nature portfolio

Peer Review File

A microengineered 3D human neurovascular unit model to probe the neuropathogenesis of herpes simplex encephalitis

Corresponding Author: Professor Jianhua Qin

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This is an interesting study from Zhang and colleagues developing a novel model of the neurovascular unit and using it to investigate the effect of herpes simplex. Despite being novel on several aspect and representing a translational alternative to other models, I have the following concerns:

1. How did the authors decide on the cell density? While the endothelial cells and the microglia are confluent it seems that the neurons and astrocyte are very sparse. In my opinion there is no physiological reason for that. What is the reason of the authors? The ratio used here with more astrocytes than neurons is also not physiological relevant as typically brain as a ratio of 1:2 or even 1:3 astrocyte:neuron.

2. It is not clear from the staining presented in the manuscript that the cells have the appropriated morphology and structure. in particular:
Endothelial cells: the Claudin 5 expression seems to be located intracellularly instead of being at the junction site. A closer view of the endothelial cells cultured with and without the other cell type would help to clarify the localization of claudin 5.
Astrocytes: at the NVU, astrocytes express Aquaporin in their endfeet facing toward the endothelial cells. Are the astrocyte polarized in this system?
Neurons: they look very round and it does not seem that they form synapses. A synapsis staining is important to confirm the neurons morphology. An additional calcium activation experiment would also prove the functionality of the neurons.

3. A consequence of long term herpes infection is the deposition of amyloid plaques within the brain. Several in vitro system demonstrate the possibility to measure Ab secretion and deposition in vitro even after a few weeks of culture. Is this system able to recapitulate this pathological feature if the cells are kept longer in culture? A quantification of Ab in the neuron-astrocyte chamber would help answering this question.

4. The experiments of infection in the absence of microglia raise question. It is expected that in the case where there are less cells in the system the remaining would be more infected. A proper experiment would have been to replace the microglia with other cell types (astrocyte-neurons for exemple) or to perform the infection directly in the astrocyte-neuron chambers. The latest would also be more relevant as Herpes virus might enter through a retrograte axonal transport.

5. Authors should include a section in the discussion where they describe the limitations of the model: non exhaustive list: no direct contact astrocyte neuron, cell line for microglia and neurons, absence of pericyte, absence of flow, use of matrigel which is rich in laminin, etc.

6. The migration of the microglia is interesting but how does the authors explain it? is due to migration or division of the cells. The latest would not be physiologically relevant however it does not seems that there are less cells in the microglia chamber to support only migration.

6. it is not clear if the EM images are performed in the chips or in monoculture. The staining quality could be better

Reviewer #2

(Remarks to the Author)
Min et al. present a novel brain-on-chip model, which they exhaustively examine using a model of HSV-1 infection. HSV-1 encephalitis remains a significant challenge in the care of children and immunocompromised individuals, with potentially devastating consequences. Studying this condition in mice remains insufficient. Human iPSCs, multicompartment, multi-cell types, and modular organoids represent the next frontier for accurately studying the initial phases of virus infection in the brain, identifying drugs, and developing protective treatments. Viral encephalitis, in general, is a major scourge, and the same system could be applied to all of them.
The system proposed by Min et al. features several unique elements, integrating microengineering and cell biology innovations. Their multimodular system comprises a combination of cell lines and primary human cells, organized into compartments representing the BBB (HBMEC and human astrocytes), microglia, and brain (neuronal cell line and human astrocytes). This arrangement facilitates the guided organization of a multi-layered organoid, which they clearly demonstrate using confocal microscopy and immunostaining. Figure 1 is very convincing. For clarity, the authors may want to add another dotted box showing the location of the cut for the HBMECs corresponding to Fig. 1d. The FITC-dextran sulfate result is very strong.
HSV-1 infection is carefully monitored at multiple levels, including virus production, cell death, and inflammatory cytokine production, faithfully recapitulating the known initial stages of HSV-1 infection (Fig. 2).
A strong aspect of this system is its modularity. For example, they conducted an elegant microglia cell migration assay in response to HSV-1 infection using cells transduced with an RFP lentivirus. They then interrogated intracellular pathways activated in these cells using scRNA sequencing (Fig. 3).
The model also studied PBMC activity in the blood channel, including transvasation through the HBMEC barrier following HSV-1 infection, representing a significant advance. The PBMCs were analyzed for chemotactic activity, and scRNA analysis of HBMECs showed dramatic changes. The data demonstrate extravasation of different cell types, including T cells, closely reflecting key aspects of human infection (Fig. 4).
The authors further examine the mechanisms of infection, showing autophagy dysregulation in astrocytes and microglia induced by HSV-1 infection on the NVU model. Using KEGG analysis of RNA sequencing data 3 days post-HSV-1 infection, electron microscopy (showing autophagic vacuoles), and confocal microscopy in different conditions (rapamycin), they demonstrate the effect of HSV-1 on autophagy and its reversal by rapamycin (Fig. 5).
Implicating autophagy as an early mechanism of dysfunction caused by HSV-1, the authors tested activators of autophagy, such as rapamycin and others, showing that activation of autophagy limits HSV-1 replication. Control of viral replication correlates with increased cell viability (Fig. 6).
Finally, they examine changes imparted by autophagy activators in microglia. Fig. 7 presents data showing decreased HSV-1 infection and restored autophagic flux in infected microglial cells treated with autophagy stimulators.
The new NVU model appears very promising as a valid alternative for studying human brain infection. However, certain questions remain:
1. In figures 1 and 2, do we know the number of cells for each of the different cell types? How close or far are these numbers from those in the human brain? This is also relevant in Fig. 2 to understand whether the percentage of infected cells represents HSV-1 cell specificity or relates to cell abundance.
2. The authors show key aspects of HSV-1 infection, including microglia migration and extravasation of immune cells, opening tremendous opportunities for study. However, how valid is the model? Although the cellular analysis is convincing, the RNA sequencing results are puzzling for observations at 3 days post-infection. Type 1 IFN is a key cytokine in HSV-1 pathogenesis, as shown in patients with inborn errors in the IFN pathway (e.g., TLR3, UNC93B, others). The defect resides mainly in neurons, which become extremely susceptible to HSV-1 infection. Could the authors comment? This point could be raised in the discussion.
3. Another question is the reproducibility of the model. The number of chips used in each experiment is relatively low (n=3). How many experiments were conducted? How have the authors tested reproducibility? This includes the number and proportions of cell types.
4. Additionally, how sensitive is the system? What is its capacity to gauge differences in cytokine levels and infections with different multiplicities of infection (m.o.i.) for example?
Regardless, while autophagy as a targetable pathway for the control of HSV-1 infection and rapamycin to counter HSV-1 has been done, the mechanism was not elucidated.
In sum, the paper presents a new approach to model a brain on a chip, using in-depth analysis of HSV-1 infection to validate the system. The model recapitulates key features of HSV-1 infection. The system's modularity allows for probing the functions of PBMCs, microglia, astrocytes, and other cell types.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors address all my concerns.

