nature portfolio

Peer Review File

Pharmacological targeting of Bunyavirus-induced synaptic impairment prevents neuronal hyperexcitability

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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 manuscript, the authors utilized a proteome-based network medicine approach to identify Gabapentin as a potential treatment for bunyavirus Tahyna (TAHV) induced neuroinfection. Specifically, the authors demonstrated that TAHV infects human cerebral organoids (hCO), induces proteomic alterations in neural pathways and synapse-associated proteins, and identifies Gabapentin as a potential agent to mitigate TAHV-induced electrical disturbances in hCO. The authors further validated the rescue effect of Gabapentin in the TAVH-infected post-mortem adult human brain (OPAB) explants.
Previous studies reported that bunyavirus infections lead to neuronal apoptosis and reduced organoid viability in hCO [1-2]. Additionally, the authors previously demonstrated that TAHV infection disrupts local field potentials in OPAB and developed a machine-learning framework to predict OPAB infection status [3]. Thus, the novelty of the study is limited.
Additionally, the major argument is not well supported by the presented data.
To improve the quality of the manuscript, the authors need to consider the following issues:
Major issues:
1. Evidence level of TAHV infections is low and based on the mRNA level.
The authors need to stain TAHV proteins using immunofluorescence or RNA FISH to provide direct evidence of the infection process.
2. It's better to co-staining TAHV with markers for various cell types (e.g., neural stem cells and neurons), which is essential to clarify TAHV tropism toward specific neural cell types.
3. The staining pattern seems inaccurate (false positive) for Bassoon and Homer1 proteins in Figure 2F-H. The authors need to provide high-resolution confocal images to enhance reliability. Additionally, I recommend the authors to perform WB of these two proteins to characterize changes in synaptic protein expression following TAHV infection. The authors can confirm this argument with PSD95 and synaptophysin staining
4. The authors need to improve the image resolution quality of Figure 2B.
5. It's better to provide proteome analysis of differentially expressed proteins in volcano plots or Venn plots.
6. In the PCA analysis (Figure 4E, S3B), the cumulative variance contribution of the first two main components was ≤ 20%, which means the identified factor is not significant enough. The authors need to discuss these results.
7. The authors only demonstrated the statistical charts of the electrophysiological data. The authors also need to provide representative neurophysiological recordings for each group and illustrate the amplitude and frequency of electrical signals. Additionally, neural organoids usually generate robust neuronal spike after three-month culture and bursts after six-month culture.
8. The authors are encouraged to conduct animal experiments to validate the reparative effects of GBP on TAHV-induced electrophysiological and motor behavior disturbances.
Minor issues:
1. In Figure 1E, the MAP2-specific expression is unclear. The authors should provide high-magnification confocal images to improve clarity.
2. Certain figures, such as Figures 2D–2E, 3A, and S2, lack clarity. The authors should enhance their overall quality to improve data readability.
3. The authors should specify whether Figure 2H was stained in the Mock group or in TAHV-infected organoids.
4. The authors should describe Figure S2 in the results section, as it is currently not mentioned in the manuscript.
References:
[1] Winkler, Clayton W., et al. "Neuronal maturation reduces the type I IFN response to orthobunyavirus infection and leads to increased apoptosis of human neurons." Journal of Neuroinflammation 16 (2019): 1-16.

[2] Ojha, Durbadal, et al. "Rottlerin inhibits La Crosse virus-induced encephalitis in mice and blocks release of replicating virus from the Golgi body in neurons." Nature Microbiology 6.11 (2021): 1398-1409.
[3] Partiot, Emma, et al. "Organotypic culture of human brain explants as a preclinical model for AI-driven antiviral studies." EMBO Molecular Medicine 16.4 (2024): 1004-1026.

Reviewer #2

(Remarks to the Author)
This study focuses on demonstrating the potential of network science in drug repurposing, in the case of viral neuroinfection. The case study they choose is significant, as it is under-studied, and it lacks comprehensive, vast datasets. The study is based on a versatile combination of experimental and computational methods (network models and machine learning). I focused in my review on the computational part of the paper. Overall, I found some major problems in that part of the study, especially a lack of explanations and details that hindered my evaluation of the significance and of the solidity of the computational results. I list my comment below.

