min post-PT stroke. An alternative to performing new in vivo experiments, if limited by logistics, could be to add NPCs at different time-points to neuronal cultures after oxygen-glucose deprivation, and measure neuronal health.

   We thank the reviewer for this suggestion and fully agree that early time points for NPC transplantation may better capture the acute stroke environment and its impact on cell survival and functional outcomes. Indeed, we have conducted additional experiments (described in a recent preprint submission: Weber et al., 2025, Delayed transplantation of neural stem cells improves initial graft survival following stroke*, BioRxiv*, **Reviewer Fig. 1**) where NPCs were transplanted 24 hours after stroke induction. In these experiments, we observed a reduced survival rate of grafted cells transplanted early compared to delayed transplantation (7dpi), which we attribute to the more hostile post-stroke environment at that time.

   While we found that the timing of transplantation does not significantly impact overall differentiation patterns, we could observe a trend: early transplantation (1dpi) results in a neuronal-to-astrocytic ratio of approximately 4.5:1, whereas delayed transplantation (7dpi) shows a higher ratio of about 7:1. Overall, our findings suggest that delaying NPC transplantation by one week may enhance the therapeutic potential of NPC-based therapies in stroke and potentially other related neurological disorders.



Reviewer Fig. 1. Delayed transplantation of NPCs improves long-term graft survival post-stroke. (A) Schematic illustration showing the experimental design. Immunodeficient Rag2$^{-/-}$ mice underwent stroke induction followed by local transplantation of rFluc-expressing NPCs at either 1 dpi (acute) or 7 dpi (delayed). (B) Laser doppler imaging confirms reduced cerebral blood flow (CBF) after stroke. (C) Quantification of CBF 2 hours after stroke induction. (D) Representative bioluminescence imaging (BLI) illustrating NPC survival over 6 weeks in both groups of selected time points. (E) Quantification of BLI signal over time and (F) within the first 3 days after transplantation in both groups. (G) Schematic timeline of proliferation assessment using EdU incorporation at 7 days post-transplantation and Ki67 staining at 42 days (acute) and 35 days (delayed) post-transplantation to track graft proliferation. (H) Representative immunofluorescence images (scale bars: 50µm) and (I) quantification of EdU^{+} NPCs at seven days post-transplantation and Ki67^{+} NPCs at 35 (delayed) and 42 (acute) days in both groups. (J) Illustration showing phenotyping panel with pluripotency marker Nanog, NPC marker PAX6, neuronal marker NeuN, and astrocytic marker GFAP. (K) Representative immunofluorescence images of grafted NPCs (HuNu+) at six weeks post-transplantation. Scale bars: 50µm. (L) Quantification of graft composition in acute vs. delayed transplantation groups. Data are shown as mean distributions where the red dot represents the mean. A total of N

= 8 animals were used, with 4 animals per group. Boxplots indicate the 25% to 75% quartiles of the data. For boxplots: each dot in the plot represents one animal. Line graphs are plotted as mean ± sem. Significance of mean differences was assessed using an unpaired Mann-Whitney-U-Test (C and E) or an unpaired t-test (I). Statistical significance was set at *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Also, from a clinical standpoint, delayed transplantation offers several advantages, including improved patient stabilization, a potentially better prediction of stroke injury progression, and the opportunity to extend the currently limited 4–24-hour therapeutic window.

We have added a brief discussion of this data in the revised manuscript and cite our preprint for further details.

2. Authors should incorporate notions from previous work published in the field of neurovascular and gliovascular plasticity after stroke in their introduction, rationale for experiments and discussion (particularly PMIDs 37816732, 33910014, 32873722, and 32848875). It is very important to put their interesting new work in the context of knowledge on endogenous brain repair mechanisms, and how exogenous cells could interfere with plasticity windows and spontaneous biological recovery.

   We thank the reviewer for highlighting these important references and the broader literature on neurovascular plasticity. We have revised the *Discussion* section to put our findings in the context of endogenous brain repair mechanisms and the potential interplay between endogenous repair processes and grafted NPCs.

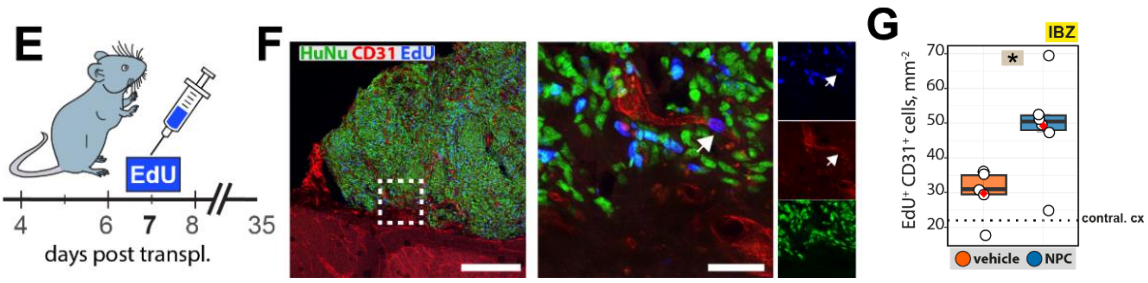
   Specifically, we have added two paragraphs discussing how stem cell-based approaches may complement or enhance the endogenous reparative pathways, while acknowledging the importance of timing to align with the natural plasticity windows post-stroke. We have cited the suggested PMIDs (37816732, 33910014, 32873722, and 32848875) where relevant to emphasize these points.

3. Biological sex may have impacts on stroke outcomes. In this study, one can find in Methods that both male and female mice were used, which is good. However, no mention is made about biological sex differences statistically. Did author find any sex-related difference? If yes, please report. If not, please mention it was not the focus of the study while acknowledging the existence of such differences.

   In our experiments, both male and female mice were included. Our statistical analyses did not reveal any significant sex-related differences in terms of CBF changes, lesion volume, functional recovery, or NPC engraftment/differentiation. These findings are consistent with observations from our earlier studies, where no clear differences emerged when both sexes were studied. We have added a statement in the *Methods* section to clarify that while both sexes were used and analyzed, no significant sex-related differences were detected.

4. How do newly-generated/incorporated cells interact with glial cells and vascular networks? Few co-staining could help add interesting insight into this question.

   This is indeed a very interesting question. To assess whether newly generated cells interact with vascular networks, we performed an additional experiment where we administered 5-ethynyl-2′-deoxyuridine (EdU) systemically on day 14 following stroke induction (which equals day 7 following NPC transplantation). We then co-labelled CD31 and EdU and identified a greater density of EdU^+ blood vessels (per mm^2) in the ischemic border zone of NPC-treated mice compared to vehicle-treated controls. These new data are now presented in **Figure 4E – 4G (Reviewer Fig. 2)** of the revised manuscript.

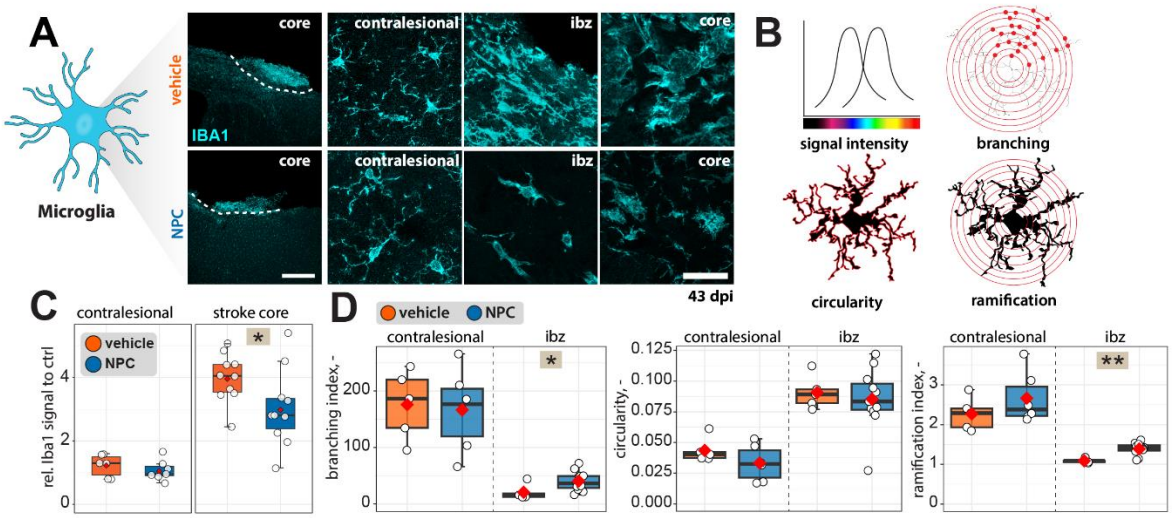


Reviewer Fig. 2: Vascular changes in ischemic stroke tissue in NPC- and vehicle-receiving animals. (E) Schematic representation of EdU injection to visualize newly-formed vessels. (F) Immunofluorescence staining of coronal sections with HuNu, CD31 and EdU. Scale bar: 100 µm (left) and 10 µm (right). (G) Quantification. Boxplots indicate the 25% to 75% quartiles of the data. For boxplots: each dot in the plots represents one animal. Line graphs are plotted as mean ± sem. Significance of mean differences was assessed using an unpaired t-test (vehicle vs. NPC). In G, n=5 (vehicle) and n=6 (NPC). Asterisks indicate significance: *p < 0.05, **p < 0.01, ***p < 0.001.

5. I am not really convinced that one can quantify any immunolabeling inside the infarct core, as it is full of dead cells and debris (which infamously alters signal from secondary antibodies). Authors should focus their quantifications on the peri-infract region. I think that, to measure anything related to infarct core, authors should use DAPI or NeuroTrace. But unless authors provide high magnifications of Iba1 (and GFAP) staining in the infract core, images in Fig.2A are not really convincing. I suggest focus on quantifying microglial density/morphology in the peri-infarct region.

We agree with the reviewer that quantification of immunolabeling inside the infarct core can be confounded by the presence of dead cells and tissue debris. Therefore, we have repeated our analysis to focus specifically on the peri-infarct region. There, we performed a more detailed assessment of microglial reactivity by analyzing morphological parameters of Iba1-positive microglia, including **branching index, ramification**, and **circularity (Reviewer Fig. 3, Manuscript Fig. 2A-D)**. These morphological features should serve as more reliable indicators of microglial activation state than simple signal intensity measurements, since resting microglia react to the inflammatory stroke milieu and change their morphology from highly ramified into a more amoeboid type.

We found that the microglial branching index, a measure of microglial branching complexity, and the ramification index, a measure of the ratio of the cell's perimeter to its area, was significantly reduced in all ipsilateral regions compared to their respective contralateral ROIs. Mice that received NPC therapy showed increased numbers in both measures compared to vehicle animals. No significant differences in branching index and ramification index were detected between the groups on the contralesional cortex, and there were no significant differences in circularity between the treatment groups in the contralesional and the ipsilesional area of interest. We have added the new results to the revised manuscript, and we have extended the methodology in the *Methods* section.



Reviewer Fig. 3: Anatomical changes after stroke induction in NPC- and vehicle receiving mice. (A) Immunofluorescent staining of coronal brain sections with Iba1. Scale bar: 50 µm (left) and 5 µm (right) (B) Scheme depicting the analysed parameters; Iba1 signal intensity and microglia morphology (branching, circularity, ramification). (C) Quantification of Iba1 immunoreactivity on the unaffected contralesional hemisphere and in the stroke core. Data is relative to unstroked control. (D) Quantification of microglia morphology parameters (i) branching index, (ii) circularity and (iii) ramification index. Data is relative to unstroked control. Data are shown as mean distributions where the red dot represents the mean. Boxplots indicate the 25% to 75% quartiles of the data. For boxplots: each dot in the plots represents one animal. Significance of mean differences was assessed using an unpaired t-test (vehicle vs. NPC). In C, n=10 mice per group. In D, n=5 animals per group. Asterisks indicate significance: *p < 0.05, **p < 0.01, ***p < 0.001.

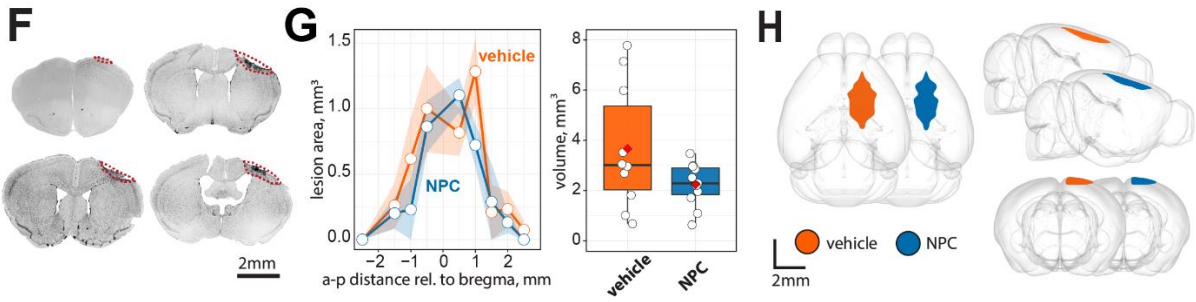
6. In Fig.3C, while the graph is interesting, one wonders why the core region length (red) would be exactly the same in control and NPC-treated mice. Was this normalized in any way? The stroke core seems a bit larger in control mice (Fig.2A,B; Fig.3B). Authors did quantify infarct lesion size and found no statistical difference (Fig.1F,G), yet is seems the lesion is slightly smaller in NPC-treated mice, which is interesting (also shown by less variability in Fig.1F, right graph). I am wondering what the p value for this graph is. The lower variability should be discussed. Authors should also indicate on Fig.1 how was infract size measured (e.g., add a staining image).

Thank you for pointing this out. We used coronal sections aligned to fixed stereotaxic coordinates from the Allen Brain Atlas. By standardizing the start and end positions of the lesion site across all animals, we ensure that all animal's "stroke length" is measured in the same anatomical region. This method, which we have detailed in a previous publication (Weber et al., A toolkit for stroke infarct volume estimation in rodents, 2024, *Neuroimage*, PMID: 38219841), makes it look as though the length is identical for both groups.

The p-value for the lesion size comparison shown in **Manuscript Fig.1F-H is p = 0.09**, which suggests a trend but does not reach statistical significance. We note the lower variability in the NPC-treated group, and we believe that it could hint at a subtle protective effect, however, our data do not allow for a conclusive difference and this question certainly requires additional follow-up studies.

To clarify how we evaluated infarct size and volume, we have now included a schematic illustration showing how we measured the infarct size (**Reviewer Fig. 4**, **Manuscript Fig. 1F**). In brief, we obtained coronal brain sections at defined stereotaxic coordinates relative

to Bregma, stained the sections with HuNu, and then imaged them using a slide scanner. We then used Fiji software (ImageJ) to outline the stroke region by drawing a polygon around the infarct. By measuring this polygonal area for each relevant section, we obtained a precise estimation of lesion size across the tissue sections. We hope that the updated figure and methodology description will help readers better understand and interpret our infarct size analysis.



Reviewer Fig. 4: Generation of hiPSC-NPCs, stroke induction and transplantation. (F) Coronal brain sections used to estimate stroke area. The dotted red line indicates the stroke-lesioned brain area. Scale bar: 2mm. (G) Quantification of stroke infarction size. Left: Lesion area plotted against the anterior-posterior (a-p) distance relative to bregma (mm). Right: boxplot displaying lesion volumes (mm^3) of both treatment groups. (H) Schematic representation of a 3-D mouse brain model depicting stroke infarction sizes. Scale bar: 2mm. Data are shown as mean distributions where the red dot represents the mean. Boxplots indicate the 25% to 75% quartiles of the data. For boxplots: each dot in the plots represents one animal. Line graphs are plotted as mean ± sem. Significance of mean differences was assessed using an unpaired t-test (vehicle vs. NPC). In G, n=11 mice per group.

7. It will be **important** to add p values to all graphs.

   We added a supplementary table with all p-values (**Suppl. Table 1**).

8. Transplantation at 7 dpi: what is the justification for this timing? Is this based on some data or the literature? If the latter, please cite.

   We appreciate the reviewer's question regarding the rationale behind the chosen transplantation timepoint of **7dpi**. Since the initial inflammatory response and excitotoxic phase is more pronounced during the first few days after stroke, the chosen time point may offer conditions that are more permissive for donor cell survival. Both prior studies in literature (Schmidt A, Minnerup J. Promoting recovery from ischemic stroke. 2016, *Expert Rev Neurotherapeutics*) and our own preliminary data (Weber et al., 2025, *Delayed transplantation of neural stem cells improves initial graft survival following stroke,* BioRxiv) indicate that delivering NPCs too early (24 hours post-stroke) can lead to extensive cell death, likely due to the hostile acute microenvironment (**Reviewer Fig. 1**).

   Thus, waiting until day 7 enables edema to partially subside and inflammatory signals to begin shifting, thereby providing a more supportive niche for cell engraftment. We have included relevant references in the revised manuscript (in the *Discussion* section) to support this timeline and further explain why a slightly delayed transplantation strategy may optimize graft survival and therapeutic potential.

9. Instead « recovery-associated responses", maybe use "tissue repair responses".

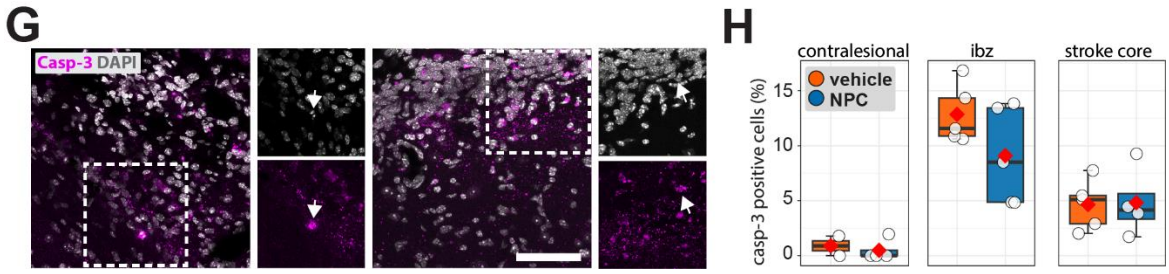
   We changed « recovery-associated responses" to "tissue repair responses" in the *Abstract*, the *Introduction* and the *Methods* section.

10. Introduction, last summary paragraph could be shorter. Instead of "(…) responses five weeks post-stroke. For instance, we observed reduced (…)" authors could focus on some examples: "(…) responses five weeks post-stroke including reduced inflammation,(…)".

We thank the reviewer for the input. We adjusted the last paragraph in the introduction according to the reviewer's suggestion.

11. Did authors see decreased cell death in peri-infarct tissues of NPC-transplanted mice?

We performed additional immunohistochemical stainings for caspase-3 to investigate apoptotic cell death in both the ischemic border zone and the infarct core (**Reviewer Fig. 5, Manuscript Fig. 2G-H**). Consistent with an active post-stroke environment, numerous caspase-3–positive cells were found in these regions compared to the contralesional side. However, we found no evidence of antiapoptopic effects after NPC transplantation in the present study ($vehicle_{IBZ}$:13±4.5, $vehicle_{STROKE}$: 4.6±2.4, NPC_{IBZ}: 9.4±4.9, NPC_{STROKE}:4.9±3, $\mathbf{p_{IBZ}=0.07}$, $\mathbf{p_{STROKE}=0.81}$).