Reviewer #3

(Remarks to the Author)

While the authors have utilized 3d human neurovascular unit (NVC) model and their application to HSV-1 is novel, the reliability of model and design is concerned.
Major Comments:
1. The authors should clearly justify the rationale for the cellular ratio used in the model. Although the initial and final number of SH-SY5Y cells are higher than other cell types, this should be explicitly addressed. A rough estimation of the final cell numbers, based on previous calculations of proliferation rates, should be provided. To accurately determine cell numbers and ratios, it is essential that the authors perform FACS analysis with proper quantification.
2. The manuscript relies predominantly on immortalized cell lines (particularly SH-SY5Y neuroblastoma cells), which differ significantly from mature, functional primary human neurons. This introduces concerns regarding the ability of the model to faithfully recapitulate the composition of the mature brain, and raises issues related to excessive proliferation at this stage, which should not occur in the context of a mature neuronal model. The authors should consider replacing these cell lines with human pluripotent stem cell-derived neurons or mature primary human cells, including neurons. Additionally, it is crucial to verify that each cell population expresses essential neuronal markers, such as Tuj1 or MAP2, as indicated in Figure 1b and Figure 6b. Moreover, the microglial morphology and activation states (e.g., as shown in PMID: 32221280) should be revisited, as these are not clearly demonstrated in the current manuscript, especially in relation to the microglial data presented.
3. There is a persistent issue with categorizing astrocytes and neurons within the same group, except for in Figure 1. While the manuscript focuses primarily on microglia as inflammatory cells, astrocytes should be analyzed separately from neurons. Astrocytes are also key players in neuroinflammation, and their inclusion as part of the neuronal population may obscure important insights.
4. In Figure 1, the overall fluorescence image of the 3D human NVU model should be shown. It is unclear how the authors' method generates a reliable model with well-distributed and appropriately formed 2D and 3D cell cultures in co-culture. Several figures, including the MOCK group in Figure 2f, show incomplete DAPI staining, suggesting issues with model integrity. In particular, Figure 2f shows lower DAPI staining (indicating fewer cells) in the MOCK group compared to the HSV-1 group, which complicates the interpretation of the cell ratio data in Figure 2g. A clearer, more consistent model should be presented to support robust conclusions.
5. In Figure 2b, the representative image of 7 dpi is inconsistent with the quantification shown in Figure 2c. This discrepancy should be addressed by performing at least three technical replicates with three biological replicates to ensure data reliability.
6. In lines 145-148, the authors mention decreased BBB integrity and increased permeability but do not provide direct evidence of HSV-1 infection in HBMECs in Figure 2c. This should be demonstrated to correlate the observed changes in BBB integrity with the infection process.
7. Figure 3e and the associated data in lines 188-199 are not convincing. The lower expression of neuronal markers in the presence of microglia (compared to their absence) suggests neuronal death, not protection. Furthermore, the HSV-1 infection ratio appears similar in the presence and absence of microglia, which contradicts the data in Figure 2c showing that microglia are more susceptible to HSV-1 infection. This inconsistency should be addressed.
8. There is a lack of consistency regarding the time points used. Some data are based on 3 dpi (e.g., Figure 4), while others use 7 dpi (e.g., Figure 1). The authors should clearly explain the rationale for choosing different time points and demonstrate the logistics behind this decision.
9. In Figure 4a, the number of down-regulated genes (415) is relatively similar to the number of up-regulated genes (634). The authors should present the top 10 up- and down-regulated genes to provide a more focused analysis.
10. In Figure 4l-n, the fluorescence expression of green and red channels is not clearly distinguished. The quantification in Figure 4o should be based on at least three technical replicates to improve the robustness of the data.
11. In Figure 5a-b, the use of mitophagy-animal and autophagy-animal data raises the question of whether these findings are directly applicable to human cells. The authors should clarify how these animal model data are relevant to the human-based 3D NVU model used in the study.
12. Figure 6 presents a key question regarding drug delivery. Since drug delivery across the blood-brain barrier (BBB) is a significant challenge, drugs should be added to the vascular (blood) side of the model, not the brain side, in order to more accurately simulate real-world drug delivery conditions. This experimental setup would allow the authors to evaluate how efficiently drugs can penetrate the BBB under MOCK vs. HSV-1 infection conditions.

Overall, the manuscript lacks sufficient technical and biological replicates to provide a convincing level of reproducibility. The authors should consider increasing the number of replicates to ensure the reliability of their findings.

Minor comments:
Figure 1b missing scale bar.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
Overall, the authors have effectively addressed the queries raised in the initial draft. However, I would like to offer the following recommendations for further improvement:

1. The authors have satisfactorily addressed the question raised in the initial draft regarding the choice of cell sources.

However, it remains critical to explicitly justify the rationale behind using a 10:1 ratio of astrocytes to SH-SY5Y cells, as stated in lines 444-445. A clear explanation for this particular cellular proportion is essential for the scientific robustness of the model. I recommend a more comprehensive discussion on why this specific ratio was selected (considering this ratio is not common in neuroscientific publication to have less neuron than other populations), particularly in relation to the experimental objectives and underlying biological relevance.

2. In alignment with previous feedback, the fluorescence imaging data could be enhanced, particularly by utilizing more advanced imaging techniques to acquire data from 3D samples. Notably, in Figures 2f and 4l-n, there appears to be a discrepancy in the DAPI staining counts, which raises concerns regarding the potential variation in cell numbers between experimental groups. To ensure consistency and accuracy in the data, I suggest revisiting these images or providing additional clarification on how these discrepancies may have arisen.

3. In reference to my prior comment regarding the data transparency (comment 5), I strongly recommend the inclusion of raw data, along with exported data for fluorescence images, for all replicates associated with the figures.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This is an interesting study from Zhang and colleagues developing a novel model of the neurovascular unit and using it to investigate the effect of herpes simplex. Despite being novel on several aspect and representing a translational alternative to other models, I have the following concerns:

We thank the reviewer for the positive comments and valuable suggestions on the manuscript.

1. How did the authors decide on the cell density? While the endothelial cells and the microglia are confluent it seems that the neurons and astrocyte are very sparse. In my opinion there is no physiological reason for that. What is the reason of the authors? The ratio used here with more astrocytes than neurons is also not physiological relevant as typically brain as a ratio of 1:2 or even 1:3 astrocyte: neuron.

In this work, the cell density was carefully considered based on several aspects, including the physiological structure of NVU in human brain, research focus in this study, and the different proliferation rates of various cell types used in this work.

As brain microvascular endothelial cells are primary anatomical component of the BBB and line the brain's capillaries, we seeded HBMECs in the vascular side to mimic a confluent endothelium. Regarding to the microglia, the reason we cultured them in a separate chamber in 2D condition is to facilitate the observation of cellular dynamic migration towards the astrocytes/neurons region. This is also one of the advantages in using this device.

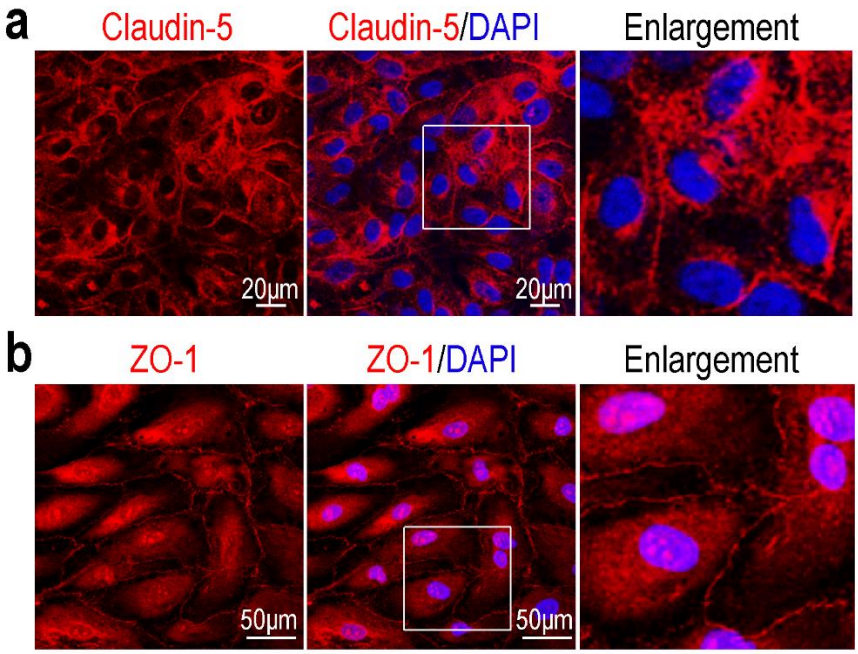
Regarding to astrocytes and neurons, we cultured them in a 3D condition in order to mimic the 3D microenvironment of brain parenchyma. This might lead to more sparse distribution of the cells as compared to those cells (brain endothelial cells and microglia) cultured in 2D conditions.