Major comments
- The data used for training the machine learning models is not described. There is a reference to it on page 22, line 562 ('available data for machine learning analysis'), but that part of the text is missing from the manuscript.
- The random forest classifier seemed to work well on 2 donor models, and less well on one. Absent a larger validation set of donor data, some discussion on what this means is needed. It seems that a good classifier may need more features than those in the current data, I wonder if the authors can speculate on what they may be.
- Line 148: "Comprehrensive network analysis of dysregulated pathways reveals…" Please add details on how this analysis was done and what were its concrete conclusions.
- Line 157: "Quantitative analysis revealed that …" Please explain the details of this quantitative analysis.
- It is unclear to me what "Mock", "Mock-infected" and "Mock/TAHV-infected" are. This is important to clarify because Mock plays a key role in the design and training of the machine learning models. I assumed in my reading that it means the control, non-infected samples.
- Figure 2D: the color code is unclear. Clarifications are needed on whether it applies both to node coloring, as well as to interaction coloring, on how to interpret multi-color coloring applied to some of the nodes, on how interactions should be interpreted, and on how the network was generated. This is better explained in Figure S2. There is a mention in the caption of Figure 2 to "String network analysis" that I am not sure how to read, did you mean that you used the STRING database? If yes, how?

Minor comments
- There is a strange change of font size in the middle of the Introduction.
- Introduce the abbreviation FC.
- Are the network models and the machine learning models available? What about the dataset used to generate/train them?

Reviewer #3

(Remarks to the Author)
In this manuscript, Gorda, Bouget et al describe the infection of human cortical organoids with bunyavirus Tahyna (TAHV) in order to identify drugs potentially able to alleviate symptomatic manifestations of the brain infection in humans. Although the concept is interesting, the presentation of the topic confuses different concepts (e.g. antiviral drugs vs symptomatic drugs, neurological manifestations due to direct neuroinfection or indirectly in post-infectious status (post-COVID syndromes), etc) and exaggerate the hazard of TAHV, especially as compared to other viruses from the same serogroup. The efforts pulled by the authors culminate with the identification of gabapentin, an anticonvulsant drug.
General comments:
This study is clearly interesting in the sense that it uses human cerebral organoids (HCO) to assess the effect of TAHV infection of neurons and test the potential of drugs. HCO represent indeed a novel and promising avenue for this type of studies. Nevertheless, the reviewer has several concerns as some conclusions are not really supported by data. First, given that other viruses from the same serogroup (California, La Crosse) as the TAHV are known to induce seizure and that anticonvulsant drugs are already used for treating those patients, the potential usefulness of gabapentine in TAHV infection as revealed by HCO does not come as a surprise. Second, important control data are lacking to support a strong effect of gabapentine on TAHV infection and its symptoms. For instance, it would be crucial to determine what the effect of gabapentine on non-infected neurons is. Another point of concern is that it is unclear how the authors came to pinpoint GBP. To correctly sustain that their proteomic-based approach led them to identify GBP, the authors should also assess the potential of other hits in their candidate list and demonstrate that most of the top hits do present some effect on the proxy of symptoms used in the in vitro model. Without this demonstration, the identification of BGP may appear as the result of chance rather than really due to this approach.
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Major comments regarding the data and interpretations:
Figure 1
- The antiviral effect of RG10b can't be classified of moderate given that only one out of the four timepoints demonstrated a significant effect. The antiviral potential is thus low.
- The authors also claim that treatment with RG10b induce little toxicity but the graph shows a higher toxicity with RG10b than TAHV alone, thus in contradiction with their conclusion of virus-induced toxicity. In addition, treatment with RG10b alone is missing to draw the proper conclusion on toxicity.

- LDH assay is used as a proxy for toxicity, yet it can be influenced by different parameters like metabolism or proliferation which are not controlled in this experimental setting. Thus the author can't present their data as cytotoxicity data. In addition, the differences are not significant thus the data do not support the sentence "some viro-induced cellular toxicity".
- The suppl figure 1 does not provide correlation between nuclei morphology and toxicity, not even for the control GA thus it is unclear what additional information this suppl figure 1 provides.
- MAP2 assessment is not enough to claim profound alterations of hCO integrity. Other neuronal and non-neuronal markers should be assessed. In addition, out of several thousands proteins tested, the relatively low number of dysregulated proteins (50 up and 87 down) and the limited toxicity (LDH readout) does not speak for profound alterations. FISH or other RNA visualization method should be used to assess the extent of infection (ratio of infected cells over total cells). Finally, since the authors use hCO, it would be interesting to have an idea of the impact of TAHV infection not only on neurons, but also on glial cells.