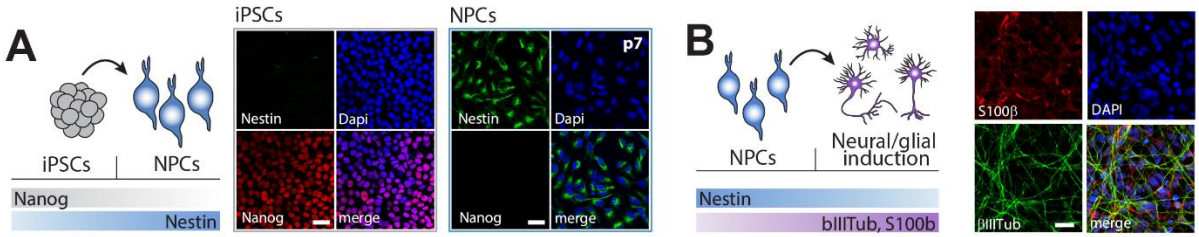


Reviewer Fig. 5: Anatomical changes after stroke induction in NPC- and vehicle receiving mice. (G) Representative histological overview of caspase-3 and Dapi. Scalebar: 10 µm. (H) Quantification of casp-3 positive cells in the unaffected contralesional hemisphere, the ischemic border and the stroke core zone. Data is relative to all counted Dapi-positive cells. Data are shown as mean distributions where the red dot represents the mean. Boxplots indicate the 25% to 75% quartiles of the data. For boxplots: each dot in the plots represents one animal. Significance of mean differences was assessed using an unpaired t-test (vehicle vs. NPC). In H, n=5 animals per group.

One possible explanation is the known temporal profile of caspase-3 activation, which peaks earlier in the post-stroke timeline, usually around 24 to 72 hours after injury (Didenko et al., 2002, *Mol Med.*, PMID: 12393932). By the time we administered NPCs (7 days post-injury), the window for capturing any potential neuroprotective effect against early apoptotic events may have closed. Thus, while NPCs may provide other benefits, such as modulating inflammation or promoting longer-term repair, they may not significantly influence the final snapshot of apoptotic cell death measured at this later time point.

12. Fig.1A: Please increase brightness of DAPI stain for NPCs.

We apologize for the poor quality of the DAPI images provided. We increased the brightness of the DAPI stain (**Reviewer Fig. 6, Manuscript Fig. 1A and 1B).**



Reviewer Fig. 6: Generation of hiPSC-NPCs, stroke induction and transplantation. (A) Left: Generation of hiPSC-NPCs. Right: iPSCs and NPCs (passage 7) stained for Nanog, iPSC marker, and Nestin, an NPC marker. Scale bar: 50µm. (B) Left: Neural differentiation of NPCs. Right: Differentiated NPCs, at d26 after differentiation (upper row), stained for βIII-Tubulin, a marker for mature neurons, S100β, a marker for astrocytes, and DAPI. Scale bar: 50µm.

13. Fig.1A legend: authors should precise what are Nanog and Nestin: "(…) stained for Nanog and Nestin, markers of… ".

    We apologize for not clarifying the methodology, and we added a description of the used IHC markers in the figure legend of **Manuscript Fig. 1**, as suggested by the reviewer (**Reviewer Fig. 6**).

14. Fig.1B panel: for the naïve readership, if might be confusing to read "neural induction" and seeing astrocyte marker S100beta. Maybe rephrase to "neural/glial induction"?

    We thank the reviewer for the input, and we adjusted the schematic illustration in **Manuscript Fig. 1B** to "neural/glial induction" (**Reviewer Fig. 6**).

15. Results for Fig.1: This sentence: "(…) one group of mice received a local transplantation of dual-reporter (red firefly luciferase and eGFP (rFluc-eGFP)) expressing iPSC-NPCs, (…)" is confusing. Please rephrase to: "(…) one group of mice received a local transplantation iPSC-NPCs expressing a dual-reporter (red firefly luciferase and eGFP, rFluc-eGFP), (…)".

    We apologize if this sentence led to any confusions. We rephrased the sentence to "(…) one group of mice received a local transplantation iPSC-NPCs expressing a dual-reporter (red firefly luciferase and eGFP, rFluc-eGFP), (…)", as the reviewer suggested.

16. Page 4: "Cell survival was confirmed (…)". Please replace by: "Transplanted cell survival was confirmed (…).

    We rephrased it accordingly, thanks for the input.

17. Stars to indicate statistical differences are too small, please increase size.

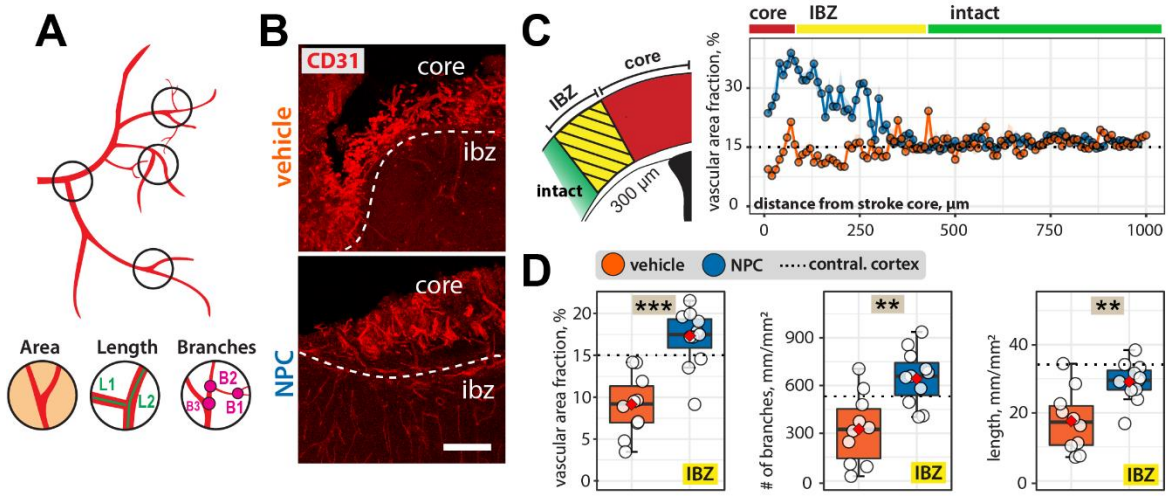
    We agree that the asterisks where a bit too small, we increased the size in all figures.

18. Writing "Iba1 expression" is not correct, as authors did not look at gene expression. It would be preferable to use "Iba1 immunoreactivity".

We agree that the term “Iba1 expression” might be misleading. We therefore changed it to “Iba1 immunoreactivity” in the revised manuscript.

19. For vascular staining images (Fig.3B) authors should add lower magnification images so one can better appreciate the vascularization of the ischemic border zone. The images are currently mainly showing the infract core.

We replaced the representative image showing the vascularization in the core/ischemic border zone of a vehicle-treated animal (**Manuscript Fig. 4B, Reviewer Fig. 7**).



Reviewer Fig. 7: Vascular changes in ischemic stroke tissue in NPC- and vehicle-receiving animals. (A) Representative image depicting blood vessels with branch-like protrusions. (B) Immunofluorescent staining of coronal brain sections with CD31. The dotted white line indicates the ischemic border zone (ibz). Scale bar: 100µm. (C) Blood vessels were analyzed in an area up to 300µm region (yellow/green) around the stroke core (red). (D) Analysis of vasculature density (vascular area fraction, number of branches, vessel length,) in the ischemic cortex. Significances refer to comparisons between NPC and vehicle group. Dashed lines represent the contralateral hemisphere. Data are shown as mean distributions where the red dot represents the mean. Boxplots indicate the 25% to 75% quartiles of the data. For boxplots: each dot in the plots represents one animal. Line graphs are plotted as mean ± sem. Significance of mean differences was assessed using an unpaired t-test (vehicle vs. NPC). In C-D, n=10 (vehicle) and n=11 (NPC) mice per group. Asterisks indicate significance: *p < 0.05, **p < 0.01, ***p < 0.001.

20. Fig.3D, left graph: please move the stars to top of the graph.

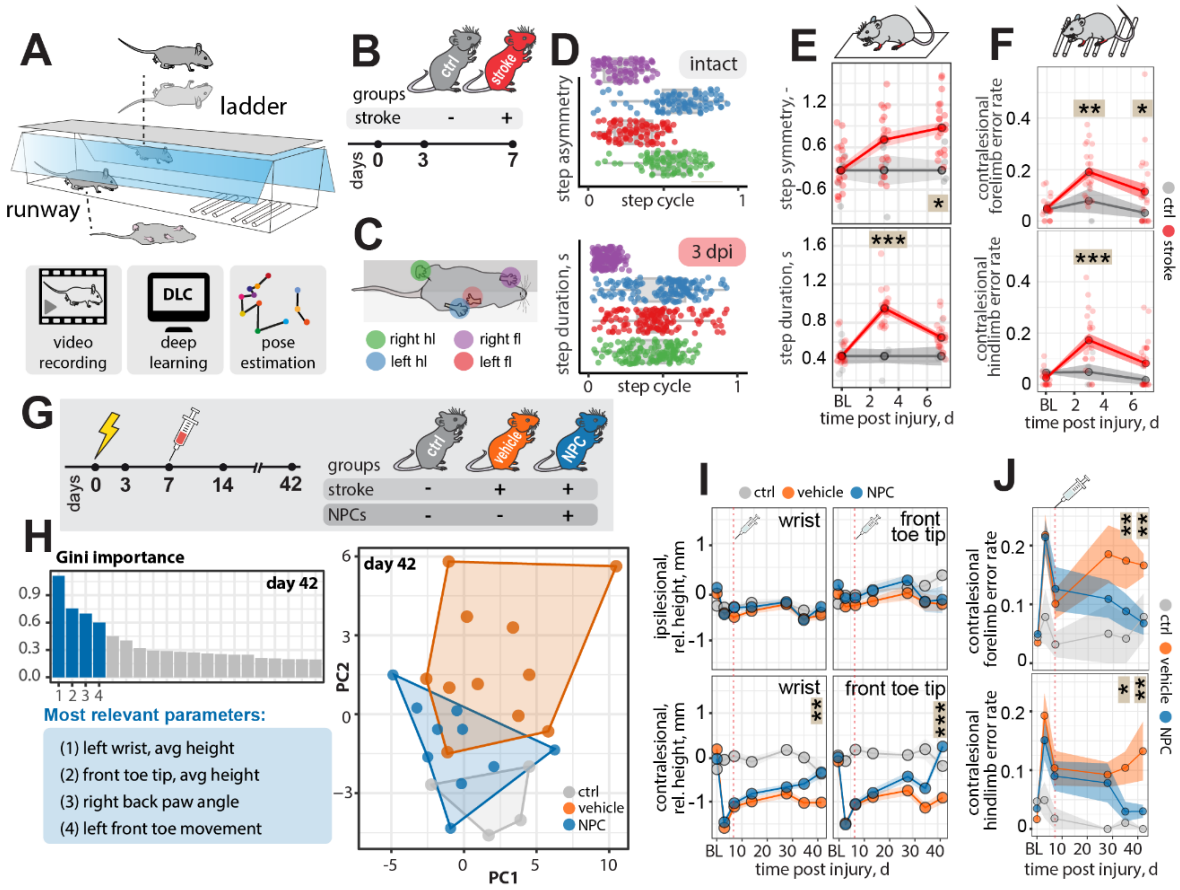
We moved the asterisk to the top of the graph **(Reviewer Fig. 7, Manuscript Fig. 4D)**

21. Fig.4 legend: “Differentiation fate”, please remove “Differentiation”.

We clarified the legend in **Manuscript Fig. 5** and replaced “Differentiation fate of transplanted NPCs 35 days post-transplantation” with “**Fate of transplanted NPCs 35 days post-transplantation**”.

22. Fig.5: current panels C and F should be move after current panels D and E (the latter are there to show stroke induces impairments, validating the approach). Validation and metric establishment should come before NPC treatment-related data are shown.

We appreciate this suggestion and agree that reorganizing the figure will improve clarity. We have accordingly rearranged **Manuscript Fig. 6 (Reviewer Fig. 8)** so that the data establishing stroke-induced impairments (previously in panels D and E) now precede the panels displaying NPC treatment-related outcomes (formerly C and F). This sequence allows readers to see the baseline validation of stroke impairments before examining the effects of NPC therapy.



Reviewer Fig. 8: Increased recovery of motor function for mice that received NPCs. (A) Schematic representation of behavioral tests and DLC workflow used to identify and label anatomical landmarks of mice for pose estimation. (B) Experimental groups. (C, D) Footfall profile of a normalized locomotor cycle showing the stance and phase start and end of an indvidual control animal (top) and stroked animal (bottom). (E) Top: Ratio of asynchronization at baseline, 3, and 7 dpi. Bottom: Duration of a step cycle at baseline, 3 and 7 dpi. (F) Overall rate of missteps in contralesional forelimbs (top) and contralesional hindlimbs (bottom). (G) Experimental treatment groups (control, vehicle, NPC) tested for motor recovery. (H) Left: Random Forest classification of most important parameters. Right: Principal component analysis of most relevant parameters at 42 dpi. (I) Relative height of wrist and front toe tip at baseline and 3, 7, 14, 21, 28 and 35 dpi. (J) Overall rate of missteps in contralesional forelimbs (top) and contralesional hindlimbs (bottom) at baseline and 3, 7, 14, 21, 28 and 35 dpi. Missteps were detected by the back toe tips and front toe tips dipping below a defined threshold (0.5cm). Dashed line shows timepoint of transplantation. Data are shown as mean distributions where the red dot represents the mean. Line graphs are plotted as mean ± sem. Significance of mean differences was assessed using an unpaired t-test (stroke vs non-stroke) or Tukey's HSD. In C-E, n=21 (stroke) and n=4 (no stroke) mice per group. In G-I, n=10 (vehicle), n=11 (NPC) and n=4 (ctrl) mice per group. Asterisks indicate significance: *p < 0.05, **p < 0.01, ***p < 0.001.

23. Fig. 7 title: I would change “Molecular communication” to “Molecular crosstalk”.

We changed the title of **Manuscript Fig. 8** according to the reviewer’s suggestion.

24. Page 17: “we performed single nucleus RNA sequencing (snRNAseq) of the mouse stroke tissue and the (??) containing human cell grafts.” I think this sentence misses a word, which makes it confusing.

We changed the sentence to “To understand the transcriptional signature and cellular identity of the grafts at cellular resolution, we performed single nucleus RNA sequencing (snRNAseq) on stroke mouse tissue containing the transplanted human cell grafts”.

Reviewer #2

The present study performed by Weber and collaborators studies a potential therapy for stroke. Induced pluripotent stem cell (iPSC)-based therapies are emerging as a promising therapeutic approach for stroke recovery. In this study, we demonstrate that local transplantation of research-graded human iPSC-derived neural progenitor cells (NPCs) induces some mechanism of brain repair and reduces neurological deficits after cerebral ischemia in mice. The study has high relevance as currently there are no therapies for stroke. A disease that affects 15 million patients worldwide. Overall, this is a very interesting research project that needs to address some major concerns before publication.

We thank Reviewer #2 for their positive overview of our work and for underscoring its relevance. We appreciate the recognition that iPSC-based therapies for stroke are a promising approach.

1. Authors indicated “NPC-grafted animals showed increased local axonal sprouting from the IBZ towards the graft, and from the graft towards the IBZ, compared to the vehicle group”. BDA labeling does not indicate axon directionality. The data provided does not support this conclusion. It will be very informative to assess if the authors determine if those axons are mouse or human.

   We thank the reviewer for highlighting this important clarification. We recognize that BDA tracing alone does not confirm the directionality of axonal projections. We also agree with the reviewer that it would be very informative to evaluate whether these axons are mouse or graft derived.

   To address this concern, we performed a 3,3'-Diaminobenzidine Tetrahydrochloride (DAB) staining using a human-specific neural cell adhesion molecule (NCAM) marker to confirm that the observed neurites originated from transplanted human NPCs rather than host mouse cells. This new analysis is presented in our revised **Manuscript Fig. 3 (Reviewer Fig. 9)**.

   We found that in the NPC-transplanted group:

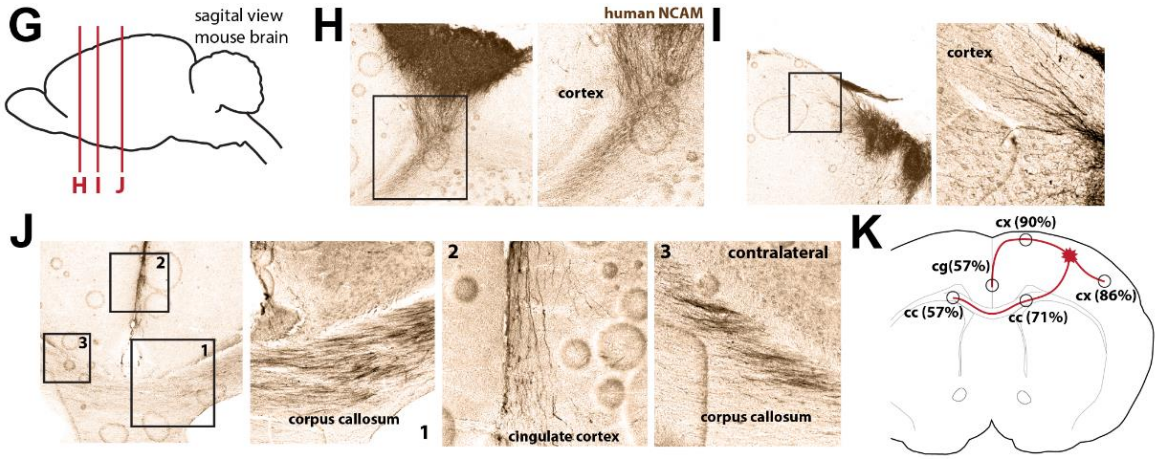
   - 71% of animals showed axonal extensions into the ipsilateral corpus callosum (cc),
   - 57% showed neurite growth along the cc to the contralateral hemisphere,
   - 57% exhibited extensions toward the cingulate cortex, and

- Nearly 90% showed neurite outgrowth extending along the ipsilateral cortex to the primary and secondary motor cortices and primary somatosensory cortex.

These findings indicate that the human NPCs contribute to new outgrowth patterns beyond the immediate graft site and may even integrate to reconstruct multiple neural circuits. Future studies should validate these functional neuronal connections by investigating trans-synaptic connections using rabies virus tracing and employing optogenetic techniques to assess the functionality of newly formed circuits.

However, we have revised our discussion to avoid implying a definitive "direction" of axonal connectivity based on BDA labeling alone, instead focusing on the presence and extent of neurite extension and potential integration in host circuitry.

Additionally, we have added a paragraph to the *Discussion* where we highlight that while we have demonstrated that grafted iPSC-derived NPCs can differentiate into mature neurons with long-range axonal projections, we did not directly assess their functional integration (e.g., via electrophysiological recordings or synaptic connectivity assays).