With regard to the ratio of astrocytes/neurons on this model, we choose it based on the consideration of in vivo-like physiological brain microenvironment, the stability of the experimental system on chip and the proliferation rates of diverse cells used in this work. Previous studies revealed, the glia/neurons ratio in the whole human brain is close 1 [1-3]. However, the glia/neuron ratio varies dramatically among distinctive brain regions, and the ratio is ~3.7 in cerebral cortex, ~0.2 in cerebellum, and ~11.3 in the rest of brain [1]. Clinically, several studies reported HSV-1 mainly attacks cortical areas of the brain in HSE patients, including temporal lobe, frontal lobe and limbic system [4, 5]. Besides, considering that astrocytes are the most abundant cells among glial cells [6, 7], we set the cell seeding ratio of astrocyte/neuron to 10 in the NVU chip after optimization of the experimental system. As SH-SY5Y cells has a higher proliferation rate than HA cells, the final ratio of astrocytes/neurons reaches to ~3 at day 3 after cell seeding on this NUV model.

2. It is not clear from the staining presented in the manuscript that the cells have the appropriated morphology and structure. in particular:

Endothelial cells: the Claudin 5 expression seems to be located intracellularly instead of being at the junction site. A closer view of the endothelial cells cultured with and without the other cell type would help to clarify the localization of claudin 5.

Thanks for the points. We indeed detected both cell periphery and cytoplasm localizations for Claudin-5 in brain endothelial cells on the NVU model (a panel in the below figure). Besides, we performed additional experiment and examined ZO-1 (another tight junction protein) in brain endothelium (b panel in the below figure). Similar to Claudin-5, although ZO-1 shows a clear localization at the cell junction site, it also has an obvious cytoplasm distribution. Actually, it is very common that in vitro culture inevitably leads to lower or changed expression of tight junction proteins in brain endothelial cells, including HBMEC (used in this study), hCMEC/D3, TY10, and BB19 [8, 9].



Recently, many groups utilized iPSC-derived BMECs (iBMECs) to construct BBB or NVU models. Although these iBMECs show relative higher expressions of tight junction proteins and TEER values than brain endothelial cell lines, much evidences revealed these iBMECs are not pure endothelial cells, with the lack of key features and expression of endothelial cell markers. It might be more like an epithelial lineage [10].

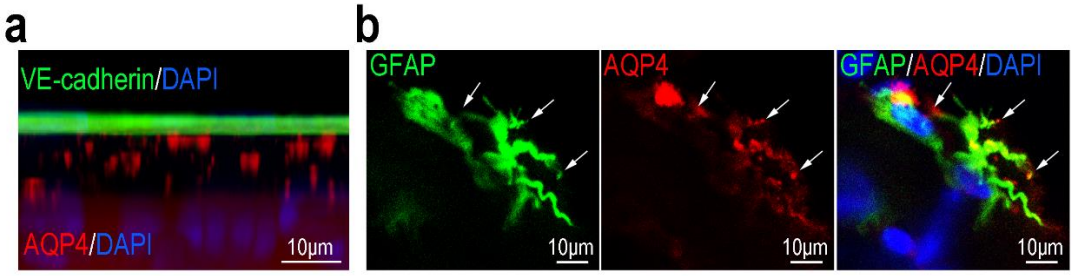
Therefore, after comprehensive consideration, we chose HBMECs to construct the NVU model in this study. Even though the expressions of tight junction proteins were lower, and some tight junction proteins were localized in the cytoplasm.

Astrocytes: at the NVU, astrocytes express Aquaporin in their endfeet facing toward the endothelial cells. Are the astrocyte polarized in this system?

We thank for the valuable suggestion. Aquaporin-4 (AQP4) is a vital water channel protein which showed concentrated expression at the end-feet of astrocytes near vasculature in human brain [11]. Ahn and colleagues have presented a very nice work regarding a BBB chip, on which polarized aquaporin-4 (AQP4) distribution was detected on the end-feet facing toward the

endothelial cells [11]. It's still difficult to reproduce the feature of polarized localization of AQP4 *in vitro* in most of studies reported till now.

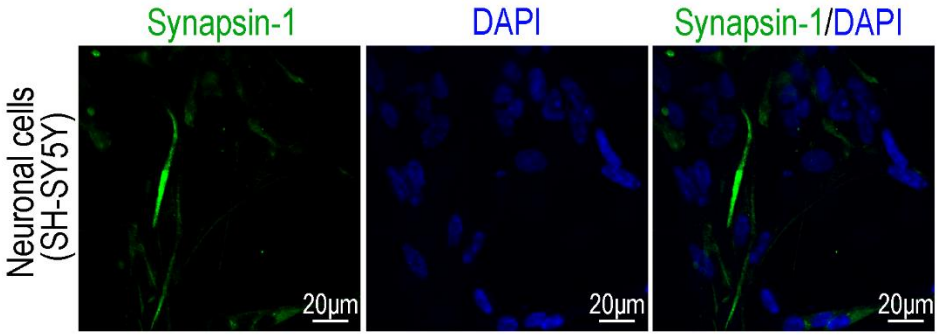
As suggested, we have tried best and performed additional immunostaining analysis to detect the subcellular localization of AQP4 on the NVU model. As Sup Fig. 3a showed, some AQP4 puncta were observed and they were located facing toward the brain endothelium. In 2D micrographs, it showed some AQP4 puncta were located at the end of the astrocytic processes (Sup Fig. 3b), indicating the partially polarized localization of AQP4 on the NVU model.



Neurons: they look very round and it does not seem that they form synapses. A synapsis staining is important to confirm the neurons morphology. An additional calcium activation experiment would also prove the functionality of the neurons.

According to the reviewer's suggestion, we examined the synapses of neuronal cells (SH-SY5Y) by staining synaptic marker (Synapsin-1). As shown in Sup Fig. 12, Synapsin-1 was expressed in the neuronal cells. However, the Synapsin-1 showed diffused distribution across the whole cell body and neurite, without Synapsin 1-positive foci detected on the long neurite, indicating no mature synapse was formed between the neuronal cells. Also, a calcium activation experiment was performed, while no functional calcium signals were detected in the neuronal cells.

We think these defects mainly resulted from the SH-SY5Y cells we used in the NVU model. SH-SY5Y cells is an immortalized neuroblastoma cell line, which is widely used as neuronal cells in in-vitro studies. Although it expresses some neuronal biomarkers, it lacked some vital neuronal features and functions. We have discussed these limitations in the revised manuscript (lines 387-391).

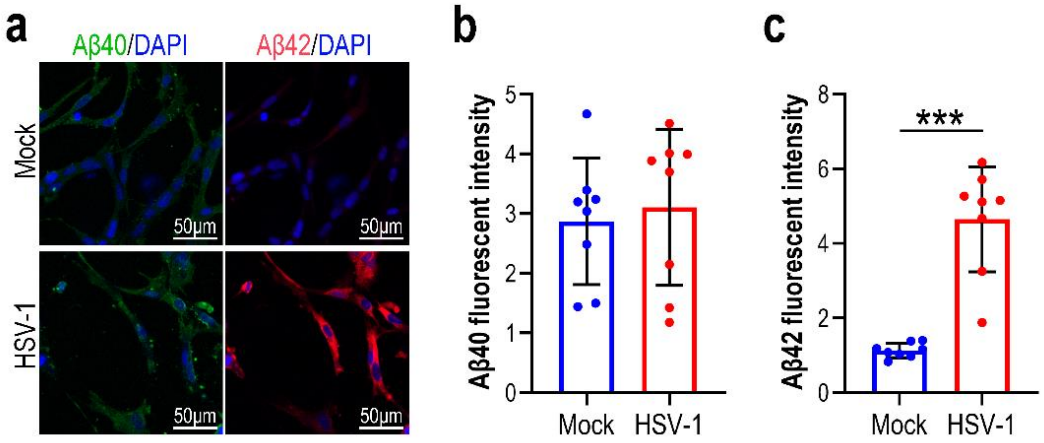


3. A consequence of long term herpes infection is the deposition of amyloid plaques within the brain. Several in vitro system demonstrate the possibility to measure Ab secretion and deposition in vitro even after a few weeks of culture. Is this system able to recapitulate this

pathological feature if the cells are kept longer in culture? A quantification of Ab in the neuron-astrocyte chamber would help answering this question.