- The authors conclude that hCO are very permissive to TAHV infection but the total absence of infectious particle production as seen with the plaque assay speaks against the conclusion which should be revised.
Figure 2
- The authors claim that "hCO showed that Bassoon signal was mostly lost at 10 dpi, while Homer-1 remained relatively unchanged" but the pictures presented do not support this sentence and there is no attempt to provide an unbiased quantification of these altreations. There is only one picture for Mock, thus one timepoint is missing and there is no information of the timpoine presented. From the pictures, Homer seems to be incresed by infection, both 4 dpi and 10 dpi compared to Mock (not unchanged), Basson seems higher in 4 dpi than in the 10 dpi and mock thus it can't be classified as "lost".
- Fig 2H should provide pictures of higer resolutions for mock as well given that the low resolution picutres from 2G do not allow differentiating TAHV from Mock
Figure 3
- The table B should present at least the top 10 drugs as per the results obtained, not just gabapentin, assimilates of gabapentine, and ivermectin in order to really display the potential of the approach used here.
- A dose-response should be performed to correctly assess the antiviral effect of both ivermectin and GBP to support the conclusions written
Figure 4
- The author mention that GBP results in misclassification of infection status by their algorithm but the data displayed still present with more than 70% of correct classification in the treated infected group thus showing at best a moderate effect of GBP in this model (about 15% of reduction, not more). In addition, there is no statistical test to assess if TAHV and TAHV+GBP results are significantly different. Importantly, LFP data should be shown, not just the results of the classification algorithm. Algorithm predictions are not enough to have an idea of the extend on perturbations triggered by TAHV infection.
- The PCA indeed shows a good overlap between TAHV GBP-treated and non-treated while the divergent points with GBP do not go cluster with the mock data points, thus the authors can't claim that GBP is protective. It is changing the LFP profile, but not getting it back to the non-infected (normal) system. As mentioned in the general remarks section, GBP alone is missing to really have all the control conditions to have a proper idea of its effectiveness
- All donors should be presented regarding the OPAB model, there is no reason that donors 1 and 3 are presented only in suppl figure 3 just because donor 2 looks the best one. This display is somewhat partial and does not reflect the data generated. At the minimum, statistics including all 3 donors should be done to support a significant effect of BGP. The variability due to the use of different donors can't be just resolved in stating that the infection had a different effect on donor 3. The algorithm still shows a correct classification score of more than 70% in the TAHV infected condition. This experiment should ideally be repeated to include at least 5 donors in order to assess whether donor 3 is a true outlier or whether GBP does not work for an important proportion of donors which would reduce its interest as a drug in this context
- Statistics are lacking on Fig 4H
Minor corrections:
Regarding the manuscript itself, some statements are not adapted or over-stretched:
- SARS-CoV-2 can't be considered neurotropic with the level of scientific evidence available at the moment. The jury is still out.
- Virus-induced and not viro-induced; virus-centered and not viro-centric
- Precise the meaning of "in cellulo", it is not clear if it is used as a synonym of in vitro or also encompass ex vivo cellular analysis;
- Neurohealth is not a validated term in clinic and claiming that organoids have the potential to serve as a surrogate for global neurological health is an overstatement, the authors should stick to the potential of organoids for electrophysiological functions

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed most of the technical and data presentation issues raised in the initial review. However, two critical scientific concerns remain unresolved, and these directly affect the robustness and experimental validity of the study's main conclusions.
Major Issues:
1. While the authors have done a reasonable job contextualizing the novelty of their viability findings and acknowledging prior work on bunyavirus cytotoxicity, a fundamental issue regarding the validity of their synaptic immunostaining data—central to their claim of "loss of neuronal and synaptic markers"—has not been addressed. This undermines the study's key conclusions. In the control (Mock) samples, the staining patterns for PSD-95 and Synaptophysin do not match what is typically seen in the literature. PSD-95, a well-known postsynaptic density marker, appears as a non-physiological, continuous reticular or mesh-like pattern, rather than the discrete punctate labeling characteristic of mature synapses. Similarly, Synaptophysin, a presynaptic vesicle protein, shows a diffuse, sheet-like signal instead of the expected clustered axonal puncta. These unusual patterns raise concerns about antibody specificity, technical artifacts (such as over-permeabilization or non-optimal antibody concentration), or insufficient neuronal maturation. Although Synaptopodin staining looks superficially consistent with dendritic spine localization, the fact that both pre- and postsynaptic markers fail to show canonical synaptic puncta means the authors' quantification of "synaptic loss" (Figures 2C–E, Suppl. Figure S2B) is unreliable and cannot be taken as evidence of TAHV-induced synaptic damage. To address this, the authors need to validate their PSD-95 and Synaptophysin antibodies using fully matured primary neuronal cultures (e.g., hippocampal neurons) to confirm that they can label characteristic synaptic puncta. Without this validation, the data do not support the conclusions regarding disrupted neural integrity and synaptic composition.
2. The authors attribute the failed TAHV–cell type co-staining to virus-induced loss of MAP2/GFAP signals, but they do not provide validation that this loss is indeed due to true protein degradation rather than staining artifacts. They also have not attempted to use alternative neural markers (e.g., NeuN, S100β, SOX2) or optimize their protocols, leaving the specific target cell types of TAHV in hCOs undefined.
Minor Issue:
Throughout the statistical figures, there is no clear distinction between biological and technical replicates, and the ambiguous "n" values (e.g., "4 organoids from 2–3 productions") compromise reproducibility. For each statistical figure in both the main text and supplementary materials, please specify in the legend whether the replicate is biological or technical, provide exact counts (n_bio, n_tech) per group, and clarify how technical replicates were incorporated into the analysis. A consistent definition of "biological replicate" should also be provided for each experimental system (hCO, mouse, OPAB) across all figures and in the Methods section.