Reviewer Fig. 9: Endo- and exogenous neurite outgrowth and neurogenesis. (G) Schematic illustration of brain sections corresponding to the following images. (H-J) Representative images of hNCAM staining at different brain levels. (K) The percentage of animals in which transplanted NPCs extended neurites to various brain regions. In K, n=10 mice.

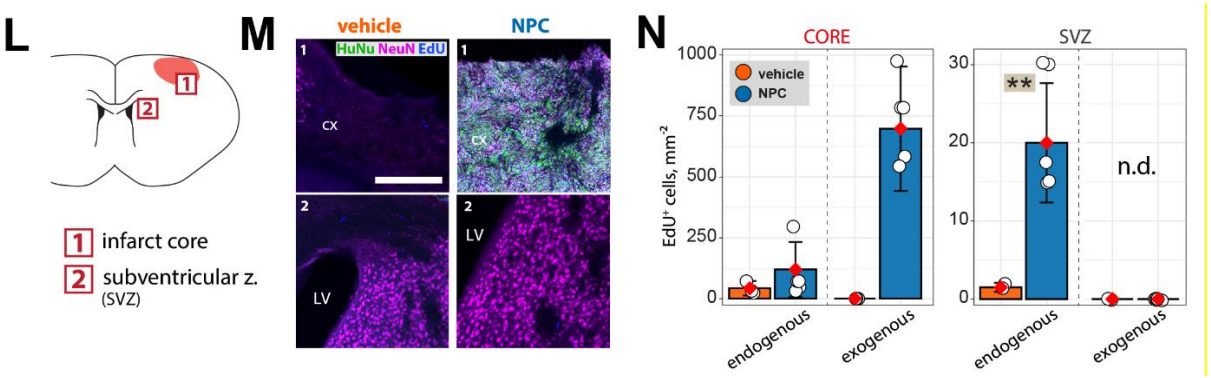
2. NeuN/EDU studies. With the data provided authors are not capable of determining if those NeuN/EDU+ cells have migrated from the SVZ. It will be very informative to assess if those NeuN/EDU+ cells are mouse or human.

We appreciate the reviewer's feedback and acknowledge the need to distinguish between endogenous and exogenous neurogenesis. To address this, we performed additional immunostainings to differentiate mouse-derived (endogenous) cells from transplanted human NPCs. We administered 5-ethynyl-2′-deoxyuridine (EdU) in both NPC- and vehicle-treated animals at day 7 after transplantation and analysed coronal brain sections to identify:

- **EdU+/NeuN+ double-positive** cells: indicative of **endogenous** neurogenesis, and
- **EdU+/NeuN+/HuNu+ triple-positive** cells: indicative of **exogenous** (human) neurogenesis.

In the infarct core, we did not detect substantial differences in endogenous neurogenesis between NPC- and vehicle-treated animals. However, in the subventricular zone (SVZ), we observed a significant increase in the number of EdU+/NeuN+ double-positive neurons in the NPC-treated group compared to the vehicle-treated group, pointing to an enhanced endogenous neurogenic response in the SVZ following NPC transplantation (**Manuscript Fig. 3L-N, Reviewer Fig. 10**).

We hope that these additional analyses clarify the contributions of both endogenous and exogenous neurogenesis and throw light on how NPC transplantation can modulate neurogenic processes in the post-stroke brain.



Reviewer Fig. 10: Endo- and exogenous neurite outgrowth and neurogenesis. (L) Illustration showing the two brain areas (infarct core, subventricular zone) analysed for EdU-positive cells. (M) Immunofluorescent staining of a coronal brain section with EdU, NeuN and HuNu and (N) quantification of EdU+ cell count relative to all counted Dapi+ cells. Scale bar = 50µm. Data are shown as mean distributions where the red dot represents the mean. Boxplots indicate the 25% to 75% quartiles of the data. For boxplots: each dot in the plots represents one animal. Significance of mean differences was assessed using an unpaired t-test (vehicle vs. NPC). In N, n=5 mice per group were used. Asterisks indicate significance: *p < 0.05, **p < 0.01, ***p < 0.001.

3. Based on IBA-1 immunostaining quantifications. The authors conclude that they observed an increase in inflammation. Without appropriate inflammation markers, these conclusions can't be supported by the provided data. IBA-1+ cells only represent a higher number of microglial cells.

We appreciate the reviewer's input regarding the interpretation of increased Iba1 signal as indicator of enhanced inflammation. We agree that quantifying the fluorescence intensity (or density) of Iba1$^+$ cells alone is insufficient to make conclusions about the inflammatory status. In response, we repeated our analysis to focus on established morphological parameters of Iba1$^+$ microglia, which are more reliable indicators of microglial activation.

Specifically, we categorized microglia based on branching index (branch complexity), ramification index (ratio of cell perimeter to area), and circularity, since resting (ramified) microglia typically transform into an amoeboid phenotype in response to inflammatory stimuli.

We found that branching and ramification indices were significantly reduced in the injured hemisphere compared to the corresponding contralateral regions, underscoring a shift toward a more activated phenotype. In contrast, in NPC-treated mice, both parameters were significantly higher compared to vehicle-treated animals, suggesting a comparatively less activated phenotype. No significant differences were detected on the contralesional side, nor were there any significant changes in circularity across treatment groups. We

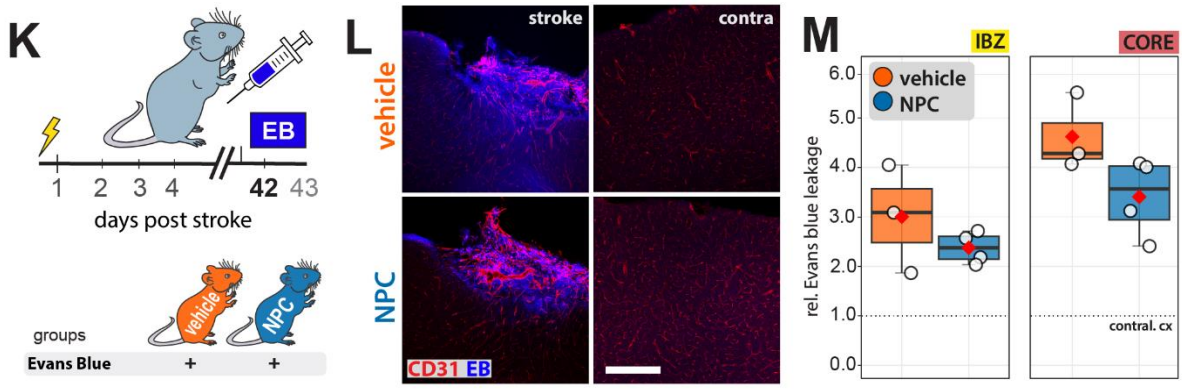
added these new findings to our revised manuscript (**Manuscript Fig. 2A-D, Reviewer Fig. 3).**

4. Authors conclude that hiPSC-NPCs transplant improved the blood-brain barrier (BBB). No data in this manuscript supports this conclusion.

   We thank the reviewer for pointing out the need for more robust evidence to support any conclusion regarding blood brain barrier integrity. In the original manuscript, we evaluated BBB integrity based on fibrinogen leakage as a marker of vascular permeability. However, to further address the reviewer's concern, we performed additional experiments using Evans blue (EB), a well-established marker of BBB permeability (Saunders et al., 2015, *Front. Neurosci*., PMID: 26578854).

   We administered EB (i.p. injection) 24 hours before perfusion (**42** days post-stroke) to both NPC- and vehicle-treated animals. In both groups, a strong EB signal was evident in the stroke core, with a lower signal in the ischemic border zone. Relative to the contralateral hemisphere, both treatment groups showed a similar increase in EB leakage in the ipsilateral hemisphere, indicating no significant difference in BBB permeability between NPC- and vehicle-treated mice (**Manuscript Fig. 4K-M, Reviewer Fig. 11**).

   Consequently, we have revised the manuscript to remove any emphasis on NPC-mediated improvements in BBB integrity. We appreciate the reviewer's feedback, and we hope we could clarify and refine our interpretation of the data.



Reviewer Fig. 11: Vascular changes in ischemic stroke tissue in NPC- and vehicle-receiving animals. (K) Schematic representation of Evans blue (EB) injection to visualize vessel leakage 24h before perfusion in control, vehicle-receiving and NPC-receiving animals. (L) Immunofluorescence staining of coronal sections with CD31 and EB. Scale bar: 100um. (M) Quantification of EB leakage relative to unaffected contralateral hemisphere in the intact cortex, the ibz and the ischemic core. Data are shown as mean distributions where the red dot represents the mean. Boxplots indicate the 25% to 75% quartiles of the data. For boxplots: each dot in the plots represents one animal. Line graphs are plotted as mean ± sem. Significance of mean differences was assessed using an unpaired t-test (vehicle vs. NPC). In M, n=3 (vehicle) and n=4 (NPC) per group were used.

5. Authors indicated: "Elevated levels of GFAP expression, indicating glial scar formation, were observed in the stroke core". In stroke, GFAP+ cells are not found in the stroke core, but in the peri-infarct area forming the glial scar.

   We apologize for the mix-up in the main manuscript. We were referring to the ischemic border zone (IBZ), as GFAP$^+$ cells are not found in the core, as correctly stated by the reviewer. We have now clarified this point in the text.

6. There are no differences in stroke size or blood perfusion between Stroke+Vehicle and Stroke+hiPSC-NPCs (Fig 1E, F, and G). It is unclear how this correlates with the behavioral deficits post-stroke and behavioral recovery observed post-transplant.

   We primarily use the perfusion imaging (Laser Doppler, performed shortly after stroke induction) to confirm successful stroke induction. It's spatial resolution and timing may not be able to capture very subtle changes in blood flow at later stages or in specific microvascular regions. Consequently, even if there are local improvements in perfusion over time, our current method would not necessarily detect them, since we did not perform CBF measurements at any time point after cell transplantation.

   Functional recovery can occur through mechanisms that are not strictly tied to reductions in the overall lesion volume. Neural plasticity, synaptic remodeling, and changes in inflammatory responses can all lead to improved functional outcomes without shrinking the infarct. Consistent with our prior work (Rust et al., 2019, *PNAS*, PMID: 31235580) stroke volume can remain unchanged, yet behavioral deficits may be improved by interventions that enhance brain repair or reorganization.

   Thus, even though NPC transplantation did not reduce infarct size,) the cells may promote recovery through other pathways, such as modulating the host immune environment, supporting neurite outgrowth, or improving synaptic connectivity.

7. There are better and more functional metrics to study vascular leakage and integrity. Fibrinogen staining is not sufficient.

   We agree with the reviewer, and as mentioned above under *Question 4*, we performed additional experiments using Evans blue as an established marker of BBB permeability (**Manuscript Fig. 4K-M, Reviewer Fig. 11)**. We administered EB (i.p. injection) 24 hours before perfusion (**42** days post-stroke) to both NPC- and vehicle-treated animals. In both groups, a strong EB signal was evident in the stroke core, with a lower signal in the ischemic border zone. Relative to the contralateral hemisphere, both treatment groups showed a similar increase in EB leakage in the ipsilateral hemisphere, indicating no significant difference in BBB permeability between NPC- and vehicle-treated mice.

   Consequently, we have revised the manuscript to remove any emphasis on NPC-mediated improvements in BBB integrity. We appreciate the reviewer's feedback, and we hope we could clarify and refine our interpretation of the data.

8. Co-localization of EDU and vascular makers should be performed to determine if there is a formation of new vasculature.

   Thank you for suggesting this additional analysis. To assess whether new blood vessels were forming in the ischemic border zone, we administered 5-ethynyl-2′-deoxyuridine (EdU) systemically on day 7 following NPC transplantation. We then co-labelled CD31 and EdU and identified a greater density of EdU^+ blood vessels (per mm^2) in the ischemic border zone of NPC-treated mice compared to vehicle-treated controls. These new data are now presented in **Manuscript Fig.4E-G** (**Reviewer Fig. 2**) of the revised manuscript.

   We also discuss these findings in the context of previous studies indicating a temporally restricted "window" of vascular plasticity within the first two weeks post-stroke (Williamson et al., 2020, *JNeurosci*.). During this period, newly formed capillary connections can help restore local perfusion, support synaptic remodelling, and improve functional recovery. Our transplantation strategy at day 7 post-stroke was specifically aligned with this critical

window, enabling pro-angiogenic cues from the transplanted NPCs to synergize with endogenous remodelling processes and likely contribute to the formation of new vessels.

9. In the transplanted iPSC-derived NPCs express neuronal markers section co-localization of HuNu/NeuN, HuNu/Glut markers, HuNu/GABAergic markers, and HuNu/OPC markers should be performed.

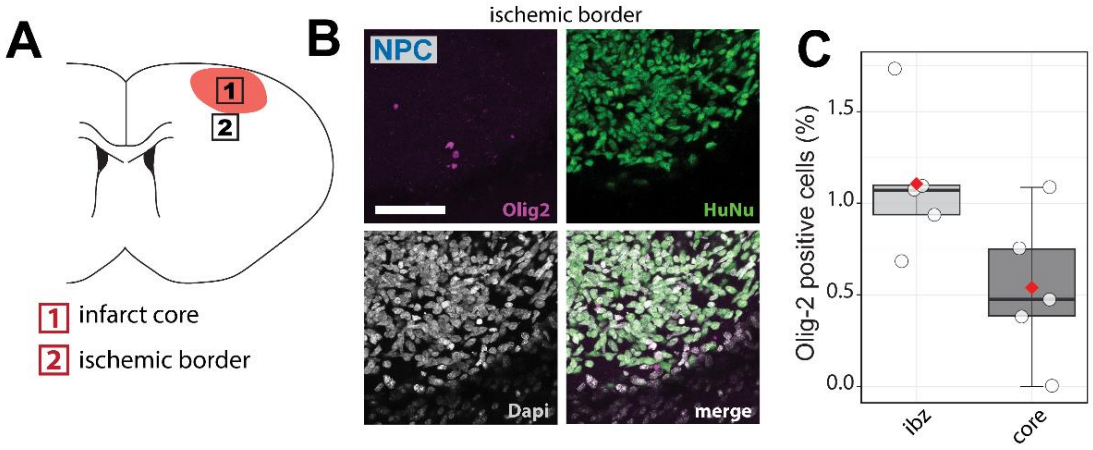
   We appreciate the reviewer's recommendation to characterize the phenotype of the transplanted human NPCs more thoroughly. To distinguish endogenous mouse cells from exogenous NPCs, we performed additional immunostaining for HuNu in combination with neuronal and glial markers:

   - **NeuN and EdU:** to differentiate between endogenous neurogenesis ($EdU^+/NeuN^+$) and exogenous (human) neurogenesis ($EdU^+/NeuN^+/HuNu^+$). In the infarct core, there was no significant difference in endogenous neurogenesis between NPC- and vehicle-treated animals. However, in the subventricular zone (SVZ), the NPC-treated group exhibited a significant increase in $EdU^+/NeuN^+$ double-positive neurons, indicating enhanced endogenous neurogenic activity (**Manuscript Fig. 3L-N, Reviewer Fig. 10**)

   - **Olig2:** to evaluate oligodendrocyte differentiation by co-staining for HuNu/Olig2 in the ischemic border zone and stroke core. We noted that 1.11% of cells in the ischemic border zone were double-positive for HuNu and Olig2, while only 0.54% of cells in the stroke core showed co-localization, suggesting some degree of oligodendrocyte lineage commitment in NPC-derived cells (**Manuscript Suppl. Fig. 5, Reviewer Fig. 12**).

   - **GABAergic markers:** we additionally assessed inhibitory interneuron differentiation by co-labeling HuNu with markers such as parvalbumin (PVALB), somatostatin (SST), vasoactive intestinal peptide (VIP), and lysosomal-associated membrane protein 5 (LAMP5). These experiments provide further insight into whether transplanted NPCs differentiate into inhibitory interneuron subtypes in the peri-infarct region (**Manuscript Fig.7E-G, Suppl. Fig. 20**).

   Taken together, these additional stainings help clarify both the fate and distribution of transplanted human NPCs, as well as the extent to which they adopt neuronal, glial or oligodendroglia lineages within the damaged tissue. We have added these findings to our revised manuscript.



Reviewer Fig. 12: Fate of transplanted NPCs 35 days post-transplantation. (A) Illustration showing the two brain areas (infarct core, ischemic border zone) analysed for Olig2/Hunu double-positive cells. (B) Immunofluorescent staining of a coronal brain section with Olig2, HuNu and Dapi and (C) quantification of Olig2/HuNu+ cell count relative to all counted HuNu+ cells. Scale bar = 50µm. Data are shown as mean distributions where the red dot represents the mean. Boxplots indicate the 25% to 75% quartiles of the data. Each dot in the plot represents one animal. Significance of mean differences was assessed using an unpaired t-test (ibz vs. core).

10. The authors described several times the transplanted cells as “good manufacturing practice (GMP)-compatible iPSC-derived neural progenitor cells (NPCs)”. This generates confusion, and it makes the reader think that these cells have been produced under cGMP conditions, which they have not. These are simply research-graded hiPSC-NPCs.

    To avoid confusion for the readers, we have removed the term “good manufacturing practice (GMP)-compatible NPCs”. Instead, we write that NPCs were generated “under transgene- and xeno-free conditions that could be smoothly adapted to GMP-grade for clinical applications as previously described”

11. Use should properly name their therapeutic product as hiPSC-NPCs. Or at least pick a single way to determine them, throughout the paper they are named NPCS, iPSC-derived NPCs, or iPS-NPCs.

    We thank the reviewer for the observation of this inconsistency. In the results section we now write that “we differentiated human iPSCs into neural progenitor cells (hiPSC-NPCs, hereafter called “NPCs”)”. For the rest of the manuscript, we use the term “NPCs”.

12. The representative image in Figure 3B1 does not show hypovascularization as the authors described in the text.

    We replaced the representative image showing the vascularization in the core/ischemic border zone of a vehicle-treated animal (**Manuscript Fig. 4B, Reviewer Fig. 7**).

13. Based on the data provided in Figures 5 D and E it seems like there is a restoration of neurological functions in stroke animals by day 6 post-stroke. While in Figures 5 G, H, and I the Stroke+vehicle group have longer-lasting deficits up to 40 days post-injury. Are stroke and stroke+vehicle cellular and behavioral distinct? And how does affect the comparison with the stroke + hiPSC-NPCs group?

    This is an important observation. It is true that some of our measured parameters (e.g. step duration, footfall pattern) return to baseline levels (pre-stroke) within a few days after stroke

. This is expected as we have shown in our previous work (Weber et al., 2022, *BMC Biology*, 2022, PMID: 36243716). We usually use those parameters to verify that stroke induction was successful and comparable in all animals. However, other measures e.g. that we used in **Manuscript Fig. 6H** (e.g. relative height of front toe tip, or wrist) are measures that show long-term deficits after stroke and do not return to pre-stroke baseline (also identified in (Weber et al., 2022, *BMC Biology*, 2022, PMID: 36243716). We use those measures to assess improved functional outcome after cell therapy. We now clarified in the results the selection of parameters more clearly.

14. The are no data in this manuscript that can sustain the following observation made by the authors: We observed a severe loss of GABAergic neurons in host stroke tissue and preferential differentiation of cell grafts into GABAergic-like phenotype after stroke.