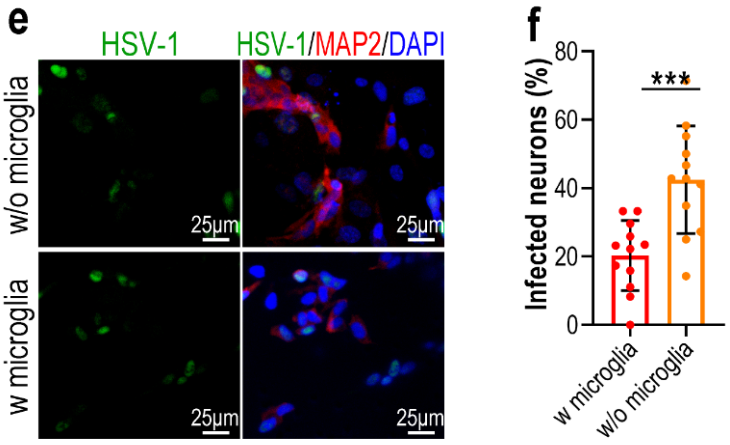
Thanks for the comments and valuable suggestions. It is true that increasing evidences implicate that HSV-1 infection is a potential risk factor for Alzheimer's disease and HSV-1 infection can induce Aβ accumulatio or deposition [12, 13].

To investigate whether this NVU model can recapitulate this amyloid pathological feature caused by HSV-1 infection, we detected the Aβ levels in the astrocytes/neurons compartment at 4 dpi by immunostaining for Aβ40 and Aβ42. The results showed, HSV-1 infection had no obvious effect on Aβ40 accumulation, while led to a significant increase in Aβ42 level (Sup Fig. 4), indicating this NVU model could also recapitulate the amyloid pathology caused by HSV-1 infection in vitro.



4. The experiments of infection in the absence of microglia raise question. It is expected that in the case where there are less cells in the system the remaining would be more infected. A proper experiment would have been to replace the microglia with other cell types (astrocyte-neurons for exemple) or to perform the infection directly in the astrocyte-neuron chambers. The latest would also be more relevant as Herpes virus might enter through a retrograte axonal transport.

We are thankful for the suggestion. To exclude the possibility that less cells lead to more infection of neurons, we replaced the microglia with the same quantity of astrocytes-neurons on the microglia-deprived NVU chip. 4 days after HSV-1 infection, we found more neurons were infected on the microglia-deprived NVU chip (Fig. 3e, f), as compared to the NVU model with microglia. This finding indicated the protective role of microglia against acute HSV-1 infection.



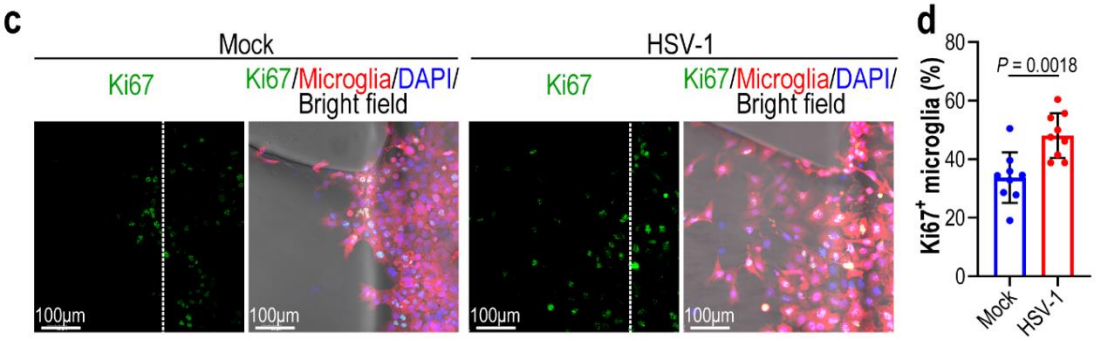
5. Authors should include a section in the discussion where they describe the limitations of the model: non exhaustive list: no direct contact astrocyte neuron, cell line for microglia and neurons, absence of pericyte, absence of flow, use of matrigel which is rich in laminin, etc.

We have added a section to address the limitations of this model in the revised version (lines 386-400).

6. The migration of the microglia is interesting but how does the authors explain it? is due to migration or division of the cells. The latest would not be physiologically relevant however it does not seems that there are less cells in the microglia chamber to support only migration.

To address whether the increased microglial migration is due to the increased cell division or not, we added the examination of proliferation state of microglia by immunostaining for Ki67. The result showed, more proliferating microglia were detected on the HSV-1-infected NVU model, compared with the mock-infected NVU model (Fig. 3c, d).

According to previous studies, proinflammatory cytokines, such as IL-1β and TNF-α, can stimulate microglia proliferation [14, 15], which is a key component of responses to damage in the brain [16]. Our data showed, HSV-1 infection triggered significant releases of IL-1β and TNF-α on the NVU model (Fig. 2h). Therefore, we speculated the increased microglia proliferation might be a result of release of proinflammatory cytokines. The enhanced proliferation and increased migration might contribute jointly to the appearance of more microglia in the astrocytes/neurons compartment following viral infection.



7. it is not clear if the EM images are performed in the chips or in monoculture. The staining quality could be better.

Thanks for the points. These TEM images were performed on the NVU chips. According to the suggestion from the reviewer, the EM images have been replaced with more clear images (Fig. 5c).

Reviewer #2 (Remarks to the Author):

Min et al. present a novel brain-on-chip model, which they exhaustively examine using a model of HSV-1 infection.

HSV-1 encephalitis remains a significant challenge in the care of children and immunocompromised individuals, with potentially devastating consequences. Studying this condition in mice remains insufficient. Human iPSCs, multicompartment, multi-cell types, and modular organoids represent the next frontier for accurately studying the initial phases of virus infection in the brain, identifying drugs, and developing protective treatments. Viral encephalitis, in general, is a major scourge, and the same system could be applied to all of them.

The system proposed by Min et al. features several unique elements, integrating microengineering and cell biology innovations. Their multi-modular system comprises a combination of cell lines and primary human cells, organized into compartments representing the BBB (HBMEC and human astrocytes), microglia, and brain (neuronal cell line and human astrocytes). This arrangement facilitates the guided organization of a multi-layered organoid, which they clearly demonstrate using confocal microscopy and immunostaining. Figure 1 is very convincing. For clarity, the authors may want to add another dotted box showing the location of the cut for the HBMECs corresponding to Fig. 1d. The FITC-dextran sulfate result is very strong.

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demonstrate the effect of HSV-1 on autophagy and its reversal by rapamycin (Fig. 5).
Implicating autophagy as an early mechanism of dysfunction caused by HSV-1, the authors tested activators of autophagy, such as rapamycin and others, showing that activation of autophagy limits HSV-1 replication. Control of viral replication correlates with increased cell viability (Fig. 6).
Finally, they examine changes imparted by autophagy activators in microglia. Fig. 7 presents data showing decreased HSV-1 infection and restored autophagic flux in infected microglial cells treated with autophagy stimulators.
The new NVU model appears very promising as a valid alternative for studying human brain infection. However, certain questions remain:
We appreciate the reviewer's very positive comments on this work.