Reviewer #2

(Remarks to the Author)
This manuscript combines proteomics, human brain organoids, electrophysiology, and computational analyses to investigate the impact of TAHV infection and to identify potential therapeutic strategies. The experimental component is extensive and, in several respects, compelling, and the link between proteomic perturbations and functional neuronal effects is of clear interest. My comments below focus specifically on the computational aspects of the study, where I have substantial concerns regarding both methodology and interpretation.

The machine learning analysis of LFP signals is presented as an "unbiased machine learning framework" capable of discriminating infection status with high accuracy. However, based on the description in the Methods, this characterization is not justified. The construction of the dataset relies on splitting each 1-minute recording into multiple 2-second segments, which are then treated as independent samples and randomly divided into training and test sets (70/30 split) . Because these segments originate from the same underlying recording, they are strongly correlated. As a result, the random split likely introduces substantial dependence between training and test data, leading to data leakage and an inflation of the reported performance. Under such conditions, classification accuracy above 90% cannot be interpreted as evidence of genuine predictive ability.

More fundamentally, the effective number of independent biological units in this analysis is the number of donors, which is four. With such a limited sample size, it is not possible to design a robust machine learning analysis that supports generalizable conclusions across individuals. The current approach, in which models are trained and evaluated separately within each donor, does not address cross-donor generalization and therefore cannot substantiate claims about consistent infection-related signatures. The variability observed across donors (e.g., lower performance in one donor) is attributed to biological heterogeneity, but the evaluation protocol itself does not allow such an interpretation. In my view, the machine learning component should be regarded as exploratory at best, and the use of the term "unbiased" is inappropriate in this context. I do not see how a robust machine learning design can be done with only 4 donors.

The implementation details of the classifier further exacerbate these concerns. The Random Forest model is used with default parameters from scikit-learn, notably with max_depth=None, which allows each decision tree to grow until leaves are pure or contain a single sample. This effectively removes depth-based regularization and permits very deep trees, increasing model capacity. While the default max_features="auto" (i.e., sqrt(p) features considered at each split) introduces some randomness and decorrelation between trees, it does not prevent individual trees from fitting highly specific patterns in the data. In the present setting, where the number of independent biological units is extremely small and many samples are

highly correlated due to segmentation, such unconstrained trees can easily memorize recording-specific structure rather than learn generalizable features of infection status. This risk is further amplified by the data splitting strategy, which likely places correlated segments from the same recording in both training and test sets. The absence of constraints on tree depth or leaf size (e.g., min_samples_leaf), and the lack of any sensitivity analysis, make it difficult to assess whether the reported performance reflects a robust signal or simply model capacity interacting with non-independent data. The evaluation is also limited to accuracy and a non-standard “confidence upon prediction” metric, without calibration analysis, baseline comparisons, or statistical validation. Altogether, the machine learning analysis, as currently designed and reported, does not provide reliable evidence supporting the claims made in the manuscript.

In contrast, the proteome-based network medicine analysis is conceptually sound and follows a standard pipeline, linking differential proteomics to functional enrichment, phenotype association, and drug repurposing. The identification of gabapentin and its subsequent experimental validation is a strength of the study. However, the methodological description remains insufficiently transparent. The manuscript does not clearly specify the underlying interactome and annotation databases used within the SAveRUNNER framework, nor their versions, which limits reproducibility. The analysis appears to rely primarily on existing tools rather than a fully specified network model, and no assessment of the robustness of the drug ranking is provided.