    We toned down the statement. We now say "We observed that the proportion of GABAergic neurons in the mouse stroke tissue was considerably reduced compared to intact mouse brain; and that half of the transplanted NPCs acquired a GABAergic-like phenotype after stroke."

15. There is no analysis or discussion on how the endogenous cells transcriptionally react to the stroke and the cell transplant. A detailed transcriptional analysis during injury and repair should be included.

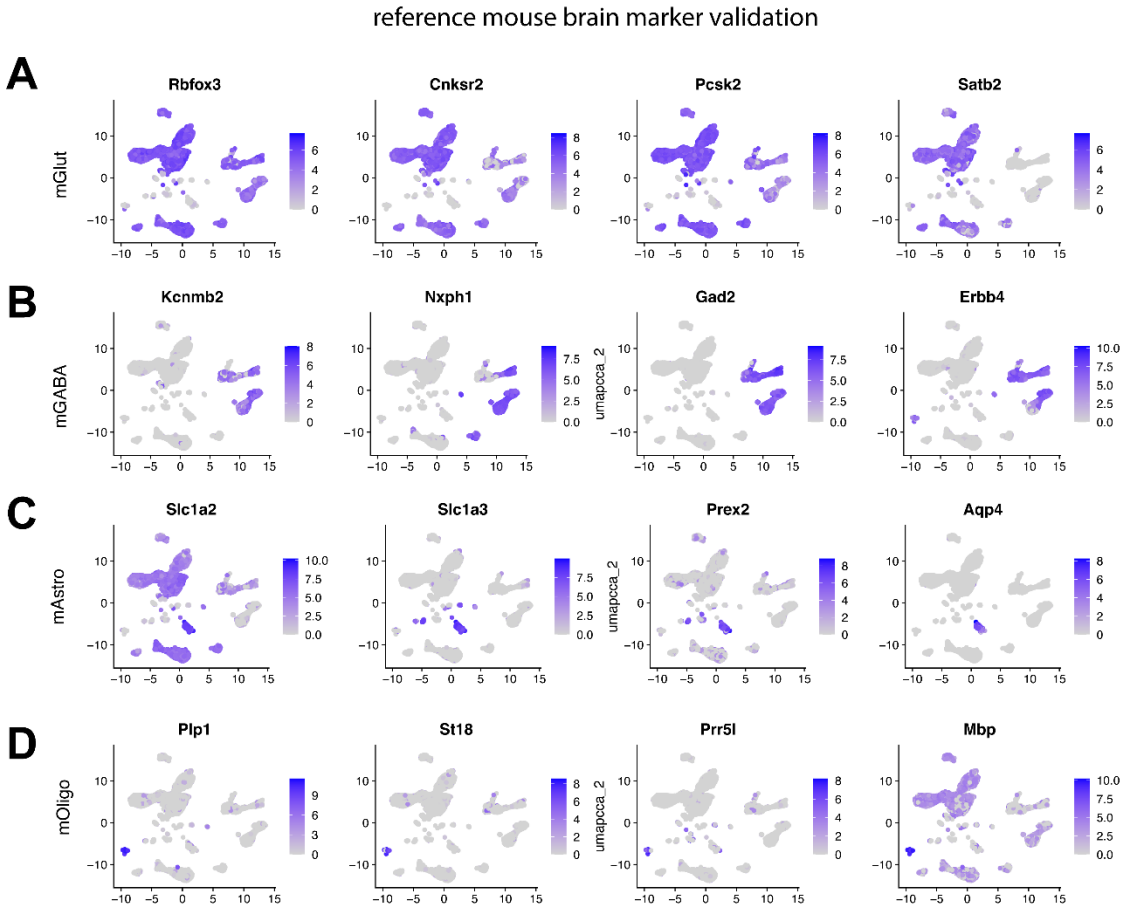
    We thank the reviewer for this important comment. We added a few analysis steps to better describe our dataset and transcriptional changes after stroke and the cell transplant. Our cell type annotation is based on reference snRNAseq datasets previously published (Callaway et al., 2021, *Nature*, PMID: 34616075; Yao et al., 2021, *Cell*, PMID: 34004146; Yao et al., 2021, *Nature*, PMID: 34616066, **Manuscript Suppl. Fig. 15**). We added the following information:

    a. The expression of marker proteins in the respective cell-types in the mouse stroke tissue and reference mouse dataset (**Reviewer Fig. 13, Manuscript Suppl. Fig. 16; Reviewer Fig. 14, Manuscript Suppl. Fig. 17**)
    b. The number of upregulated and downregulated genes for all cell-types mAstro, mGABA, mGlut, mNN, mOligo (**Reviewer Fig. 15, Manuscript Suppl. Fig. 18**). We also added a **Suppl. Table 2** with the top 100 up-and downregulated genes for each cell type)

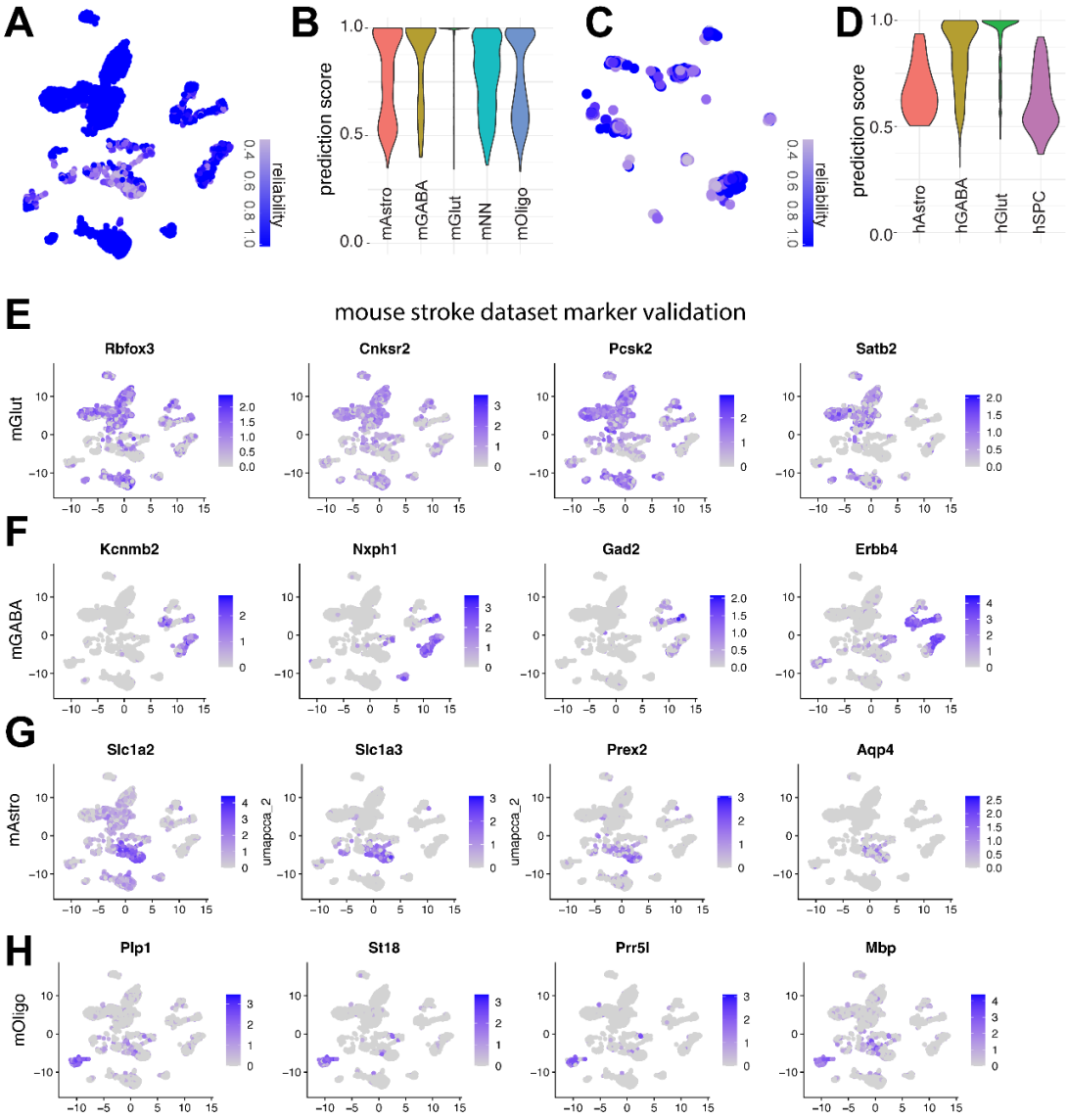
    We have now performed gene set enrichment analysis (GSEA) for each cell type showing a broad suppression of key metabolic, transport and signaling pathways in the majority of host cell types compared to the intact mouse brain cells (**Suppl. Fig 19**). We also provide top GSEA terms for GO Biological process in **Suppl. Table 3.** We added and described the results in the result and discussion section.

    Additionally, we have recently investigated a related question in a separate study (available as a preprint, Weber et al., 2024, **A molecular brain atlas reveals cellular shifts during the repair phase of stroke**, *bioRxiv*). In that work, we used single-cell RNA sequencing to generate a comprehensive transcriptomic atlas of various brain regions 4 weeks after photothrombotic stroke induction. Our findings revealed distinct cell- and region-specific responses within the ischemic border zone and the stroke-injured tissue, including upregulated axon guidance and synaptic plasticity pathways in both GABAergic and glutamatergic neurons. Additionally, we found that key

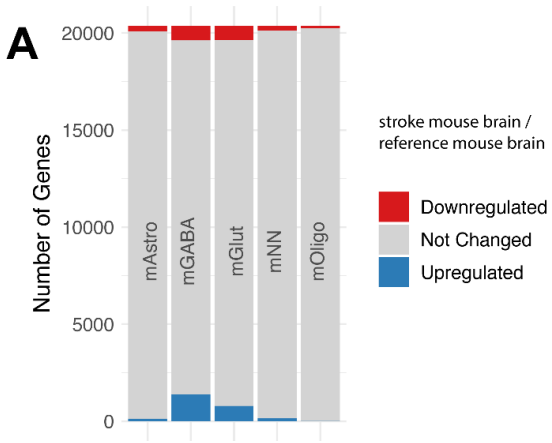
inflammatory, extracellular matrix, and angiogenic pathways were common to both mouse and human stroke lesions, suggesting translational relevance. Using cell–cell communication analyses, we identified signaling pathways (e.g., laminin, collagen, and certain cell adhesion molecules) potentially involved in post-stroke remodeling. We further discuss this data in the revised manuscript (*Discussion*).



Reviewer Fig. 13: Mouse reference dataset marker validation. (A-D) Feature plots of gene expression for specific cell type markers in reference mouse brain dataset: glutamatergic neurons (mGlut, A), GABAergic neurons (mGABA, B), astrocytes (mAstro, C), and oligodendrocytes (mOligo, D).



Reviewer Fig. 14: Mouse stroke dataset marker validation. (A, B) Reliability feature plot and prediction score of 83'058 stroke mouse tissue (n =5), (C, D) Reliability feature plot and prediction score of 1149 transplanted human cell grafts (n = 5). (E-H) Feature plots of gene expression for specific cell type markers in mouse stroke brain: glutamatergic neurons (mGlut, E), GABAergic neurons (mGABA, F), astrocytes (mAstro, G), and oligodendrocytes (mOligo, H).



Reviewer Fig. 15: **Changes in gene expression in host brain cells after stroke**. Number of genes upregulated (blue), downregulated (red), and unchanged (gray) in mouse astrocytes (mAstro), GABAergic neurons (mGABA), glutamatergic neurons (mGlut), non-neural cells (mNN), and oligodendrocytes (mOligo) in the stroke mouse brain compared to the reference mouse brain.

16. The meaning of Abbreviations like I.e. mGABA, hGlut, etc should be included.

    We agree with the reviewer, and we introduce all the abbreviations in the manuscript the first time we use them.

17. The authors indicated that the majority of grafted cells showed hGABA characteristics. But 44% of grafted cells showed hGABA characteristics and 42% of grafted cells showed hGlut characteristics. This is a 50-50 split. It is unclear why authors have decided to solely focused their transcriptional analysis of the GABAergic neurons. Based on the data it seems like the authors should expand their focus.

    We thank the reviewer for this feedback and agree. Our general hypothesis to focus on hGABA cells was primarily because we observed a 50% reduction of GABAergic neurons in the host tissue (29% drop to 14%, while only observing minor changes for Glutamaterigc neurons (68% drop to 58%). Previous studies have also reported that the loss of GABAergic neurons contributes to stroke pathology, as GABAergic neurotransmission can mitigate excitotoxic damage in the acute phase, and optogenetic activation of inhibitory neurons at 24 hours post-injury has been shown to improve stroke outcomes (Pellegrini-Giampietro et al., 2003, *Trends Pharmacol Sci*, PMID: 12967771; Balbi et al., 2021, *Cell Rep*, PMID: 33535035).

    We have also added some additional analysis and description of the Glutamatergic-like neural grafts in the interaction of our key pathways that we identified (NRXN, NRG, NCAM, SLIT). See **Suppl. file 22-25**.

18. Authors should limit the discussion, to discussion and omit any repetition of results.

    We agree with the reviewer; we removed all paragraphs where we repeat our results.

19. Methods. The cell differentiation protocol lacks all relevant details. A methods section should be able to be reproduced by other investigators, in this case in impossible.

We thank the reviewer for pointing out the need for comprehensive details regarding our cell differentiation protocol and we apologize for the lack of clarity. In the revised *Methods* section, we have included a step-by-step outline of the generation and maintenance of our iPSC-derived NPCs, along with the neural induction process. We also included media compositions, growth factors, small molecules, and other reagents used at each step. These additions should now provide sufficient detail for other investigators to reliably reproduce our protocols.

20. Abbreviations should not be used in the abstract (NRXN, NRG, NCAM, and SLIT).

We agree with the reviewer, and we changed the text in the abstract to "Interaction between graft and host transcriptome indicated that GABAergic cell grafts were primarily involved in graft-host communication through the regeneration-associated **neurexin (NRXN), neuregulin (NRG), neural cell adhesion molecule (NCAM)** and **SLIT** signalling pathways".

Reviewer #3

Weber et al. have demonstrated the engraftment and therapeutic effects of iPSC-NPCs in MCAO mice. Graft-derived GABAergic neurons communicate with stroke-injured host tissue potentially through neuronal protective signaling pathways including NRXN, NCAM, SLIT and NRG. In general, this study provides some novel insights for cell therapy to treat stroke. However, there are still some major points about the experimental design and data quality that compromised the scientific contributions.

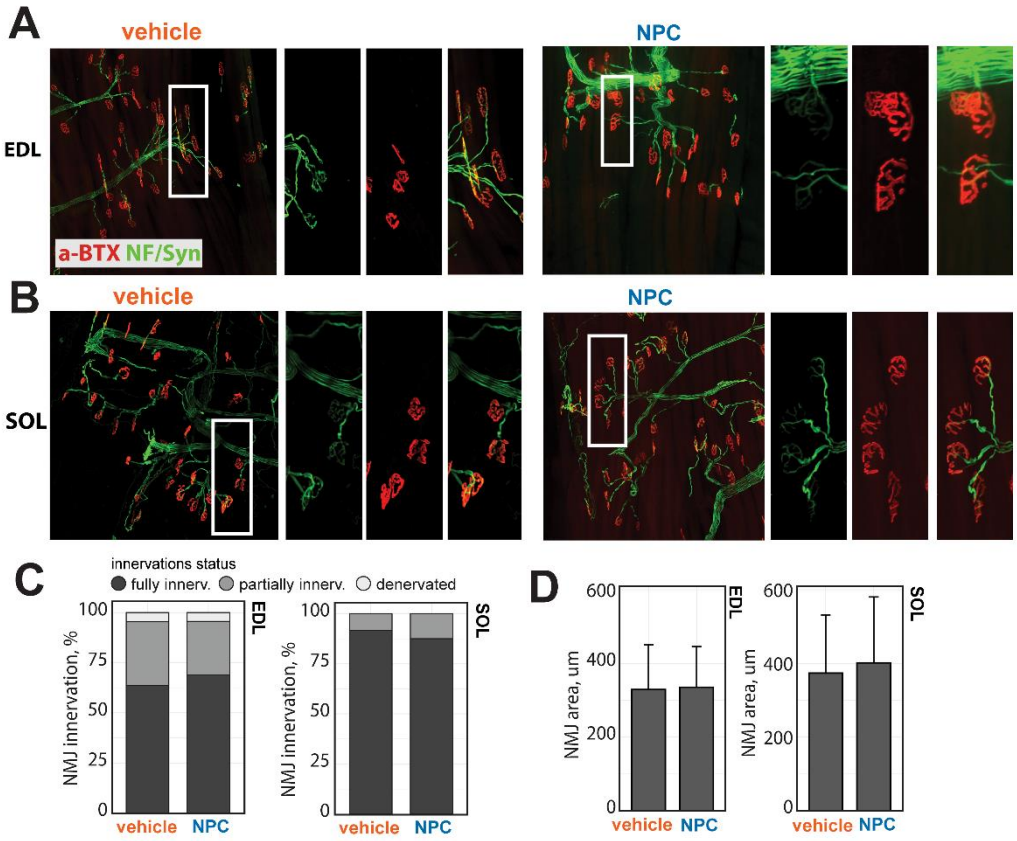
1. The novelty of this work is limited since previous studies have reported the therapeutic effects of iPSC-derived NPCs transplantation in stroke animals. In addition, many researchers have devoted considerable efforts to improve the overall viability, promote the differentiation and migration of transplanted cells to augment their efficacy. Transplantation of iPSC-derived neural progenitor cells (NPCs) has been demonstrated to enhance functional recovery in animal models of stroke.

   We thank the reviewer for raising the issue of novelty. Although previous studies have demonstrated that transplantation of iPSC-derived NPCs can improve aspects of structural and functional recovery after stroke, our work introduces several novel contributions, which we list below. We have strengthened these points in our revised manuscript by adding multiple *in vitro* and *in vivo* experiments and analyses, as suggested by the reviewer, that further strengthen the novelty of our study. The major points highlighting the novelty of our study are as follows:

   - The majority of stroke and cell therapy studies use transient stroke models and focus on acute time points (**1 dpi – 7 dpi**). In contrast, our study employs a permanent stroke model and evaluates the effects of cell therapy over a 6-week period, in line with STAIR/STEPS preclinical testing guidelines. Moreover, by using a delayed transplantation (at 7 dpi), our approach addresses key translational hurdles and reflects a more clinically relevant treatment strategy. We also recently confirmed improved graft survival after delayed transplantation (Weber et al., 2025, Delayed transplantation of neural stem cells improves initial graft survival following stroke*, BioRxiv*, see **Reviewer Fig. 1**). And added this information to the revised manuscript.

   - Most stroke and cell therapy studies do not provide a comprehensive analysis of both neuronal and non-neuronal brain tissue responses, covering aspects such as inflammation, scarring, BBB integrity, neurogenesis, axonal tracing, and stroke volume. Following the reviewer's important suggestion, we have now expanded our study to

include non-CNS changes by assessing structural alterations in neuromuscular junctions. (**Reviewer Fig. 16, Manuscript Suppl. Fig. 13**).