1. In figures 1 and 2, do we know the number of cells for each of the different cell types? How close or far are these numbers from those in the human brain? This is also relevant in Fig. 2 to understand whether the percentage of infected cells represents HSV-1 cell specificity or relates to cell abundance.
   Regarding to the cell number, it was carefully considered based on several aspects, including the physiological structure of NVU in human brain, research focus in this study, and the different proliferation rates of various cell types used in this work. The number of cells in Fig. 1 and 2 for each of the different cell types are counted before seeding them on the chip (lines 442-455). After seeding cells, different types of cells were further co-cultured for 3 days to form a functional NVU model. As SH-SY5Y cells has a higher proliferation rate than HA cells, and the ratio of astrocytes/neurons commonly reaches to 3, 3 days after cell seeding.
   As brain microvascular endothelial cells are primary anatomical component of the BBB and line the brain's capillaries, we seeded HBMECs in the vascular side to mimic a confluent endothelium. Regarding to the microglia, the reason we cultured them in a separate chamber in 2D condition is to facilitate the observation of cellular dynamic migration towards the astrocytes/neurons region. This is also one of the advantages in using this device. Regarding to astrocytes and neurons, we cultured them in a 3D condition in order to mimic the 3D microenvironment of brain parenchyma.

2. The authors show key aspects of HSV-1 infection, including microglia migration and extravasation of immune cells, opening tremendous opportunities for study. However, how valid is the model? Although the cellular analysis is convincing, the RNA sequencing results are puzzling for observations at 3 days post-infection. Type 1 IFN is a key cytokine in HSV-1 pathogenesis, as shown in patients with inborn errors in the IFN pathway (e.g., TLR3, UNC93B, others). The defect resides mainly in neurons, which become extremely susceptible to HSV-1 infection. Could the authors comment? This point could be raised in the discussion.
   We are thankful for the comments. HSE is an acute brain infectious disease, which can cause severe brain injury quickly after HSV-1 invades the CNS. To gain an overall insight of molecular basis underlying HSE and develop new countermeasure therapeutics at early time point, we performed RNA-seq analysis on the NVU model at 3 dpi. The RNA-seq

results revealed HSV-1 infection induced obvious immune responses in host cells and elicited activation of several inflammation-related pathways, such as TNF signaling pathway, IL-17 signaling pathway and NF-kappa B signaling pathway. However, type I interferon signaling pathway, which plays pivotal roles in defense against viral infection [17, 18], are not activated by HSV-1 infection on the NVU model, according to RNA-seq analysis.

With regard to this finding, we thought it might be due to the immune evasion mechanism exploited by HSV-1. Previous studies reported HSV-1 has evolved several strategies to interfere with IFN responses at multiple levels [19]. For example, HSV-1 ICP0 protein was reported to degrade the host deubiquitinase BRCC36 and further down-regulate IFN-I receptor IFNAR1 [20], and HSV-1 ICP27 protein was found to interfere with STAT-1 activation and hamper the downstream transcription of ISGs (interferon-stimulated genes) [21]. Moreover, another study revealed the levels of IFN antiviral responses to HSV-1 is different among distinctive tissues, for instance, IFN induction is more robust in skin than in brain [22]. In summary, we suggested that the immune evasion mechanism of HSV-1 is responsible for the failure to trigger a significant IFN response on the NVU model, which in turn increases the injury degree of brain.

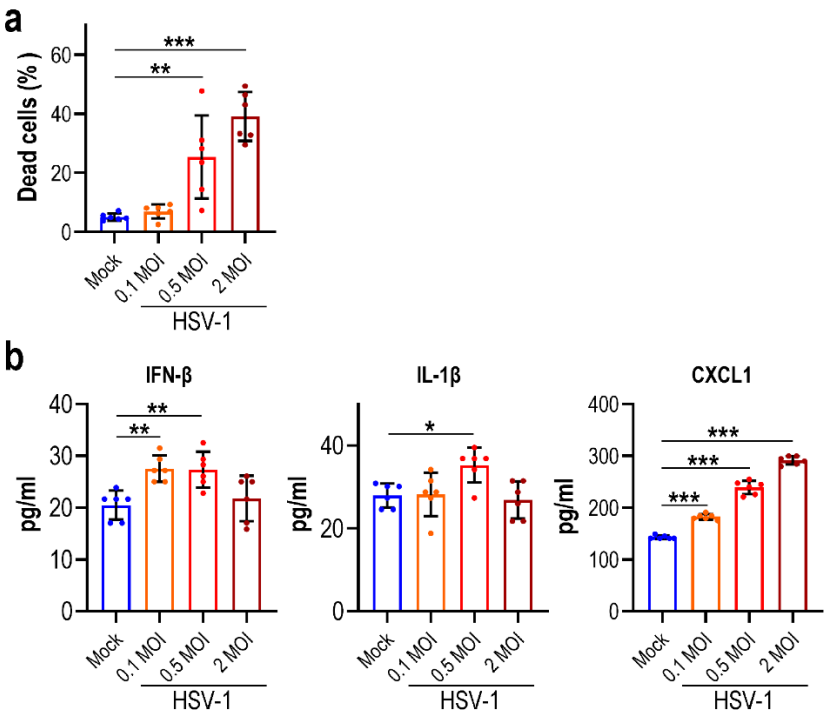
According to the suggestion from the reviewer, we discussed this point in the revised manuscript (lines 347-363).

3. Another question is the reproducibility of the model. The number of chips used in each experiment is relatively low (n=3). How many experiments were conducted? How have the authors tested reproducibility? This includes the number and proportions of cell types.

   We are thankful for the comments. In all experiments of this study, at least 3 chips were utilized for each group, and the exact sample size was introduced in the figure legends. To verify the experimental reproducibility, all experiments were repeated at least 3 times, excepting RNA-seq analysis (n = 3; 1 time).

4. Additionally, how sensitive is the system? What is its capacity to gauge differences in cytokine levels and infections with different multiplicities of infection (m.o.i.) for example? Regardless, while autophagy as a targetable pathway for the control of HSV-1 infection and rapamycin to counter HSV-1 has been done, the mechanism was not elucidated.

   We are thankful for the suggestions. To test the sensitivity of the model, we infected it with different multiplicities of infection, including 0.1 MOI, 0.5 MOI and 2 MOI. Cell viability assay using Calcein AM/EthD-1 kit revealed, cell death level was positively correlated with the dose of infection (a panel in the below figure). Also, levels of INF-β, IL-1β and CXCL1 were determined by ELISA experiments (b panel in the below figure), which showed infection doses had different effects on these cytokines. For CXCL1, its level was positively correlated with the dose of infection. For INF-β and IL-1β, the highest levels were detected at the moderate infective dose (MOI = 0.5), while it decreased at the highest infective dose (MOI = 2), which may result from the severe cell death caused by the highest infective dose.



With regard to the association between autophagy and HSE pathogenesis, it appeared a large number of autophagic vacuoles were accumulated in glial cells of HSV-1 infected NVU model by TEM examination. RNA-seq analysis revealed HSV-1 infection caused down-regulation of autophagy pathway in glial cells, especially in microglia. Consistent with the RNA-seq data, autophagic flux assay showed HSV-1 infection severely blocked autophagy flux in microglia, while treatment with autophagy activators significantly restored autophagic flux and decreased the viral load in the microglia. Collectively, we concluded that, HSV-1 infection can disturb autophagy pathway, and autophagy can be utilized as a targetable pathway for the control of HSV-1 infection. In this study, our research focuses mainly on the construction of the 3D NVU model, disease modeling and evaluation of new therapeutics for HSE treatment, while less attention was paid on the molecular basis of HSV-1 induced autophagy disturbance. In future, more work is needed to clarify how HSV-1 interferes with autophagy pathway in the host cells, especially in microglia.

In sum, the paper presents a new approach to model a brain on a chip, using in-depth analysis of HSV-1 infection to validate the system. The model recapitulates key features of HSV-1 infection. The system's modularity allows for probing the functions of PBMCs, microglia, astrocytes, and other cell types.

We thank the reviewer for the positive comments on the manuscript.