The data availability statement reflects this imbalance. The proteomics dataset is appropriately deposited in ProteomeXchange (PXD058349) and made accessible to reviewers, which is commendable and ensures reproducibility at the data level. In contrast, the dataset underlying the machine learning analysis is not made available; only a GitHub repository for the analysis pipeline is provided, and it is described as still being developed . Given the concerns about data splitting and sample independence, access to the underlying LFP data is essential to evaluate the validity of the analysis. As it stands, the machine learning results cannot be independently reproduced or verified.

Overall, while the experimental and proteomics components of the study are strong, the computational analyses, particularly the machine learning component, are methodologically weak and over-interpreted. I recommend that the authors substantially revise this part of the manuscript, either by redesigning the analysis with appropriate evaluation protocols (if feasible), or by clearly reframing it as exploratory and tempering the associated claims. Clarification of the network analysis pipeline and improved transparency regarding computational methods would further strengthen the work.

Reviewer #3

(Remarks to the Author)
Gorda, Bouget et al have made a remarkable effort to address most of the concerns regarding their work. In particular, it is very appreciable to see that the study emphazises much more on the potential of gabapentin as a symptomatic drug for TAHV infections and their conclusions strictly stick to this effect without over interpretation. The novel presentation of the work together with the additional data (huge amount of work really appreciated, congrats) is now really convincing regarding the authors claims. I don't have further concerns.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The revised manuscript has been substantially improved, and the authors have addressed many of the previous technical concerns. The new data, including direct viral staining, improved tropism analysis, synaptic marker validation, proteomic visualization, representative OPAB electrophysiological traces, clearer replicate information, and an in vivo mouse experiment, all strengthen the study.
However, I still have a major conceptual concern regarding the coherence of the evidence across the three experimental systems. At present, the authors use different readouts in different models: in human cerebral organoids, they show viral infection and changes in synaptic protein expression; in human adult brain explants, they show electrophysiological changes; and in mice, they show transcriptional changes in neurotransmission-related genes. Although these findings are broadly consistent, they are not linked by a common functional endpoint. As a result, the central conclusion that TAHV induces synaptic dysfunction and neuronal hyperexcitability, and that gabapentin counterbalances this process, is supported by separate lines of evidence rather than by a unified cross-model demonstration.
To strengthen the manuscript, I recommend that the authors add electrophysiological characterization in both human cerebral organoids and mice. For the cerebral organoids, MEA recordings or another appropriate electrophysiological approach should be used to determine whether the observed loss of synaptic proteins is accompanied by functional changes in neuronal network activity, such as altered firing rate, burst frequency, network synchrony, oscillatory activity, or other relevant parameters. This would directly connect the proteomic and synaptic marker changes in hCOs with the

hyperexcitability phenotype observed in OPABs.
For the mouse model, in vivo electrophysiological assessment, such as EEG or LFP recordings, should be added to determine whether the transcriptional changes in grm1, gria1, or other neurotransmission-related genes correspond to measurable alterations in brain activity. This is particularly important because the infected mice do not show overt neurological symptoms, and the current mRNA data alone cannot establish functional neuronal dysregulation or functional rescue by gabapentin in vivo.
A unified electrophysiological endpoint across models would substantially improve the logic of the study. It would allow the authors to test whether TAHV infection consistently produces neuronal hyperexcitability or related network-level dysfunction in hCOs, OPABs, and mice, and whether gabapentin mitigates the same type of functional abnormality across these systems. Without such data, the manuscript should avoid presenting the hCO synaptic protein changes, OPAB electrophysiological alterations, and mouse transcriptional changes as a single continuous mechanistic chain.
Therefore, I recommend major revision. The authors should either provide electrophysiological validation in hCOs and mice, or substantially temper the main claims to state that TAHV infection is associated with synaptic protein remodeling in hCOs, hyperexcitability in OPABs, and altered neurotransmission-related transcripts in mice, while the functional connection among these observations remains to be established.

Reviewer #2

(Remarks to the Author)
The authors have substantially improved the machine learning analysis and, importantly, have revised both the methodology and the interpretation of the results. The use of held-out recordings instead of randomly split time windows addresses my main concern regarding data leakage, and the manuscript now clearly acknowledges the donor-specific and exploratory nature of the models. The additional reporting of performance metrics and the release of the underlying dataset further improve transparency.