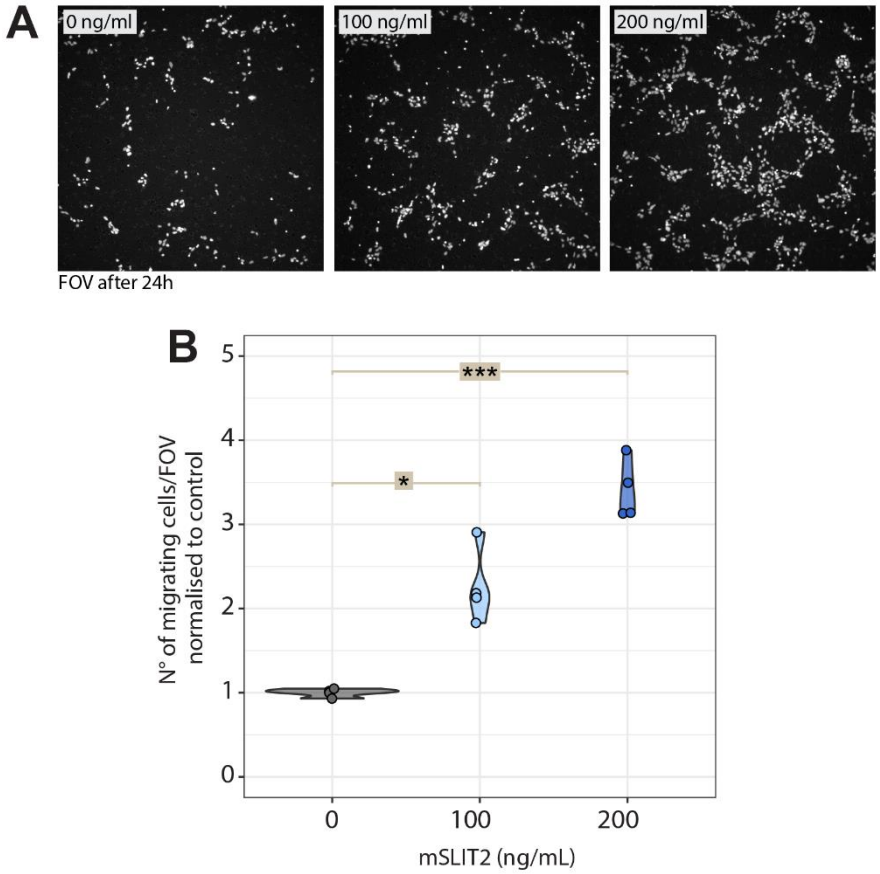
Reviewer Fig. 16: Structural alterations in NMJs after vehicle or NPC treatment. (A) Representative images of NMJs from EDL (A) and SOL (B) from vehicle- and NPC-treated mice. Immunofluorescence assay with α-bungarotoxin (BTX) (red, for acetylcholine receptor) and anti-synaptophysin (Syn) and anti-neurofilament (NF) antibodies (both green, for nerve terminals) used for analysis of NMJ area and innervation status. (C) Barplot showing the proportion of fully innervated, partially innervated, and denervated endplates in EDL (left) and SOL (right). (D) Quantification of NMJ area in EL and SOL muscles of vehicle- and NPC-treated animals. The area of BTX-labelled AChR represents the NMJ area. Data are plotted as mean ± sem. In C and D, we used N=3 mice per group (total N=6).

While many studies use conventional behavioral tests to assess efficacy of cell therapies after stroke, these methods often lack the sensitivity to capture subtle yet meaningful improvements in motor function. In our study, we implemented a novel deep learning-assisted approach that identifies key gait parameters and other movement measurements. This advanced method enables a more comprehensive and precise assessment of functional recovery, which is more reliable than conventional behavioral tests.

The long-term fate of the grafted cells following transplantation after stroke remains one of the most important yet poorly understood aspects in this field. Our study provides, to our knowledge, the first single-cell transcriptomic characterization of the graft and elucidates the mechanisms by which it could communicate with the host tissue. In response to the reviewer's feedback, we have now also added more detailed information on differentially expressed genes in the stroke tissue (**Reviewer Fig. 15, Manuscript Suppl. Fig. 18, Suppl. Table 2)** and performed gene set enrichment analysis, further strengthening the novelty of our work (**Suppl. Fig 19**). We also provide top GSEA terms for GO Biological

process in **Suppl. Table 3.** We added and described the results in the *Result* and *Discussion* section.

As the reviewer also suggested we performed in this revision a functional validation of one of the proposed signaling pathways showing migration of the cell grafts toward mouse Slit2 (**Reviewer Fig. 17, Manuscript Suppl. Fig. 26**).



Reviewer Fig. 17: Migration of hNPCs. Boyden chamber assays of hNPCs towards 0nM, 100 ng/ml, or 200 ng/ml of recombinant SLIT2 protein. (A) Representative images showing cells that successfully migrated towards different concentrations of SLIT2 protein. (B) Quantification. Violin graphs are plotted as mean ± sem. Significance of mean differences was assessed using an unpaired t-test. Asterisks indicate significance: *p < 0.05, **p < 0.01, ***p < 0.001.

2. What is the significance and innovation of this research? The analysis of the molecular crosstalk between the host and the graft is not comprehensive. Single nucleus profiling of the cell transplants and host stroke tissue was performed in this study and GABAergic cell grafts were primarily identified as involving in graft-host communication and preferentially differentiating towards GABAergic cells. The authors hypothesized that GABAergic grafts interact with damaged host tissue to initiate recovery-associated processes. However, the ligands or receptors mentioned in this study are not exclusively expressed in graft. The NRXN, NRG, NCAM, SLIT signaling pathways are dominantly expressed in mGABA. Given the large number of local host cells compared to the engrafted cells, the communication strength of host between host is much stronger than host between graft. Does secretion from transplanted cells mediate regeneration of the ischemic core? More validation experiments (i.e. loss of function experiments) are necessary to support the purposed molecular mechanisms

We thank the reviewer for this important comment. We agree that many of the identified ligands and receptors (NRXN, NRG, NCAM, SLIT) are not exclusively expressed by the grafts, and that there is substantial molecular interaction among local host cells, as clearly observed in our CellChat analysis. In the revised Discussion, we now clarify that host–host communication is dominant in the stroke regions and likely much stronger than the signaling between transplanted cells and the host, and we toned down our conclusions regarding the direct contribution of the transplanted cells.

However, we believe it is important to understand whether those predicted graft-host signaling pathways have functional implications. To further understand the functional role of the molecular graft-host interactions, we performed a proof-of-concept validation experiment to assess whether, for example, mouse Slit2 affects the migration of our transplanted human NPCs as predicted by our CellChat analysis (**Reviewer Fig. 17, Manuscript Suppl. Fig. 26)**. In this experiment, we observed a dose-dependent migration of NPCs toward mouse Slit2, supporting the proposed contribution of this pathway to graft–host interactions. We also added to the *Discussion* that future studies could perform loss of function experiments to identify direct *in vivo* evidence.

3. Did the transplantation of iPSC-derived NPCs increase the concentration of ligands mentioned in the manuscript, such as slit2 and slit1.

This is a great point, we checked some of the ligands mentioned in the manuscript and observe that the majority of ligands were upregulated in the mouse stroke tissue (**Reviewer Table 1**):

Reviewer table 1:

Gene	avg_log2FC	p_val_adj	Status	Dataset
SLIT1	-0.3564857	1.546E-268	Downregulated	mGABA
NRXN2	0.76941504	1.002E-259	Upregulated	mGABA
SLIT2	-1.1299389	5.286E-217	Downregulated	mGABA
NRG1	0.79140379	6.482E-178	Upregulated	mGABA
NRG3	0.5518612	3.788E-177	Upregulated	mGABA
NRXN1	0.35452043	1.706E-111	Upregulated	mGABA
NCAM2	-0.5951095	6.186E-10	Downregulated	mGABA
NCAM2	1.41810455	0	Upregulated	mGlut
NRG1	1.85743792	0	Upregulated	mGlut
NRG3	2.13126391	0	Upregulated	mGlut
NRXN1	1.26131486	0	Upregulated	mGlut
SLIT2	1.21406418	1.8703E-71	Upregulated	mGlut
NCAM1	0.6036225	1.9739E-46	Upregulated	mGlut
NRXN1	1.33103239	1.1818E-62	Upregulated	mAstro
NCAM2	2.23061293	0.04540928	Upregulated	mOligo
NRXN1	1.18755742	0.00087588	Upregulated	mNN
NCAM1	1.13078754	0.03218991	Upregulated	mNN

Of note, these observations may be expected because we used the mouse stroke tissue Seurat file and the human cell graft Seurat file as input for CellChat. Therefore, most of the ligands and receptors inferred in CellChat are likely enriched in the original datasets.

Therefore, we additionally performed a proof-of-concept in vitro experiment to validate that one of our predictor ligand-receptor pairs induced NPC migration towards the ligand Slit2. We show a dose-dependent migration of NPC towards increasing amounts of mouse-Slit2 (**Reviewer Fig. 17, Manuscript Suppl. Fig. 26)**.

4. Why transplant iPSC-NPCs cells 7 days after stroke? When is the most effective transplantation time point for the treatment of stroke?

We appreciate the reviewer's question regarding the rationale behind a 7-day post-injury transplantation. Since the initial inflammatory response and excitotoxic phase is more pronounced during the first few days after stroke, the chosen time point may offer conditions that are more permissive for donor cell survival. Prior studies in the literature (Schmidt & Minnerup, 2016, *Expert Rev Neurotherapeutics*, PMID: 26689223) indicate that delivering NPCs too early (24 hours post-stroke) can lead to extensive cell death, likely due to the hostile acute microenvironment.

To further assess this question, we have conducted additional experiments (described in a recent preprint submission: Weber et al., 2025, Delayed transplantation of neural stem cells improves initial graft survival following stroke*, BioRxiv*, **Reviewer Fig. 1**) where NPCs were transplanted 24 hours after stroke induction. In these experiments, we observed a reduced survival rate of grafted cells transplanted early compared to delayed transplantation timepoints, which we attribute to the more hostile post-stroke environment at that time. Interestingly, despite the reduced survival, the differentiation pattern/potential of the transplanted NPCs remained similar, with comparable neuron/glia/NPC ratios to delayed transplantation timepoints.

Thus, waiting until day 7 enables edema to partially subside and inflammatory signals to begin shifting, thereby providing a more supportive niche for cell engraftment. We have included relevant references in the revised manuscript (in the *Discussion* section) to support this timeline and further explain why a slightly delayed transplantation strategy may optimize graft survival and therapeutic potential.

5. The detailed protocol to differentiate human iPSCs to NPCs should be presented in the method section.

We thank the reviewer for pointing out the need for comprehensive details regarding our cell differentiation protocol and we apologize for the lack of clarity. In the revised *Methods* section, we have included a step-by-step outline of the generation and maintenance of our iPSC-derived NPCs, along with the neural induction process. We also included media compositions, growth factors, small molecules, and other reagents used at each step. We hope that these additions now provide sufficient detail for other investigators to reliably reproduce our protocols.

6. The efficacy of human iPSC-derived NPC transplantation could be evaluated in immunodeficient Rag2-/- mice, which lack functional T cells and B cells, thereby reducing immune rejection of the transplanted cells. However, to fully understand the complex interaction between the host immune system and the transplanted cells. The therapeutic effects and NPCs differentiation towards GABAergic cells should also be determined and validated in normal C57BL/6 mice.

We appreciate the reviewer's suggestion to investigate both immunodeficient and immunocompetent mouse models. Prior to the present study, we characterized stroke pathology in (the most) popular genetic immunodeficient strains, including NOD scid gamma (NSG) and Rag2$^{-/-}$ mice, as well as in pharmacologically immunosuppressed (Tacrolimus) and wild-type (WT) C57BL/6J animals (Weber et al., 2022, *Front. Immunol*., PMID: 36569903). At three weeks post-injury, these strains exhibited some anatomical and genetic differences, but we did not observe a significant impact on functional recovery over this timeline.

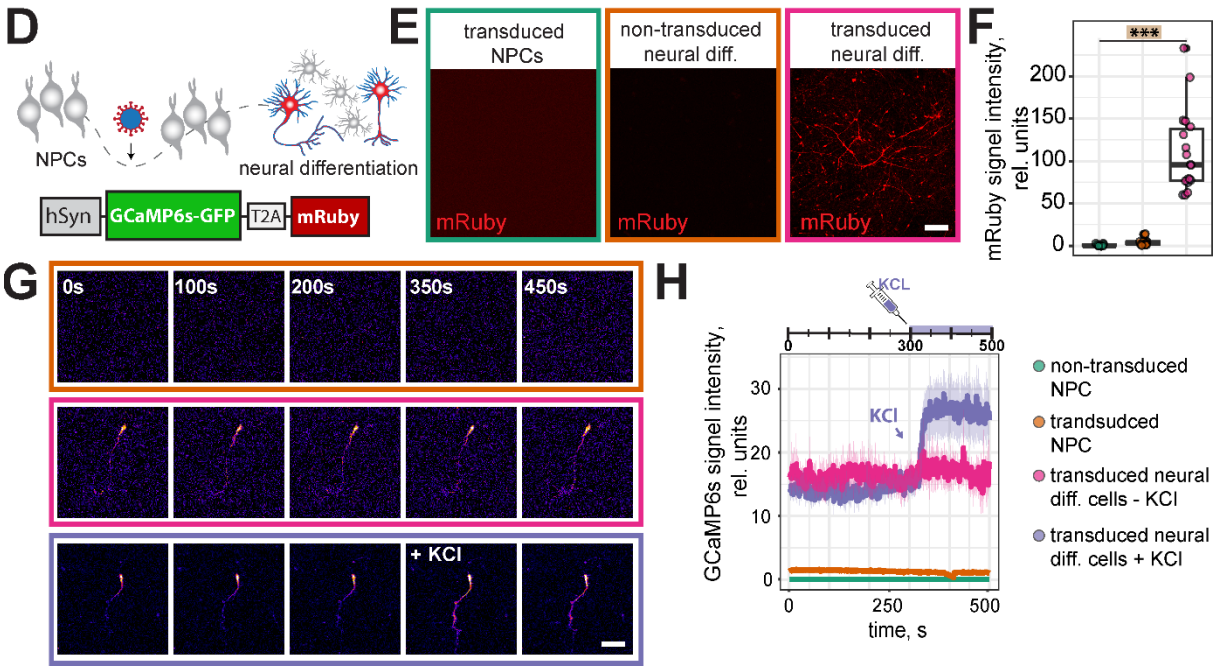
In parallel, we transplanted our NPCs into all models, including WT mice immunosuppressed with tacrolimus delivered via osmotic mini pumps.

Although this approach provided a continuous delivery previously validated in other studies, we remain uncertain about the precise spatiotemporal distribution and efficacy of tacrolimus within the injured brain tissue. Consequently, we were unable to obtain robust, long-term graft survival data in immunocompetent hosts, which would be a prerequisite for fully assessing differentiation potential (such as into GABAergic lineages) over an extended period. As a next step, we plan to explore alternative immunosuppression regimens, including daily intraperitoneal injections, to maintain stable drug levels and support long-term survival of transplanted NPCs in WT stroke models to explore the therapeutic effects of NPCs in mice with a C57BL/6J background, as the reviewer suggested.

However, we recognize that evaluating our cell line in genetically modified Rag2$^{-/-}$ mice represents a limitation and therefore added a paragraph in the *Discussion* section where we discuss the current limitations of our study.

7. The authors only demonstrated that transplanted NPCs could differentiate into neurons and express MAP2 but have not been proven whether the neurons formed by the differentiation of this group of cells exhibit corresponding functions, such as transmitting action potentials.

We agree that confirming the functional maturity of transplanted neurons is crucial. In our revised *Discussion*, we highlight this point as a key limitation of our current study. While we have previously shown that our NPCs become electrically active *in vitro* (using mRuby2 and the calcium sensor GCaMP6s under the human synapsin 1 promoter to confirm activity-dependent signaling), further experiments are needed to verify whether the same functionality is retained *in vivo* following transplantation (Rust et al., 2022, *J Transl Med*, PMID: 36114512, **Reviewer Fig. 18**).



Reviewer Fig. 18: Neural differentiation of NPCs (from Rust et al., 2022, *J Transl Med*, PMID: 36114512). (D) Experimental setup for determining the activity of differentiated NPCs and AAV construct for the expression of GCaMP6s and mRuby under the human synapsin promoter. (E) Human synapsin promoter-based expression of mRuby in NPCs and differentiated neural cells. Confocal images of NPCs transduced with the AAV-mRuby construct (left), differentiated NPCs at d21 after differentiation (middle) and differentiated NPCs at d21 after differentiation and transduced with AAV-mRuby construct (right). (F) Quantification of mRuby signal in transduced NPCs (left), non-transduced differentiated neural cells (middle) and transduced neural cells (right). (G) Confocal time-lapse images of non-transduced, differentiated neural cells (upper row) and neural cells transduced with AAV-GCaMP6s construct (middle row). After 350 s, cells were treated with 60 mM KCL (lower row). Scale bars: 50 µm. (H) Quantification of GCaMP6s signal intensity from confocal time-lapse images. H-Syn: human synapsin. Boxplots indicate the 25–75% quartiles of the data. Each dot in the plots represents one cell culture well and significance of mean differences between the groups was assessed using Tukey's HSD. Line graphs are plotted as mean ± sem. Asterisks indicate significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

8. Stroke can lead to sarcopenia and worsening of motor function. Muscle mass should be assessed in the limbs of stroke mice. Does iPSC-derived NPC transplantation improve motor function by preventing sarcopenia.

We appreciate the reviewers' input and we think it's a great suggestion to analyse the potential role of sarcopenia in post-stroke motor impairment. Given that neuromuscular integrity is critical for muscle maintenance and function (Moreira-Pais et al., 2022, *GeroScience*., PMID: 34981273; Ham et al., 2020, *Nat Commun*, PMID: 32908143), we performed an in-depth analysis of neuromuscular junctions (NMJs) and their innervation status in two hindlimb muscles. We decided to look at the **extensor digitorum longus (EDL)** and the **soleus** in both NPC- and vehicle-treated groups.

Despite the functional improvements observed in NPC-treated mice, we found no significant differences in NMJ occupancy between the two groups.

Also, postsynapses of both groups show normal pretzel-like shapes and were comparable in size ($vehicle_{EDL}$: 329±121.87, $vehicle_{SOL}$: 373±158.1, NPC_{EDL}:335.1±110.3, NPC_{SOL}: 401.2±178.97, $p>0.05$ for all comparisons, **Reviewer Fig. 16, Manuscript Suppl. Fig. 13**).

Although we did not specifically quantify muscle mass in these experiments, our NMJ findings imply that NPC transplantation does not exert a significant effect on the peripheral neuromuscular interface within the time window examined.

We added the new results to the revised manuscript. Methodology is described in a new section in *Methods*.

9. Are experimental behavioral improvements in stroke mice related to changes in the structure or quantity of neuromuscular junctions within the skeletal muscle? If so, are transplanted NPCs involved in the innervation of neuromuscular junctions?