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Reviewer #3 (Remarks to the Author):

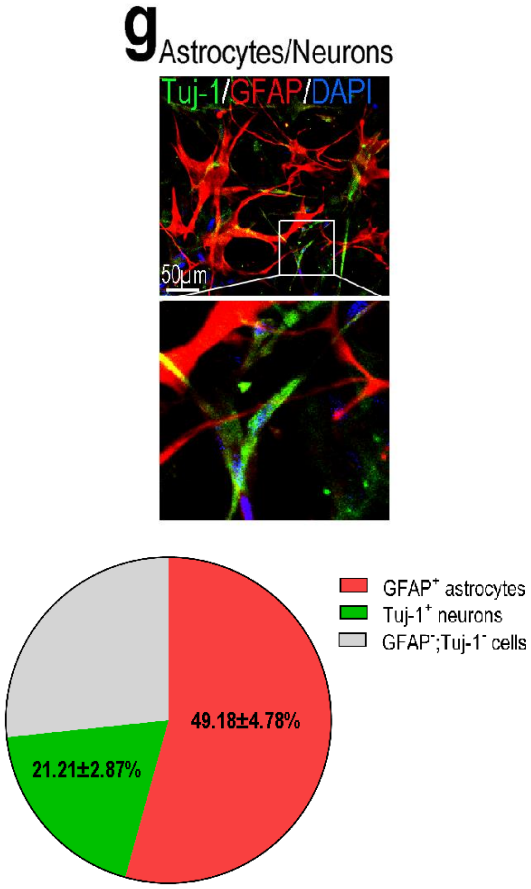
While the authors have utilized 3d human neurovascular unit (NVC) model and their application to HSV-1 is novel, the reliability of model and design is concerned.

Major Comments:

1. The authors should clearly justify the rationale for the cellular ratio used in the model. Although the initial and final number of SH-SY5Y cells are higher than other cell types, this should be explicitly addressed. A rough estimation of the final cell numbers, based on previous calculations of proliferation rates, should be provided. To accurately determine cell numbers and ratios, it is essential that the authors perform FACS analysis with proper quantification.

The reasons for the cellular ratio used in the model have been explained in the last round of response letter, please check it (Question 1 for the #1 reviewer).

Before FACS analysis, cells are required to be digested from the Matrigel. However, both astrocytes and neurons have cell processes, and the digestion process can damage the cells and affects the accuracy of cell counting. Instead, we quantified astrocytes and neurons by immunofluorescent imaging using astrocytic marker (GFAP) and neuronal marker (Tuj-1) on the NVU model (Figure 1g), The result showed, after co-culture for 3 days, the ratio of GFAP+ astrocytes to Tuj-1+ neurons was ~2.3 (Supplementary Fig. 3). We have described it in the revised manuscript (line 121-123).



2. The manuscript relies predominantly on immortalized cell lines (particularly SH-SY5Y neuroblastoma cells), which differ significantly from mature, functional primary human neurons. This introduces concerns regarding the ability of the model to faithfully recapitulate the composition of the mature brain, and raises issues related to excessive proliferation at this stage, which should not occur in the context of a mature neuronal model. The authors should consider replacing these cell lines with human pluripotent stem cell-derived neurons or mature primary human cells, including neurons. Additionally, it is crucial to verify that each cell population expresses essential neuronal markers, such as Tuj1 or MAP2, as indicated in Figure 1b and Figure 6b. Moreover, the microglial morphology and activation states (e.g., as shown in PMID: 32221280) should be revisited, as these are not clearly demonstrated in the current manuscript, especially in relation to the microglial data presented.

We acknowledge that SH-SY5H cells have some limitations compared with primary cells or hPSC-derived neurons, but they are widely used in various aspects of neuroscience research (Cell. 2022 May 26;185(11):1943-1959.e21; Cell. 2024 Dec 26;187(26):7603-7620.e22; Nat Cell Biol. 2021 Jan;23(1):40-48; Nature. 2022 Mar;603(7899):131-137). The limitations of the two types of immortalized cells (SH-SY5Y, HMC3) have been discussed in the manuscript (line 388-392), and the reviewer #1 who raised this question also approved the response.

With regard to the expression of neuronal markers in SH-SY5Y cells, these neuronal cells showed expression of Tuj-1 and MAP2, as shown in Figure 1f-g. In the subsequent experiments, we mainly focused on detection of infection and damage of neuronal cells following HSV-1 infection, and did not repeat examinations of these neuronal markers.

With regard to microglial morphology and activation states, as HMC3 is a commonly used human microglial cell line, and has been described in many published papers (Int J Mol Sci. 2023 Oct 18;24(20):15288; Neuroscience. 2022 May 10:490:131-143; Mol Immunol. 2021 Mar:131:171-179; J Neuroinflammation. 2018 Sep 10;15(1):259).

3. There is a persistent issue with categorizing astrocytes and neurons within the same group, except for in Figure 1. While the manuscript focuses primarily on microglia as inflammatory cells, astrocytes should be analyzed separately from neurons. Astrocytes are also key players in neuroinflammation, and their inclusion as part of the neuronal population may obscure important insights.

We tried to explain it although this question is beyond the scope of the work.

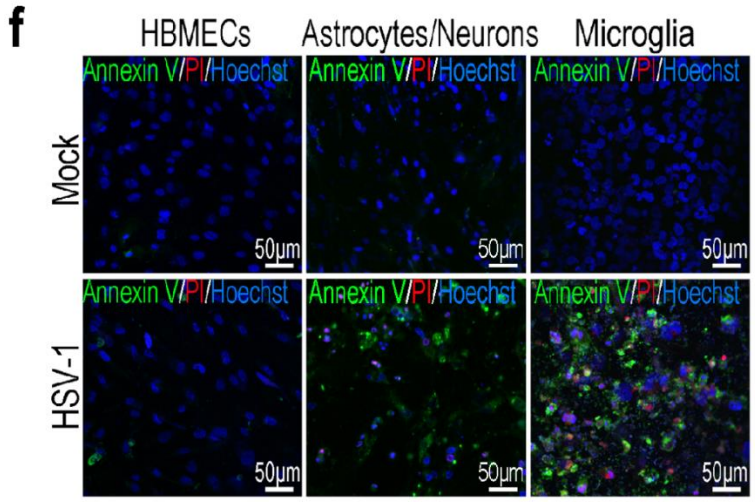
As mentioned by the reviewer, astrocytes are also vital players in neuroinflammatory responses. However, as the resident immune cells in the central nervous system, it's well known that microglia play a more important role in mediating neuroinflammation. So we mainly focused on the responses of microglia in this study. There are various types of cells in the central nervous system, each of which might play distinctive roles in the disease pathogenesis, and it is impossible to clarify the responses of all cell types in HSE in one single study. The roles of astrocytes in the HSE pathogenesis may need to be explored in depth in subsequent works.

4. In Figure 1, the overall fluorescence image of the 3D human NVU model should be shown. It is unclear how the authors' method generates a reliable model with well-distributed and

appropriately formed 2D and 3D cell cultures in co-culture. Several figures, including the MOCK group in Figure 2f, show incomplete DAPI staining, suggesting issues with model integrity. In particular, Figure 2f shows lower DAPI staining (indicating fewer cells) in the MOCK group compared to the HSV-1 group, which complicates the interpretation of the cell ratio data in Figure 2g. A clearer, more consistent model should be presented to support robust conclusions.

Thanks for the points. The area of the NVU interface is 27mm^2, which is too large to get an overall fluorescent image under a confocal microscope. In Figure 1, we have characterized the NVU structure by immunofluorescent images, and verified the barrier function by dextran permeability assay. As demonstrated in this work, we are confident that we have established a functional NVU model in vitro. The functions of our models are similar to those BBB or NVU chip work published previously in the higher quality journal. (Nat Biomed Eng. 2021 Aug;5(8):830-846; Nat Commun. 2021 Oct 8;12(1):5907; Nat Biomed Eng. 2021 Aug;5(8):847-863; Adv Sci (Weinh). 2023 Apr;10(11):e2205752).