We appreciate the reviewer's suggestion to investigate the potential role of neuromuscular junctions (NMJs) in mediating the functional recovery we observed after NPC transplantation. To address this, we performed a systematic evaluation of NMJ structure and innervation in two representative hindlimb muscles commonly assessed in rodent models of neurological impairment: the extensor digitorum longus (EDL) and the soleus in *N*=6 animals. These muscles were harvested from both NPC-treated and vehicle-treated groups at the end of the study (**Reviewer Fig. 16, Manuscript Suppl. Fig. 13)**.

Despite the observed functional improvements in the NPC-treated group, we did not observe significant changes in NMJ innervation status compared to vehicle-treated control animals in either the EDL or soleus muscles. This suggests that the functional benefits of NPC transplantation may not be directly attributable to enhanced innervation at the NMJ level, at least in the muscles we examined. However, it is possible that other muscle groups or different time windows might show treatment-related changes.

We added the new results to the revised manuscript.

10. Extravasation of tracing dye and quantification of tight junction proteins should be performed to assess the permeability of cerebral blood vessels.

We thank the reviewer for pointing out the need for more robust evidence to support any conclusion regarding blood brain barrier integrity. In the original manuscript, we evaluated BBB integrity based on fibrinogen leakage as a marker of vascular permeability. However, to further address the reviewer's concern, we performed additional experiments using Evans blue (EB), a well-established marker of BBB permeability (Saunders et al., 2015, *Front. Neurosci.*, PMID: 26578854).

We systematically administered EB 24 hours before perfusion (**42** days post-stroke) to both NPC- and vehicle-treated animals. In both groups, a strong EB signal was evident in the stroke core, with a lower signal in the ischemic border zone. Relative to the contralateral hemisphere, both treatment groups showed a similar increase in EB leakage in the ipsilateral hemisphere, indicating no significant difference in BBB permeability between NPC- and vehicle-treated mice (**Manuscript Fig. 4K-M, Reviewer Fig. 11**).

Consequently, we have revised the manuscript to remove any emphasis on NPC-mediated improvements in BBB integrity. We appreciate the reviewer's feedback, and we hope we could clarify and refine our interpretation of the data.

11. In Figure 7F, the authors grouped different signaling pathways via functional and structural similarity. What is the definition of functional and structural similarity? In Figure7I-L, what is the meaning of mediator and influencer. Please describe in detail.

In Figure 7F (in the revised manuscript: **Fig. 8F**), signaling pathways were grouped based on functional and structural similarity as defined in CellChat. Functional similarity in CellChat refers to pathways that regulate related biological processes, whereas structural similarity is

based on shared ligand-receptor families or overlapping downstream signaling components. We added a description to the *Methods* section.

In Figure 7I-L (in the revised manuscript: **Fig. 8I-L**), the terms *Sender, Receiver, Mediator,* and *Influencer* represent distinct roles in intercellular communication inferred from CellChat's network analysis:

- **Sender (Source) Cells**: Cells that secrete ligands, initiating the signaling process.
- **Receiver (Target) Cells:** Cells that express the corresponding receptors and respond to the signals.
- **Mediator Cells**: Cells that facilitate or amplify communication between sender and receiver cells, acting as intermediaries in the network.
- **Influencer Cells**: Cells that modulate the strength or efficacy of a signaling pathway, shaping how communication occurs even if they are not direct senders or receivers.

We added a description to the *Methods* section.

Reviewer #2 (Remarks to the Author):

Weber et al. have made a commendable effort in addressing the reviewers' concerns. They have incorporated several new experiments and analyses, added new figures, and expanded the discussion with important new insights. Additionally, they have appropriately downplayed points that are no longer strongly supported by the new data. Overall, these revisions have improved the manuscript. However, several points still remain to be addressed.

We thank the reviewer for their positive feedback.

Based on the new results, NPC transplantation does not affect BBB integrity. The abstract must be revised accordingly. NPCs do not primarily differentiate into GABAergic interneurons. Instead, 44% differentiate into GABAergic interneurons, while 42% become glutamatergic neurons. The abstract must be revised accordingly. A more accurate statement would be that the graft preferentially differentiates into neurons.

We agree that the abstract might be misleading, and we changed the sentence in the abstract to: "We identified graft differentiation preferentially towards neurons with GABAergic and glutamatergic phenotypes in similar proportions, with the remaining cells acquiring astrocyte and NPC-like phenotypes".

Two out of three reviewers requested a justification for the selected transplant day. The authors cited their own BioRxiv manuscript, which supports 7-day over 24-hour transplantation, but did not acknowledge the potential for chronic-phase transplantation—a stage where most stroke patients reside and where intervention may be more clinically relevant. There is extensive literature, including clinical trials, on therapeutic windows for stem cell-based therapies in stroke and other types of brain/spinal cord injury models, and failing to discuss this represents a missed opportunity to address a critical issue in the field.

We thank the reviewer for the important input. We agree that the potential for stem cell therapies in the chronic phase of stroke is a critical area of both clinical and preclinical research. In response, we have added a brief discussion to the revised manuscript (in the *Discussion* section) acknowledging the significance of chronic-phase transplantation.

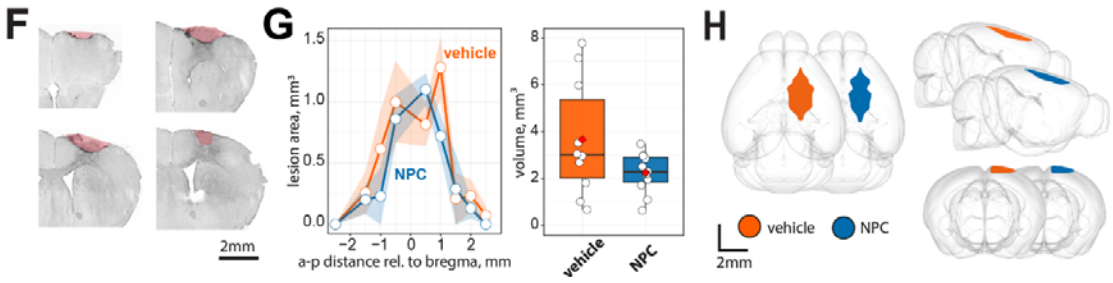
Specifically, we now highlight that many stroke patients reside in the chronic phase, where treatment options remain limited. We discuss evidence supporting an extended/prolonged window of neuroplasticity after stroke, including recent preclinical data showing that e.g. hMSC therapy can reopen adaptive plasticity even at chronic stages (Klein et al., 2025, *Mol Ther*., PMID: 39668560). We also reference clinical trials demonstrating functional recovery in patients treated with stem cell interventions more than 90 days post-stroke. Additionally, we note the challenge of transplanting into mature infarcts, which may already be undergoing spontaneous repair, and briefly mention emerging strategies (such as biomaterial scaffolds) to reduce mechanical disruption during chronic-stage transplantation.

We hope that this added discussion helps to contextualize our choice of the 7-day transplant time point while acknowledging broader therapeutic windows in the field.

What markers were used to assess stroke size? In the rebuttal letter (Reviewer 1, Question 6), the authors imply that HuNu was used, but GFAP, IBA-1, and NeuN would be more appropriate for this purpose. Clarification is necessary.

We thank the reviewer for pointing out this clarification and we agree with the reviewer that markers such as GFAP, IBA-1, and NeuN are more appropriate for assessing stroke size. We confirm that we used both HuNu (to measure **graft size**) and GFAP (to accurately delineate

the **stroke lesion**). We apologize for initially only mentioning HuNu in our rebuttal, leading to confusion. We have now clarified this detail in the *Methods* section of the revised manuscript and updated the representative images in **Reviewer Fig. 1** (**Manuscript Fig. 1F**) accordingly.



Reviewer Figure 1: Generation of hiPSC-NPCs, stroke induction and transplantation. (F) Coronal brain sections used to estimate stroke area. The dotted red line indicates the stroke-lesioned brain area. Scale bar: 2mm (G) Quantification of stroke infarction size. Left: Lesion area plotted against the anterior-posterior (a-p) distance relative to bregma (mm). Right: boxplot displaying lesion volumes (mm3) of both treatment groups. (H) Schematic representation of a 3-D mouse brain model depicting stroke infarction sizes. Scale bar: 2mm. Data are shown as mean distributions where the red dot represents the mean. Boxplots indicate the 25% to 75% quartiles of the data. For boxplots: each dot in the plots represents one animal. Line graphs are plotted as mean ± sem. Significance of mean differences was assessed using a paired t-test (baseline vs. stroke) or an unpaired t-test (vehicle vs. NPC). In E-J, n=11 mice per group; in M, n=9 animals per group. Asterisks indicate significance: *p < 0.05.

At what time point was the caspase-3 experiment performed? Clarification is needed.

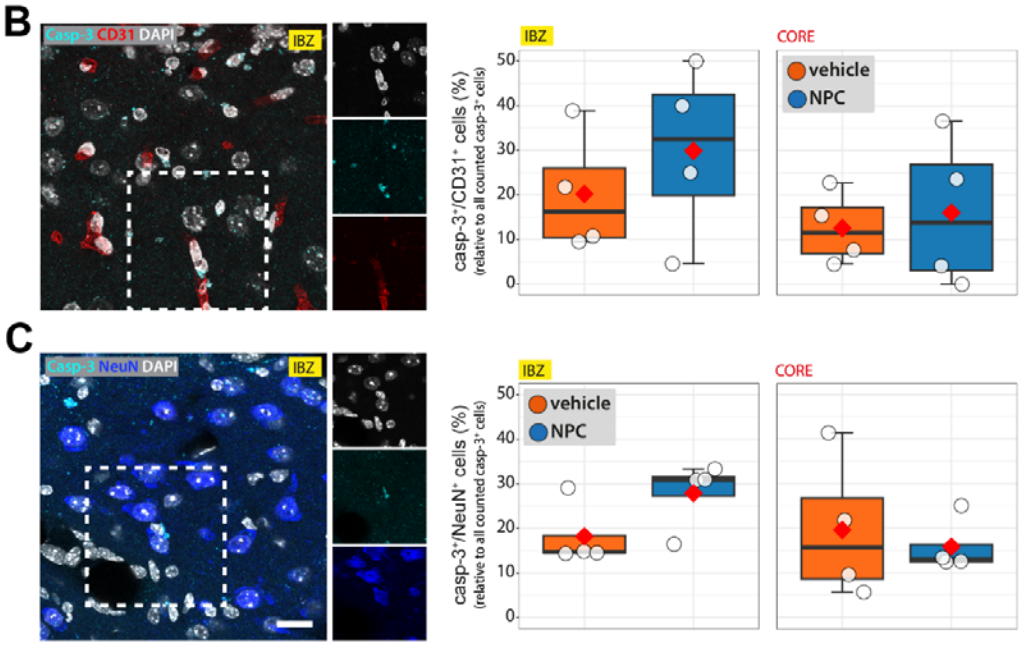
Caspase-3 immunohistochemical stainings were performed on day 43 after stroke induction. We added this information to the main manuscript and to the figure.

The authors suggest that NPC therapy does not affect apoptosis, but they did not perform colocalization with caspase-3. Was caspase-3 expressed in transplanted NPCs? If so, in what proportion? In which host cell types was caspase-3 expressed? Are different cell populations undergoing apoptosis in stroke alone versus stroke + NPCs?

This is a good point! Indeed, we did assess caspase-3 expression in transplanted NPCs by co-labeling with human nuclear antigen (HuNu). However, only a very small fraction of transplanted NPCs were positive for caspase-3 (1–3%), indicating only minimal apoptosis within the grafted population.

But as the reviewer suggested, we further characterized the caspase-3$^+$ cell population to determine which host cell types were undergoing apoptosis and whether differences existed between the two treatment groups (vehicle vs. NPC, **Reviewer Fig. 2, Manuscript Suppl. Fig. 3B, C**). We performed co-labeling of caspase-3 with CD31 (an endothelial cell marker) and NeuN (a neuronal cell marker). In both the ischemic border zone and stroke core, we found comparable proportions of caspase-3/CD31 and caspase-3/NeuN double-positive cells in NPC- and vehicle-treated animals.

Notably, there was a trend toward fewer apoptotic neurons in the stroke core of NPC-treated animals, although this did not reach statistical significance. These findings are now included in the *Results* section of the revised manuscript-

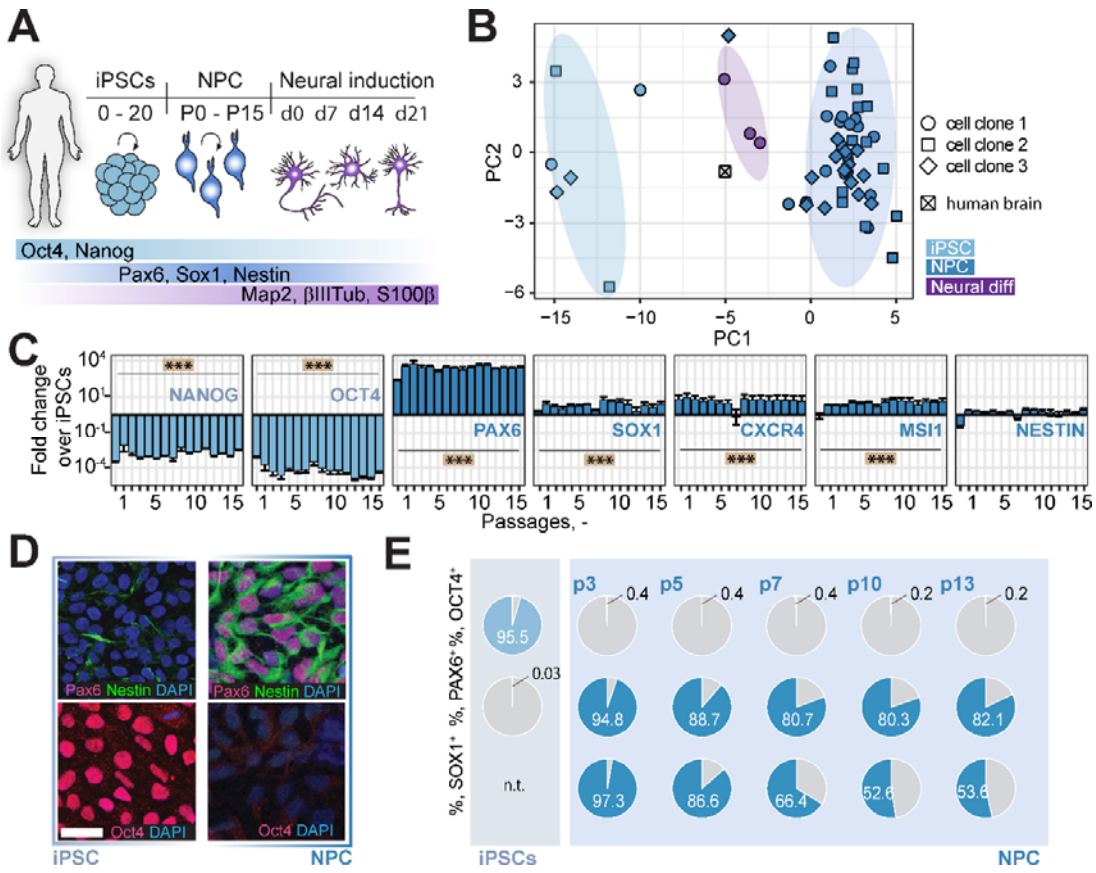


Reviewer Figure 2: Histological analysis of microglia, astrocyte expression in the contralesional hemisphere and characterization of Casp-3+ cells in the IBZ and the core region. (B) Immunofluorescent staining of a coronal brain section with Casp-3, CD31 and DAPI and (right) quantification of Casp-3/CD31+ cell count relative to all counted Casp-3+ cells in the IBZ and the core region. (C) Immunofluorescent staining of a coronal brain section with Casp-3, NeuN and DAPI and (right) quantification of Casp-3/NeuN+ cell count relative to all counted Casp-3+ cells in the IBZ and the core region. Scale bar = 10µm. Data are shown as mean distributions where the red dot represents the mean. Boxplots indicate the 25% to 75% quartiles of the data. Each dot in the plot represents one animal. Significance of mean differences was assessed using an unpaired t-test (vehicle vs. NPC).

What is the cellular composition of the final therapeutic product? A substantial proportion of S100β+ cells appears to be present. How reproducible is this differentiation protocol across batches? Are the authors consistently transplanting the same proportions of cell types? This is a crucial consideration for potential clinical translation.

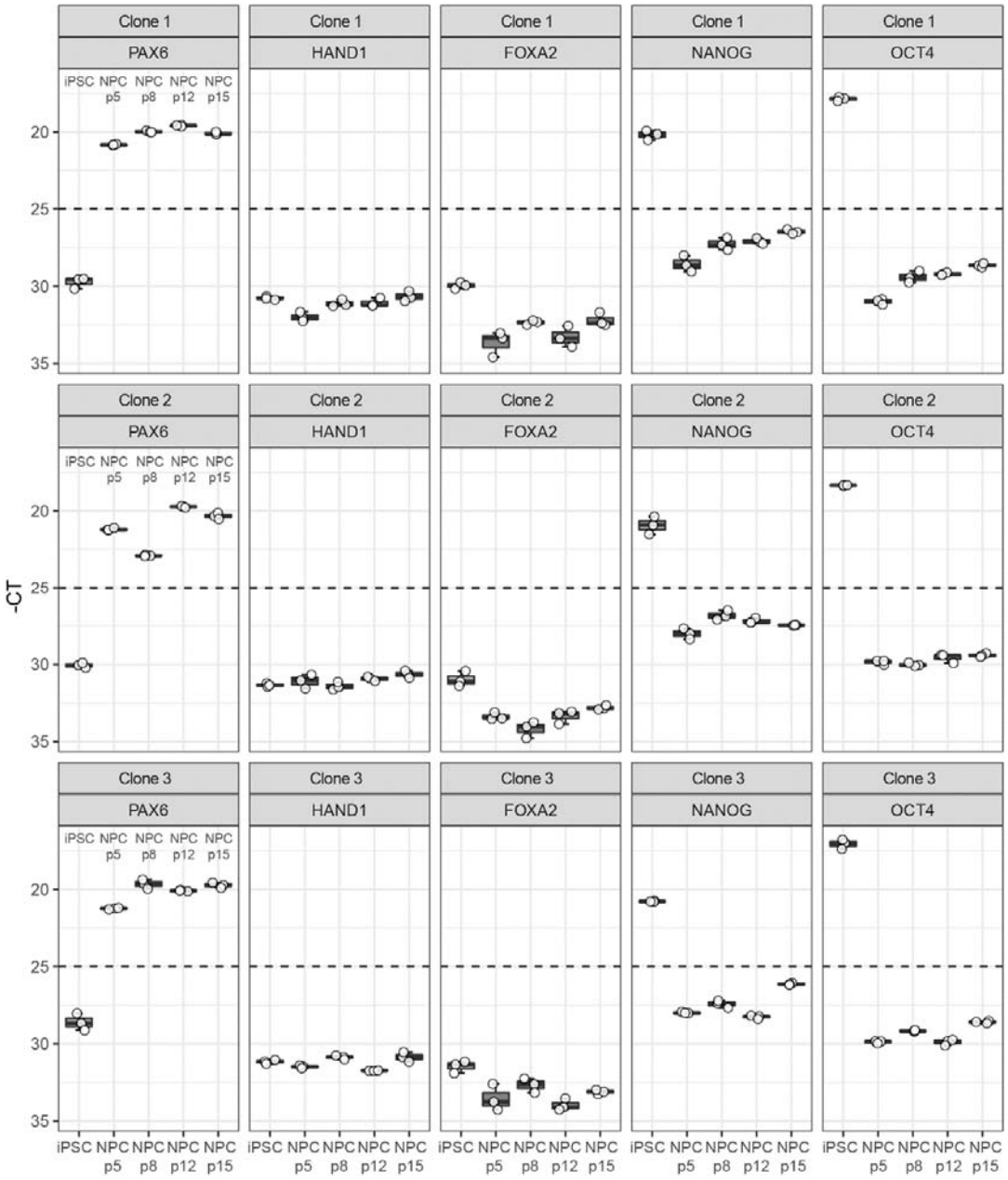
We thank the reviewer for highlighting this important consideration. Indeed, we consistently transplant the same therapeutic product (with reproducible cellular composition). Our differentiation protocol reliably generates functional and pure iPSC-derived NPC populations.