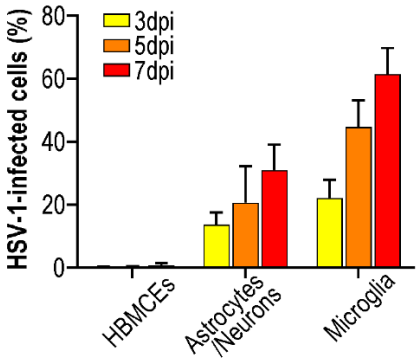
Regarding the resolution of images in Figure 2f, we cultured the neurons and astrocytes in 3D Matrigel, therefore, it was more difficult to gain clear images as brain endothelial cells and microglia cultured in 2D culture. In Figure 2f, we counted the proportion of dead cells, and the nuclei number didn't affect the final statistical results. To avoid misunderstanding, we replaced the unclear image with more clear images.



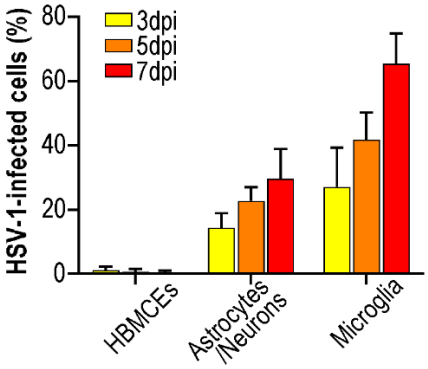
5. In Figure 2b, the representative image of 7 dpi is inconsistent with the quantification shown in Figure 2c. This discrepancy should be addressed by performing at least three technical replicates with three biological replicates to ensure data reliability.

Figure 2b are representative images from 3 NVU models (3 biological replicates), and Figure 2c is the quantification result of the 3 NVU models. This experiment has been repeated for three times (3 independent technical replicates), and repeated experiments showed similar results, verifying the reproducibility and reliability of this NVU model. Results of 3 repeated experiments were shown below:

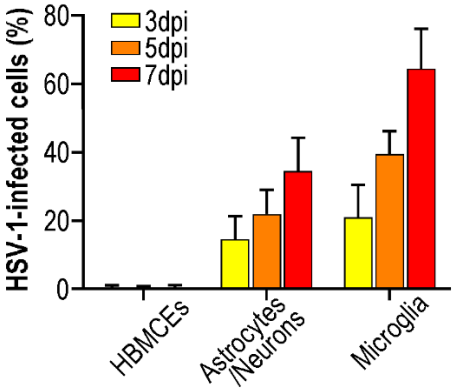
Technical replicate #1:



Technical replicate #2:



Technical replicate #3:



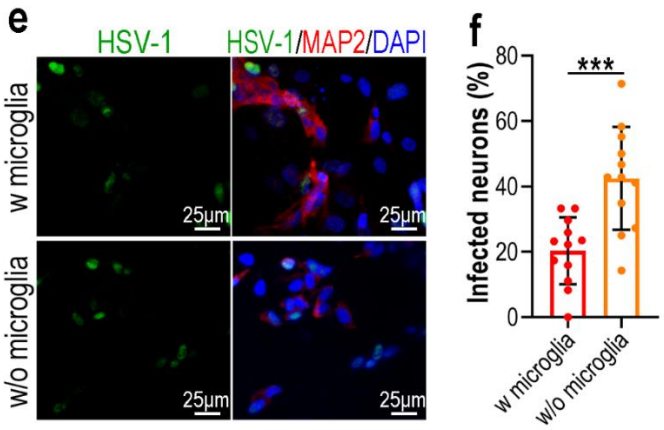
6. In lines 145-148, the authors mention decreased BBB integrity and increased permeability but do not provide direct evidence of HSV-1 infection in HBMECs in Figure 2c. This should be demonstrated to correlate the observed changes in BBB integrity with the infection process.

It appears the reviewer misunderstood our findings regarding the damage of brain endothelial cell following HSV-1 infection on the NVU model. Our results showed HSV-1 rarely infected endothelial cells (Fig. 2b). We assumed brain endothelium disruption was not caused by direct HSV-1 infection in endothelial cells, but it might be induced by the inter-cellular communication between brain cells and endothelial cells indirectly after viral infection in the brain side.

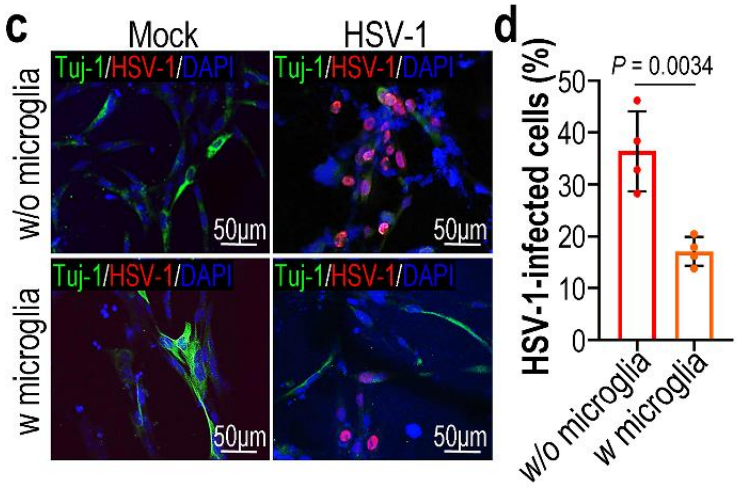
7. Figure 3e and the associated data in lines 188-199 are not convincing. The lower expression of neuronal markers in the presence of microglia (compared to their absence) suggests

neuronal death, not protection. Furthermore, the HSV-1 infection ratio appears similar in the presence and absence of microglia, which contradicts the data in Figure 2c showing that microglia are more susceptible to HSV-1 infection. This inconsistency should be addressed.

Thanks for the point. We apologized for the mislabeling in Fig 3e. The group labels in Fig 3e were reversed, and the group labels in Fig 3f were correct. We have corrected them in this new version.



The figure below was the result from another set of parallel experiments, which also showed deprivation of microglia resulted in increased neuronal infection, suggesting a protective role for microglia in the disease.



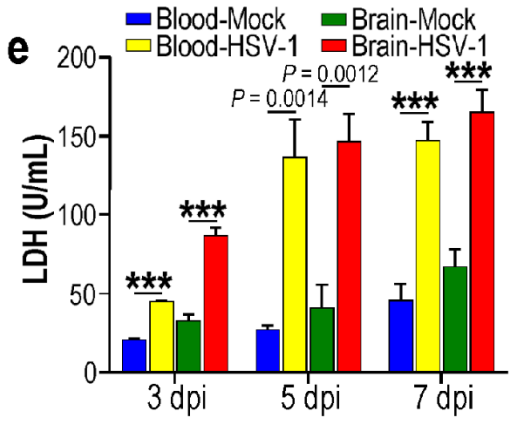
8. There is a lack of consistency regarding the time points used. Some data are based on 3 dpi (e.g., Figure 4), while others use 7 dpi (e.g., Figure 1). The authors should clearly explain the rationale for choosing different time points and demonstrate the logistics behind this decision.

Thanks for the comments. In order to comprehensively assess viral infection and disease progression on the NVU model following HSV-1 infection, we detected the NVU models at multiple time points in Figure 2. At 3 dpi, we have detected obvious viral infection and NVU injury. At 7 dpi, the NVU injury was very severe. We think it is quite important to study the disease progression and intervention evaluation in a timely and dynamic manner. It is obvious, the pathological changes of NVU in vivo are dynamic following the viral exposure. Also, this

is the unique advantage of this in vitro 3D NVU model for disease study, which is not easily achieved by animal models.

From the point of view of disease treatment, it is more important to analyze the pathogenesis mechanism at early time. Because early intervention helps prevent patients from getting worse and is conducive to patients' recovery. This is the main reason why we examined the NVU samples mainly at 3 dpi in the following study, including recording the responses of immune cells, RNA-seq, drug evaluation, and et al.

According to the reviewer's suggestion, we adjusted the detection time points of some experiments in Figure 2. We re-analyzed the LDH level for the medium samples at 3, 5 and 7 dpi. Consistent with previous results, LDH in the infected group had risen significantly at 3 dpi, and the LDH level continued to rise at the following time points (Fig 2e). It indicated that HSV-1 infection has caused severe cell damage at 3dpi.