Gene expression analysis of our cell product demonstrated clear cell type-specific clustering across NPCs (3 different clones) without any significant clone-specific effects (Rust et al., 2022, J Transl Med, PMID: 36114512, **Reviewer Fig. 3, 4**). Key pluripotency markers, NANOG and OCT4, were significantly downregulated in NPCs (all $p < 0.001$), while neural progenitor markers (PAX6, SOX1, CXCR4, MSI1) remained stably upregulated across multiple passages. Markers associated with non-neural lineages (HAND1, FOXA2) remained consistently low.



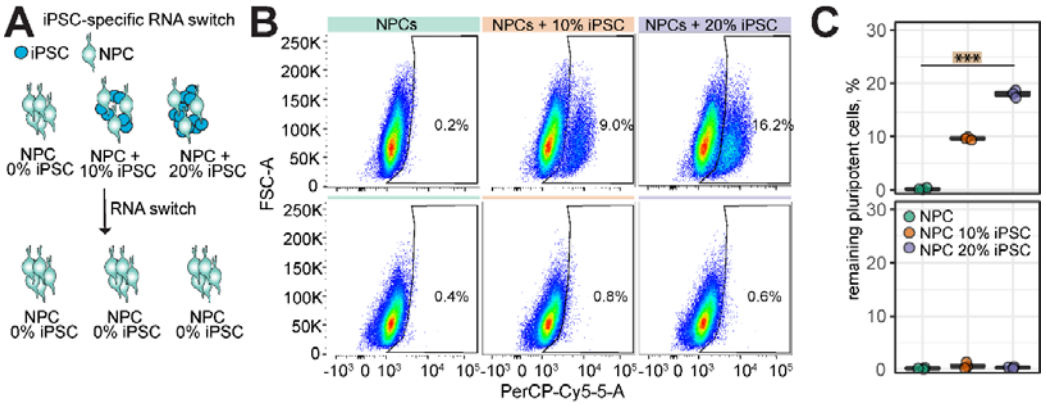
Reviewer Figure 3: **Generation and characterization of iPSC-derived NPCs**. A: Schematic representation of the analyzed cell types, time frame and the main marker proteins used for characterization. B: Principal component analysis of qPCR data for NPCs (3 clones, dark blue) iPSCs (3 clones, light blue), NPCs 21d after neural differentiation (clone 1, violet) and human total brain RNA. Each symbol illustrates data from RNA extracted from one cell culture well. C: Gene expression of pluripotency marker (NANOG and OCT4) and NPC marker (PAX6, SOX1, CXCR4, MSI1, NESTIN) in NPCs over the course of 15 passages, measured by qPCR. D: iPSCs (left panel) and NPCs at passage 7-10 (right panel) stained for Pax6, Nestin, Oct4 and DAPI. E: Flow cytometry analysis of iPSCs (upper row) and NPCs at different passages (lower rows) for Oct4, Pax6 and Sox1. Pie charts illustrate percentage positivity (light/dark blue) for the respective marker and cell type. n.t.: not tested. Neural diff: NPCs after neural differentiation; CXCR4: CXC-motif chemokine receptor-4; MSI1: Musashi-1; Scale bars: 50µm. Significance of mean differences between the groups was assessed using Tukey's HSD. Asterisks indicate significance: *** $P < 0.001$.

Immunostaining and flow cytometry analyses confirmed these findings, with NPCs consistently positive for Pax6 and Nestin and negative for Oct4. Further, we performed karyotype analyses across various passages and confirmed stable 44, XX karyotypes. Additionally, reproducibility was confirmed using two additional iPSC clones, yielding similar robust NPC differentiation results (Rust et al., 2022, J Transl Med, PMID: 36114512, **Reviewer Fig. 4**).



Reviewer Figure 4: Expression of different cell lineage markers in NPCs. Gene expression of markers for ectodermal (PAX6), mesodermal (HAND1) and endodermal (FOXA2) cell lineages, as well as for pluripotent cells (NANOG and OCT4), in iPSCs and NPCs from three clonal lines at different passages, measured by qPCR. Boxplots show –CT values.

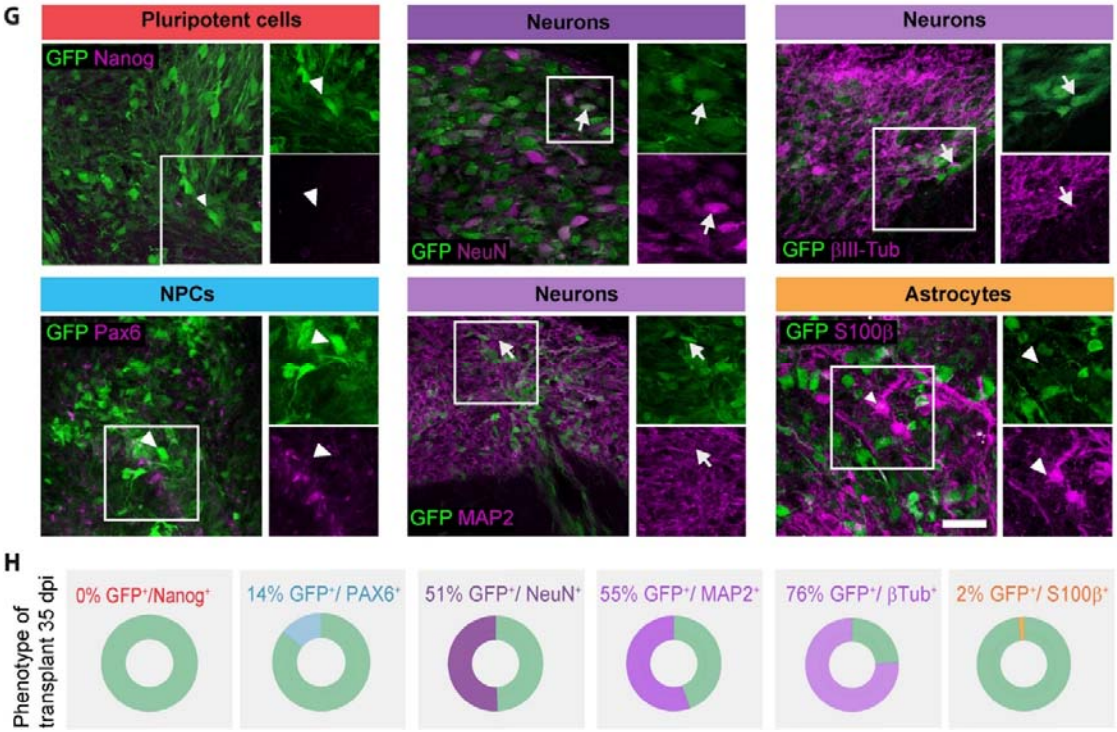
In our lab, we have established an RNA switch technology specifically targeting miR-302a-5p, a marker which is highly expressed in hiPSCs, to address the safety issue of residual pluripotent cells (Rust et al., 2022, J Transl Med, PMID: 36114512, **Reviewer Fig. 5**). Our differentiation protocol already produced highly pure NPC populations, yet we validated the RNA switch efficacy by adding 10% and 20% iPSCs to NPC cultures. The RNA switch successfully reduced the residual iPSC content to below 1%, as validated by flow cytometry. While we did not apply this RNA switch technique to the cells used in the current manuscript, we have discussed the potential in the manuscript's *Discussion* section.



Reviewer Figure 5: RNA switch for iPSC elimination. A: Schematic representation of RNA switch technology. RNA switch was applied to NPCs without iPSC supplementation (0% iPSC) and with supplementation of 10% and 20% iPSCs. B: Flow Cytometry dot plot of NPCs with negative control switch (upper row) and with RNA switch (lower row). C: Quantification of the remaining percentage of iPSCs in NPC cultures without (top) and with RNA switch (bottom). Boxplots indicate the 25–75% quartiles of the data. Each dot in the plots represents one cell culture well and significance of mean differences between the groups was assessed using Tukey's HSD. Asterisks indicate significance: *** $p < 0.001$

We would also like to emphasize that we have transplanted the same cell line in a previous study and evaluated the differentiation pattern 35 days post-transplantation (42 days after stroke, Rust et al., 2022, J Transl Med, PMID: 36114512, **Reviewer Fig. 6**). The results from that study revealed comparable ratios of differentiated cell types, with the majority of grafted GFP+ cells expressing neuronal markers such as Map2 (55%), βIII-Tubulin (76%), and NeuN (51%). Only 14% of transplanted cells expressed the neural progenitor marker Pax6, and a very small fraction (2%) were positive for the astrocytic marker S100β. Importantly, no Nanog-positive cells were detected, confirming the absence of residual pluripotent cells.

In the current manuscript, we similarly observed that Nanog was not detectable in any brain sections post-transplantation. A small number of transplanted NPCs (12%) expressed Pax6, while a significant proportion (78%) differentiated into MAP2-positive neurons. Only 10% of cells were GFAP-positive astrocytes. These results highlight the consistency of the differentiation outcome across independent experiments.



Reviewer Figure 6: Analysis and in vivo tracking of transplanted NPCs. G: Representative histological stainings of transplanted NPCs, 35d post transplantation in stroked Rag2$^{-/-}$ mice. Arrowheads indicate GFP-expressing cells that are negative for the respective maker. Arrows indicate positivity for marker and GFP. Scale bar = 50um. H: Quantification of cells positive for the respective marker and for GFP. BLI bioluminescence imaging, SNR Signal-to-noise ratio, WT wild-type, NSG: NOD scid gamma. Line graphs are plotted as mean ± sem

The authors state that “the majority of ligands were upregulated in the mouse stroke tissue” and cite their own BioRxiv manuscript (Weber et al., 2024) to describe transcriptional changes during stroke repair. Since most of these upregulated ligands are in host cells, how do the current findings relate to the stroke-induced transcriptional shifts in the previous study? A direct transcriptional comparison between stroke and stroke + NPCs appears necessary.

There are a few limitations in this comparison that we would like to point out first:

- This study focused specifically on graft–host interactions within the stroke tissue, whereas the Weber et al. bioRxiv manuscript examined transcriptional changes in the peri-infarct and stroke core regions compared to the contralesional site. Our references datasets (non-stroke) are therefore different. For this study the reference was obtained from Yao et al., 2021 Nature.
- In the current study, we performed the analysis at 42 days post-stroke, guided by the observed behavioral improvements. In contrast, Weber et al. analyzed tissue at 1 month post-stroke, aiming to capture the earlier repair phase.

That said, the reviewer raises an important point—there is indeed some overlap in upregulated cell–cell communication pathways across both studies, including PTPR, NEGR, SEMA, and COLLAGEN signaling. Similarly, we observe gene set enrichment changes that are consistent with stroke-induced alterations, such as reduced metabolic activity in neurons and changes in synaptic plasticity and axon guidance. However, due to the differences in experimental design, timing, and regional focus, we believe a direct transcriptional comparison may be limited in

interpretability. Still, this points to a promising direction for future studies specifically designed to compare stroke versus stroke + NPC conditions under more suitable parameters.

Reviewer 3 raised concerns about the study's novelty, and the authors do not provide compelling evidence to counter this critique. i.e., NPC transplantation has been extensively studied in permanent stroke rodent models.

We responded in the previous revision round in quite detail to the novelty aspects of our study. While we agree that NPC transplantation in stroke models has been studied before, we believe our work introduces several important advances, we revised our previous response:

1. First single-cell atlas of graft–host interactions at the long-term/chronic phase after stroke. We performed a single-cell transcriptomic analysis of over 45,000 cells, identifying major ligand–partner interactions mediating graft–host communication, including NRXN, NCAM, SLIT, NRG, PTPR, and NEGR signaling pathways.
2. Mechanistic validation *in vitro*. We functionally validated one of the predicted pathways by showing that human NPCs migrate toward SLIT2 in vitro, supporting the relevance of the SLIT–ROBO signaling axis.
3. Advanced functional recovery assessment. Instead of relying only on conventional behavioral tests, we used a deep learning-assisted analysis to detect subtle but biologically relevant improvements in motor function, providing a more sensitive and objective readout of graft effects.
4. Tranlational experimental set-up. Most previous studies use transient stroke models and transplant cells acutely (within 24 hours). Here, we used a permanent MCAO model and performed delayed transplantation at 7 days post-stroke, reflecting a more clinically relevant treatment window. We also provided a rationale with the same cells why 7 days post stroke is a good time point for transplantations approach (Weber et al., 2025, bioRxiv) and added this information to the revised manuscript.
5. Comprehensive analysis of brain and peripheral tissue changes: We expanded the study beyond brain tissue by analyzing neuromuscular junction structure, in addition to assessing inflammation, blood–brain barrier integrity, axonal spouting, and stroke volume etc.

Taken together, we believe that the combination of clinically relevant transplantation timing, long-term multi-level analysis, high-resolution functional assessment, single-cell profiling, and pathway validation provides a significant advancement over previous NPC transplantation studies.

It is unclear why SLIT2 was chosen as a proof-of-concept ligand for the in vitro assay. SLIT signaling is primarily associated with neuroinflammation rather than migration, and the transplanted cells exhibited minimal migration to the peri-infarct area.

We agree with the reviewer that SLIT signaling has indeed been extensively studied in the context of neuroinflammation. However, multiple studies also demonstrate the significant role

of SLIT signaling in regulating cell migration in various neurological contexts, including stroke and other injury models.

Specifically: (1) Kaneko et al. (2018) demonstrated that neuroblast migration toward stroke lesions involves Slit1-Robo2 signaling, wherein transplanted Slit1-overexpressing neuroblasts showed enhanced migration towards the lesion compared to control cells, by disrupting the cytoskeletal arrangement of reactive astrocytes (PMID: 30547091). (2) Enomoto et al. (2016) used a Boyden chamber assay, similar to our approach, to show that Slit2 induces migration of endothelial cells (HUVECs) through Robo1 receptor signaling (PMID: 26713366), highlighting the broader migratory function of Slit proteins beyond neuronal cell types. (3) Guerrero-Cazares et al. (2018) demonstrated that Slit2 exerts chemorepellent effects on the migration of human fetal neural progenitor cells, and that interference with Robo1 prevents their proper migratory pattern toward the olfactory bulb, further underscoring the role of Slit-Robo signaling in directional migration of neural progenitor cells (PMID: 28406573). And (4) Deboux et al. (2020) showed that Slit1 regulates the ectopic migration of subventricular zone-derived neural progenitor cells in response to demyelination injury, suggesting its crucial involvement in NPC mobilization and oligodendrocyte renewal at injury sites (PMID: 32670024).

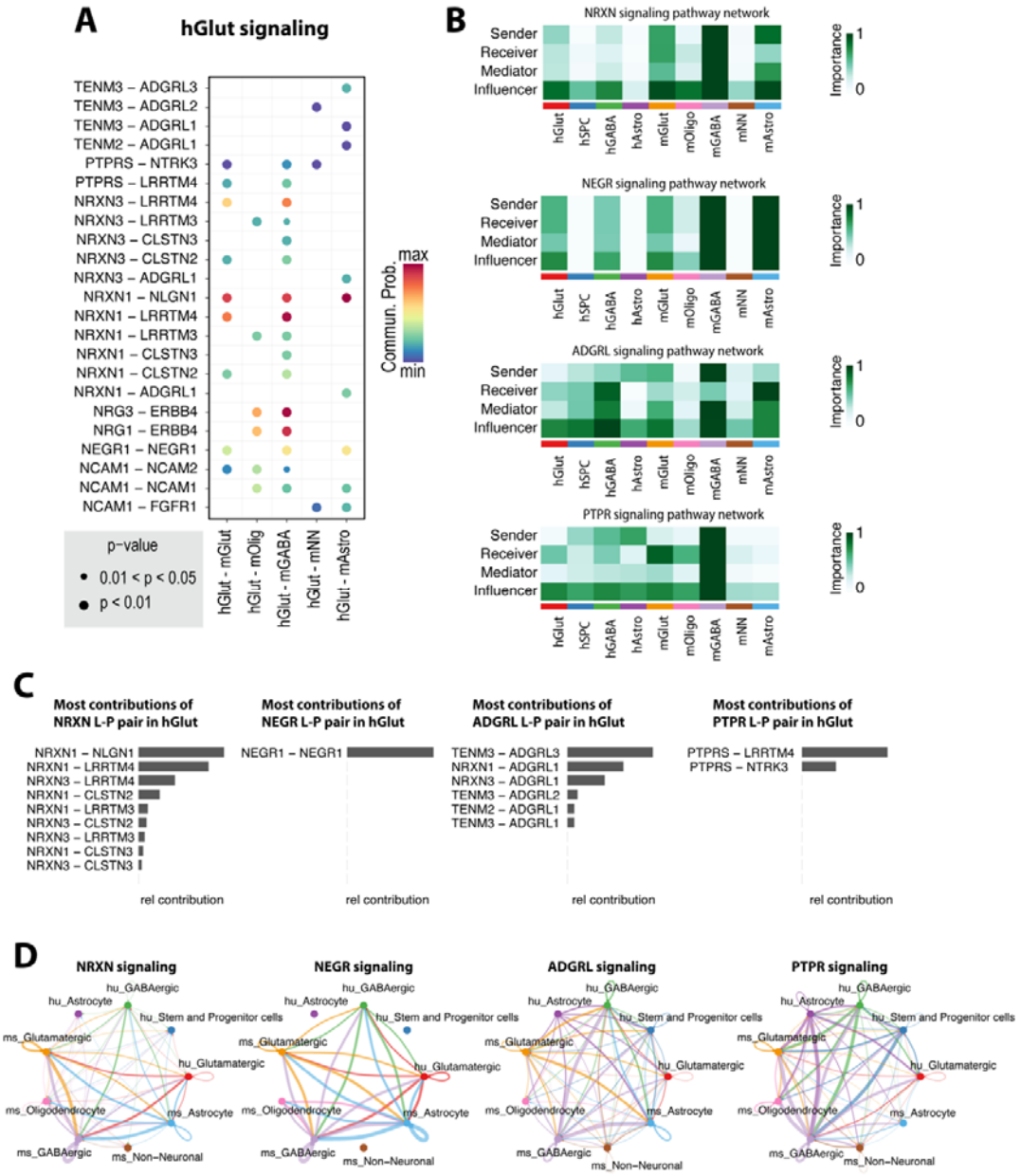
Additionally, we selected SLIT2 for our *in vitro* migration assay primarily because recombinant purified, high-quality SLIT2 protein is commercially available. Furthermore, our laboratory had already established and optimized the Boyden chamber migration assay, making SLIT2 an ideal ligand choice for this proof-of-concept experiment.