9. In Figure 4a, the number of down-regulated genes (415) is relatively similar to the number of up-regulated genes (634). The authors should present the top 10 up- and down-regulated genes to provide a more focused analysis.

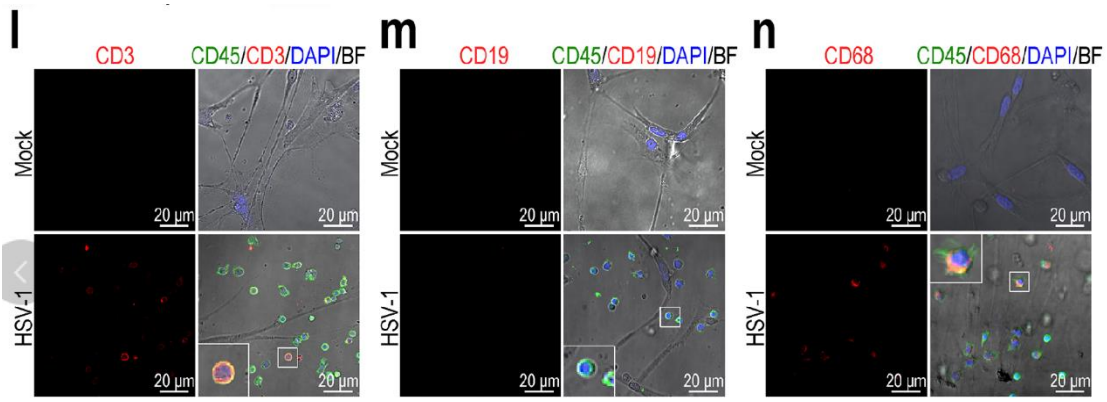
Thanks for the point. We think the close number of down-regulated and up-regulated genes in a study is not an issue that needs special explanation, because it is the fact. Following HSV-1 infection, many genes were modulated in the host cells, while some of which were not particularly relevant to our research focus or have been reported previously, therefore we didn't not specifically show these genes.

In fact, few studies present all regulated genes, even those with the most significant changes. As the top-regulated genes do not necessarily means that they play the most critical roles in the pathogenesis of disease. Therefore, we didn't present the top 10 up-regulated and down-regulated genes in the manuscript. In Figure 4, we focused on the up-regulated genes related with immune responses (pro-inflammatory cytokines, chemokines), because inflammation is a major factor contributing to the pathogenesis of HSE.

In addition, we have uploaded the raw data of RNA-seq to the public database, and the reviewer is welcome to download and check it if he/she is interested.

10. In Figure 4l-n, the fluorescence expression of green and red channels is not clearly distinguished. The quantification in Figure 4o should be based on at least three technical replicates to improve the robustness of the data.

Thanks for the points. As suggested, we revised the Figure 4l-n to distinguish the green and red channels. This experiment has been repeated for 3 times. We obtained the same results that T cells and macrophages infiltrated the diseased tissue following HSV-1 infection.



11. In Figure 5a-b, the use of mitophagy-animal and autophagy-animal data raises the question of whether these findings are directly applicable to human cells. The authors should clarify how these animal model data are relevant to the human-based 3D NVU model used in the study.

It is generally known that the terms of "mitophagy-animal" and "autophagy-animal" are two annotation terms for signaling pathways based on KEGG database, which doesn't mean these data were obtained from animal experiment. The mitophagy and autophagy are two very conserved biological pathways across different animal species. If the reviewer still has any question, welcome to check these terms on the KEGG database. mitophagy-animal (ID: hsa04137; https://www.genome.jp/pathway/hsa04137); autophagy-animal (ID: hsa04140; https://www.genome.jp/pathway/hsa04140).

12. Figure 6 presents a key question regarding drug delivery. Since drug delivery across the blood-brain barrier (BBB) is a significant challenge, drugs should be added to the vascular (blood) side of the model, not the brain side, in order to more accurately simulate real-world drug delivery conditions. This experimental setup would allow the authors to evaluate how efficiently drugs can penetrate the BBB under MOCK vs. HSV-1 infection conditions.

It seems this question is out of the scope and focus of this work. We acknowledge that the BBB is an obstacle for the entry of drugs in treatment of brain diseases, and it is a technical challenge that needs to be overcome. But the key point is, to solve this problem, the first step is to find a drug that has therapeutic effectiveness. The drug delivery across BBB is an issue that should be considered next, such as by structural modification of drug, or by drug delivery system. The reviewer cannot ask us to address all aspects of a disease in a single study, from disease modeling, pathogenesis study, drug screening, to drug delivery. This is clearly beyond the scope of this study.

In addition, there have been a lot of published papers, focusing on brain disease and drug development. In these studies (Nat Neurosci. 2023 Sep;26(9):1489-1504; Nat Med. 2020 Jun;26(6):952-963.), it is not required to solve the problem of drug delivery across BBB in one single study, and drugs were also added on the brain side of organ chip.

Overall, the manuscript lacks sufficient technical and biological replicates to provide a convincing level of reproducibility. The authors should consider increasing the number of replicates to ensure the reliability of their findings.

Actually, we conducted sufficient technical replicates ($n \geqslant 3$) and biological replicates ($n \geqslant 3$) throughout this study, which has been explained in the revised manuscript . The repeated experiments verified the reliability of our findings. We are very confident with the data that we provided in this work.

Minor comments:

Figure 1b missing scale bar.

Figure 1b is the real image of NVU chip. Actually, in most of the published papers on organ-on-a-chip, there is usually no scale bar in the real image of chips. Moreover, the size of chip has been described in detail in the manuscript (line 432-437; Supplementary Fig 2). While, as suggested, we have added the scale bar in Figure 1b in this revised version.

REVIEWERS' COMMENTS

Reviewer #3 (Remarks to the Author):

Overall, the authors have effectively addressed the queries raised in the initial draft. However, I would like to offer the following recommendations for further improvement:

1. The authors have satisfactorily addressed the question raised in the initial draft regarding the choice of cell sources. However, it remains critical to explicitly justify the rationale behind using a 10:1 ratio of astrocytes to SH-SY5Y cells, as stated in lines 444-445. A clear explanation for this particular cellular proportion is essential for the scientific robustness of the model. I recommend a more comprehensive discussion on why this specific ratio was selected (considering this ratio is not common in neuroscientific publication to have less neuron than other populations), particularly in relation to the experimental objectives and underlying biological relevance.

We thank for the suggestion from the reviewer. We have discussed the rational of using a 10:1 ratio of astrocytes to SH-SY5Y cells in the revised manuscript (line 380-389).

2. In alignment with previous feedback, the fluorescence imaging data could be enhanced, particularly by utilizing more advanced imaging techniques to acquire data from 3D samples. Notably, in Figures 2f and 4l-n, there appears to be a discrepancy in the DAPI staining counts, which raises concerns regarding the potential variation in cell numbers between experimental groups. To ensure consistency and accuracy in the data, I suggest revisiting these images or providing additional clarification on how these discrepancies may have arisen.

We thank for the commets from the reviewer. As mentioned by the reviewer, it is technically more difficult to acquire clear images from a 3D samples. In addition to the fact that the 3D sample is thicker than the 2D sample, which may affect the penetration of the antibodies or fluorescent dyes, the PDMS material can also affect the imaging clarity.

With regrad to the differences in the DAPI staining counts in Figures 2f and 4l-n, it's because the magnification of the two sets of images is different. The size of peripheral blood immune cells is very small, in order to see them clearly, we photographed Figure 4l-n at a higher magnification. So the number of cells in Figure 4l-n looks smaller than in Figure 2f. Please check the scale bar in these figures.

3. In reference to my prior comment regarding the data transparency (comment 5), I strongly recommend the inclusion of raw data, along with exported data for fluorescence images, for all replicates associated with the figures.

We thank for the suggestion from the reviewer. The raw data of Figure 2c has

been depositedin the Source Data file. As the data size of fluorescent images (3 technical replicates) is too big, and we have deposited them on Figshare database (https://doi.org/10.6084/m9.figshare.28517246). Please check them.