In their rebuttal letter, the authors acknowledge that BDA tracing alone does not confirm axonal projection directionality. The main manuscript must be revised accordingly, particularly in the second paragraph of the “Increased neurite outgrowth and endogenous neurogenesis in NPC-receiving animals” section.

We agree, and we changed the sentence to “NPC-grafted animals showed increased axonal connection density within the IBZ, compared to the vehicle group (1.83% ± 0.342%; NPC: 3.4% ± 0.36%, p = 0.0055, **Fig. 3F**).”

While graft-derived GABAergic neurons communicate with stroke-injured host cells, it is well established that stroke disproportionately affects GABAergic interneurons compared to glutamatergic neurons. Given that an equal proportion of NPCs differentiate into glutamatergic and GABAergic neurons, it is important to determine how graft-derived glutamatergic neurons interact with host cells and which pathways they influence.

This is an important point. We performed an entirely new analysis of glutamatergic neurons and their communication with the host tissue. We indeed observe that glutamatergic grafts communicate via distinct pathways with the host cells. Among others, pathways were predicted to contribute to outgoing communication of hGlut including neuronal growth regulator (NEGR), adhesion G protein-coupled receptor-like (ADGRL), and protein tyrosine phosphatase receptor (PTPR) signaling pathways (which have been previously described in cell adhesion and communication). Please see our analysis here (reviewer Fig 7, Suppl. Figure 26).



Reviewer Fig. 7: Molecular crosstalk between Glutamatergic cell grafts and host. (A) Major ligand-receptor pairs that contribute to the signaling sending from human (hGlut) cells to host cell types from stroked tissue **(B)** Heatmap show the relative importance of each cell type based on the computed four network centrality measures (sender, receiver, mediator, or influencer) of major hGlut-related pathways: NRXN signaling, NEGR signaling ADGRL signaling, and PTPR signaling. **(C)** Barplots showing major ligand-partner pairs with most relative contributions in hGlut cells. **(D)** Number of cell-cell interactions between individual cell types from graft and host for pathways NRXN, NEGR, ADGRL and PTPR.

Methods sections should be reviewed. As an example, DAB experiments are not included. Make sure all new and old methodologies are included in this section.

We apologize for the missing details in the *Methods* section, and we added the corresponding protocol/steps to the section. We further adjusted the *Antibody list* and added all the missing antibodies.

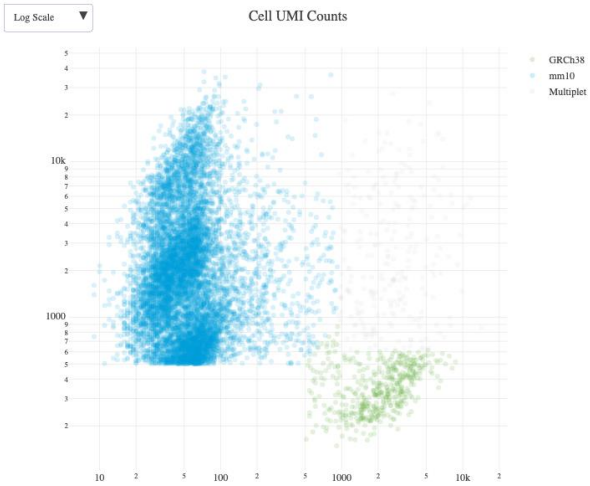
Reviewer #4 (Remarks to the Author):

The study by Weber et al presents a detailed investigation into the effects of transplanted neural progenitor cells (NPCs) on stroke pathophysiology. One major component of this study is to explore graft-host interactions using snRNA-seq. While this approach is innovative and potentially informative, several concerns arise, such as the lack of methodological details and validation, which may undermine robustness of the conclusions.

We thank the reviewer for his feedback and recognizing that our work presents “a detailed investigation into the effects of transplanted neural progenitor cells (NPCs) on stroke pathophysiology” and that our approach is “innovative and potentially informative”. We now carefully added the requested methodological details and validations to support our data in the response below, new data and explanations are highlighted in yellow.

1. It is unclear how the host and transplanted cells were separately isolated for sequencing. Cell sorting?

We isolated nuclei from microdissected tissue samples without prior sorting and performed species-specific transcriptomic separation using dual-species reference genomes (human: GRCh38, mouse: mm10 2020-A) with Cell Ranger v7.1.0 (10x Genomics). Reads were aligned to a combined reference allowing clear discrimination between mouse host and human graft-derived cells based on species-specific mapping with high accuracy (as previously described e.g. here with an accuracy of >99%: PMID: 29304755). We chose not to use cell sorting to preserve potential host-graft interactions and the more native microenvironment, which we believe in such a study is very important. We added this information in more details to the methods “Single-nucleus RNA sequencing” and “Clustering and annotation of mouse and human cell types”. Our QC report confirms high accuracy in separation between mm10 /GCH38 (reviewer Fig 1)



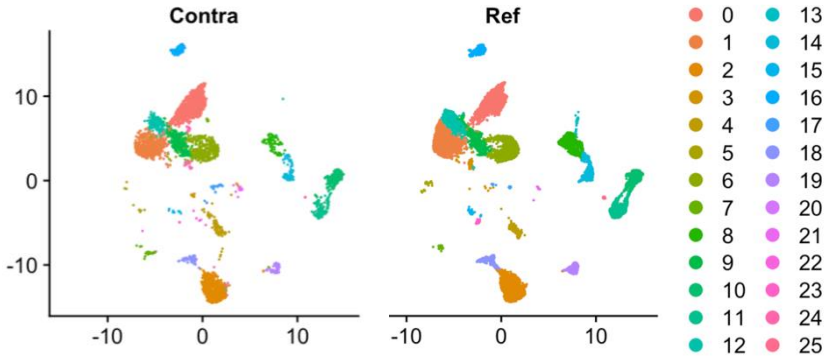
Reviewer Fig 1: QC from mouse and human species-specific transcriptomic separation.

2. The Methods section indicates that non-stroke mouse cortices were collected and sequenced. However, such data do not appear to be presented or used as a reference. Instead, the authors relied on a previously published dataset for reference, which may not be optimal due to potential technical variations. This needs to be clarified.

We thank the reviewer for highlighting this inconsistency and have clarified the Methods sections accordingly. The study was designed to primarily investigate the graft-host interactions in the stroke environment, and therefore no samples were processed from contralesional sites. To obtain a robust cell-type annotation framework that already incorporates multiple laboratories and technical batches, we aligned our stroke dataset to the well-curated brain atlas (of non-stroke mouse motor cortex, which is the region we introduced our strokes, PMID: 34616066). We first transferred cell-type labels with Seurat v5 mapQuery, followed then integrated the datasets using Seurat v5 reciprocal PCA + Harmony batch correction. This is a well-established workflow that which preserved biological variance for cross-study comparisons.This is now added in more detail to the methods.

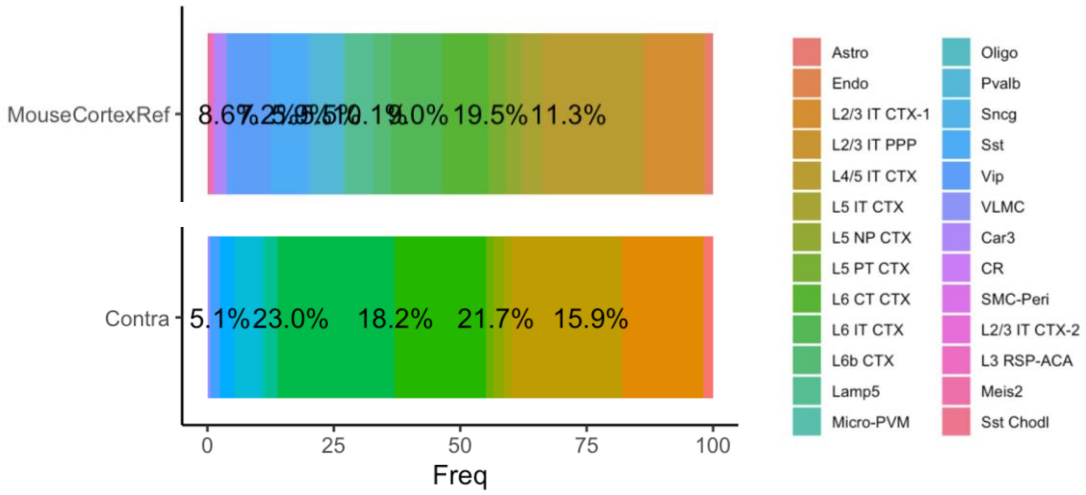
As a proof of concept, we show here some validation experiments between the used reference dataset (PMID: 34616066) and a dataset from a more recent study performed in our lab (PMID: 40251566), cluster identities and many differentially expressed genes/pathways were consistent between those datasets, demonstrating robustness despite cross-study integration (see some examples in reviewer Fig 2-4):

The UMAP clustering shows similar Seurat cluster distribution and similar cell types:



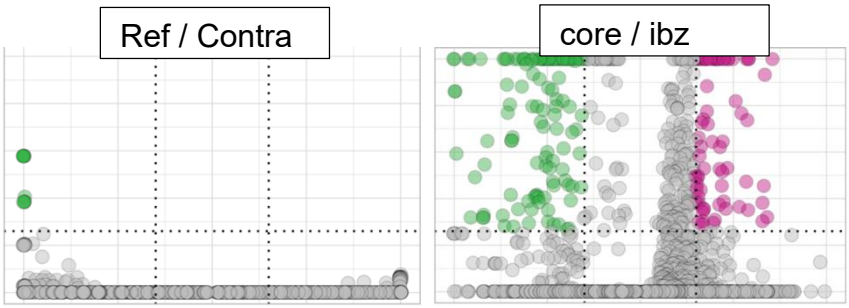
Reviewer Fig 2: Integrated UMAP embedding of Seurat cluster between contralesional dataset (Contra, from a recent study performed in our lab, PMID: 40251566) and reference dataset (PMID: 34616066, used as a reference in this study).

Also assigned sub-cell proportions were comparable between contralesional and reference datasets:



Reviewer Fig 3: Assigned cell type frequencies are similar between Contra (from a recent study performed in our lab, PMID: 40251566) and reference dataset (PMID: 34616066, used as a reference in this study).

To validate that gene expression differences in individual cell types were lower between reference and contralesional dataset than e.g., between stroke core and ischemic border zone (ibz), we show here a representative volcano plot for one cell type:



Reviewer Fig 4: Volcano plots showing gene expression changes. Left: Minimal differences between the reference dataset and Contra (PMID: 40251566). Right: Marked transcriptional changes between stroke Core and ischemic border zone (IBZ).

We also want to clarify that the primary focus of this study was on graft–host interactions. The analysis of host-specific gene expression changes was added in response to a reviewer's suggestion and, while informative, was not part of the primarily original study design. We would prefer not to include the here shown analysis as supplementary data as it might be confusing for the reader, and would like to acknowledge that these changes, although valuable, are not the main focus of the manuscript. The Discussion now explicitly acknowledges these limitation of technical variation.

3. The authors should provide unbiased clustering data files, containing top differentially expressed genes (DEGs) for each cluster. Such data are important for interpreting their analyses.

We added an unbiased clustering data file for DEG for each cluster containing top differentially expressed genes for each cluster for the mouse host and human graft now in Supp. Table 4 and Supp Table 5.
(Additionally, we also have two tables showing DEG and GSEA between individual host cell types before and after stroke Supp. Table 2-3).

4. Suppl. Fig. 17. It is unclear how reliability and prediction analyses were performed.

Reliability and prediction scores in Suppl. Fig. 17 were generated with Seurat v5 MapQuery/TransferData workflow. Briefly, anchors were computed between our stroke dataset (query) and reference atlas (reference); cell-type labels were then transferred, and prediction.score.max (range 0–1) served as a reliability metric for each cell that was plotted for each cluster. We added this information to the methods.

5. Only 1149 nuclei from transplanted cells were sequenced. Were these cells pooled from 5 mice as well? The cell number seems relatively low.

Yes, those 1,149 nuclei represent the pooled graft-derived nuclei recovered from all five transplanted mice, which passed the strict quality control. The absolute number is modest because the human grafts are small relative to host tissue and we intentionally avoided FACS sorting to preserve native ligand-receptor profiles, which can be altered by surface-antibody binding and other extended processing. Although yield was lower, sequencing saturation exceeded 60% and we retained >1,000 high-quality graft nuclei with a mean of 25’233 reads per nucleus (and 1’500 – 2’000 median genes per nucleus) similar to previous studies (e.g. PMID: 33618539), providing sufficient statistical power for downstream differential-expression and interaction analysis. We added our rationale to avoid sorting to the methods under “Single-nucleus RNA sequencing” and “Clustering and annotation of mouse and human cell types”.

6. For Supplemental Table 2, how was this DEG analysis performed? It seems that a public reference dataset was used as a reference. If so, again, this comparison is not convincing.

Differential expression in Supplementary Table 2 was calculated with Seurat’s FindMarkers (Wilcoxon test, Bonferroni FDR < 0.05) after mapping our nuclei onto the public brain atlas (non-stroke mouse motor cortex, which is the region we introduced our strokes, PMID: 34616066) via MapQuery + Harmony batch correction to minimise batch effects before testing. Please also consider our more detailed response in Point 2. We expanded now on the details in the methods.

7. Fig. 7 F. Given the emphasis on GABAergic neurons, it would be important to include common markers such as GAD65 (GAD2) and GAD67 (GAD1) for immunostaining.

We appreciate the suggestion and fully agree that GAD65/67 are important pan-GABAergic markers. We did test GAD67 (GAD1) immunostaining, but the signal

appeared mostly as punctate presynaptic labeling, which didn't reliably co-localize with the human nuclear marker HuNu. This made it hard/impossible to confidently assign those signals to grafted cells; therefore we focused on VIP, SST and PV, whose somatic labelling was easier to colocalize with HuNu-positive in grafts. Also, we found it useful to see whether we find specific subtypes of GABAergic neurons. We believe it is relatively well established that VIP, SST and PV are important GABAergic subtypes that account to the majority (some studies say nearly to 90%) of GABAergic neurons in the cortex (e.g., PMID: 21154909). This approach also allowed us to assess whether specific GABAergic subtypes were represented in the graft.

8. Another major concern is related to their data regarding graft-host interactions. The authors conducted an extensive analysis of cell-cell communication using several bioinformatics tools. While this can be valuable, such analysis can sometimes lead to overinterpretation, particularly when not supported by repeated sequencing or validation experiments. Here, it appears that only a single pooled snRNA-seq sample was analyzed for each group. Although an in vitro study exploring Slit2 signaling was included as a proof-of-concept, its relevance is questionable because the snRNAs-seq study used cells one month after transplant, when most transplanted NPCs showed mature properties. An in vivo validation would provide stronger support for their conclusions.

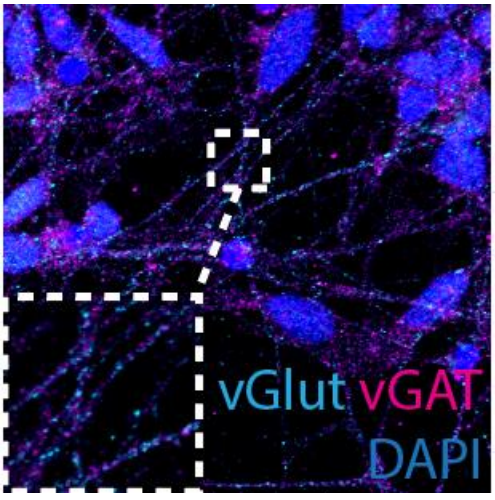
We acknowledge the limitations of interpreting cell–cell communication from a pooled snRNA-seq sample. However, the dataset includes biological replicates from five transplanted mice, enabling robust statistical inference using CellChat.
We validated one of the top predicted pathways (Slit2–Robo) in vitro, and while our grafted cells show mature properties at one month, Slit–Robo signaling is well-documented in post-stroke tissue, supporting its relevance.
Also Slit-Robo pathways have been reported in NSC in regulating their proliferation, migration/guidance and differentiation (e.g., PMID: 30547091). We now toned down the inferred interactions and added a sentence with this limitation and outlook for future studies in the discussion. Of note, *in vivo* interference with Slit-Robo pathways in cell grafts is planned in future studies (if we receive the relevant funding), but is beyond the scope of this present work.

REVIEWER COMMENTS
Reviewer #4 (Remarks to the Author):

The detailed response is greatly appreciated. However, given the focus on GABAergic neurons, staining with a pan-GABAergic marker still seems necessary. The authors note that “VIP, SST, and PV are important GABAergic subtypes that account for the majority (with some studies suggesting nearly 90%) of GABAergic neurons in the cortex”; however, these three subtypes together represent only ~15% of all $HuNu^+$ cells in their study (Fig. 7F,G), which is substantially lower than the ~44% revealed by the snRNA-seq analysis. This discrepancy needs to be clarified.

We thank the reviewer for their feedback and for highlighting this important point. We fully agree that further discussing the discrepancy between our snRNA-seq analysis and the histology will be helpful. Below we discuss the potential reasons for the observed discrepancy and describe our practical limitations of adding a pan-GABA staining:

1. Specificity of available pan-GABA antibodies: We tested three widely used clones during early method development in vitro and in vivo; but all antibodies produced non-specific staining in pilot sections. The only related antibodies that reliably worked for us were vGlut and vGAT (that label the vesicular Glut/GABA transporters, **see Reviewer Fig 1**). However, since these markers exhibit punctate vesicular staining, we could not use them for any useful quantifications in vivo.



Reviewer Fig 1: Representative image of *In vitro* differentiated neurons from neural progenitor cells after 14 days stained for vGlut (cyan) and vGAT (magenta), counterstained with DAPI (blue). The image illustrates that quantification of co-localization with vGAT is not feasible due to the punctate vesicular staining pattern.

Also, some of the discrepancy might also be explained as we stained for GABAergic subtypes (PV, VIP, SST) in immunostaining, while the transcriptomic data reflects all GABAergic-like neurons, including unassessed subtypes and immature or low-expressing populations. We now mention these limitations in the revised discussion.

2. Protein (histology) versus transcript detection (snRNAseq)

We also highlight in the revised Discussion that transcript abundance does not always predict protein levels, particularly for neuronal transmitter systems where post-transcriptional control and peptide transport may influence the expression. This additionally could contribute to the observed difference between the histological and RNAseq readouts.

We added the following limitation to our study to reflect both mentioned points (limitation paragraph of discussion before summary):

"Notably, the proportion of graft-derived nuclei classified as GABAergic by snRNA-seq (~44% of $HuNu^+$ cells) exceeded the fraction we detected histologically with PV, VIP and SST (~15 % of $HuNu^+$ cells). This gap may reflect immature or non-canonical interneuron subclasses that lack these peptide markers at the protein level, and may also be influenced by the known differences in mRNA and protein abundance.[1]"

References:

1. Wegler, C. *et al.* Global variability analysis of mRNA and protein concentrations across and within human tissues. *NAR Genomics and Bioinformatics* **2**, lqz010 (2020).