One caveat remains. The manuscript reports 10-fold cross-validation metrics, but given the small number of recordings per donor it is unclear how these folds were constructed. If the cross-validation was performed on segmented windows rather than independent recordings, the resulting accuracy estimates should be interpreted with caution and should not be viewed as an independent assessment of generalization performance. In my view, the held-out recording results provide the more meaningful evaluation.

Most importantly, the main conclusions of the manuscript are supported by multiple independent experimental approaches and do not rely critically on the machine learning analysis. I consider my major concerns to have been adequately addressed.

Point-by-point response to editor and reviewers

Reviewers' comments:

Reviewer #1

In this manuscript, the authors utilized a proteome-based network medicine approach to identify Gabapentin as a potential treatment for bunyavirus Tahyna (TAHV) induced neuroinfection. Specifically, the authors demonstrated that TAHV infects human cerebral organoids (hCO), induces proteomic alterations in neural pathways and synapse-associated proteins, and identifies Gabapentin as a potential agent to mitigate TAHV-induced electrical disturbances in hCO. The authors further validated the rescue effect of Gabapentin in the TAVH-infected post-mortem adult human brain (OPAB) explants. Previous studies reported that bunyavirus infections lead to neuronal apoptosis and reduced organoid viability in hCO [1-2]. Additionally, the authors previously demonstrated that TAHV infection disrupts local field potentials in OPAB and developed a machine-learning framework to predict OPAB infection status [3]. Thus, the novelty of the study is limited.

We thank the reviewer for highlighting the novel and "less novel" aspects of our work. We fully agree with the reviewer that the decrease of viability upon bunyavirus infection has been previously shown, and we do not claim that this is an important message of our manuscript. It is actually not mentioned in the title nor the abstract, and it represents a single panel in former **Figure 1B** (new **Figure 1C**). In particular, the decrease of viability is not significant, so we cannot claim important neuronal death upon infection. We considered that it was important to characterize this in our model, as the studies cited by the reviewers have not shown TAHV cytotoxicity in organoids. We might have overemphasized the toxicity effect in the previous manuscript, and according to the reviewer's suggestion, we are now citing the two articles mentioned, to clearly state what is already known about it. Again, viability is a small aspect of this study and should not be considered as a lack of novelty but rather a necessity to characterize the system. However, our data demonstrates that even without detectable neuronal death, we could notice a loss of neuronal markers (**Figure 1E**), synaptic markers (**Figure 2C-E and Suppl. Figure S2B**), and glial markers (**Supp Figure S1A**), which together reveal an important impact of TAHV infection on neural integrity and composition.

The second point of the reviewer mentions that it was already shown that TAHV disrupts local field potential in OPAB, based on our previous work (Partiot, Gorda, et al, EMBO Mol Med, 2024), and we fully acknowledge it. However, this new manuscript does not aim to show TAHV-induced perturbation, but instead: 1) to characterize the TAHV infection of organoids (fully new), 2) proteomic analyses of TAHV-infected organoids (fully new), 3) that TAHV diminishes the expression of synaptic components in organoids (fully new), 4) to identify and characterize GBP as a symptom-oriented therapeutic strategy (fully new), and 5) to show that GBP rescues TAHV-induced electrical perturbation, including firing activity (fully new). Indeed, the only overlap with the previous study is the fact that we took advantage of a machine learning framework developed in the previous manuscript, to serve a fully novel question: whether and how GBP restores TAHV-induced synaptic dysfunction. Furthermore, we have now added new data performed in mice to highlight that TAHV perturbs neurotransmitter receptor expression, which can be rescued upon gabapentin treatment.

Given the above-mentioned facts, we would like to respectfully disagree with the reviewers' comment and propose that our work provides significant novel information. Furthermore, the network medicine pipeline and identification of a symptom-oriented neuromodulatory strategy

are contributions that we consider to be of significance and new, in particular in the context of virus-induced neurological dysfunction. We hope that these clarifications and new data will convince the reviewer as well.

Additionally, the major argument is not well supported by the presented data. To improve the quality of the manuscript, the authors need to consider the following issues: Major issues:

1. Evidence level of TAHV infections is low and based on the mRNA level. The authors need to stain TAHV proteins using immunofluorescence or RNA FISH to provide direct evidence of the infection process.

We believe that there is a misunderstanding here. We might have not presented clearly enough the data, and we therefore have remastered the **Figure 1A** to better highlight the high permissivity of brain organoids to TAHV. As you can now see, the inoculum (day 0), is at normalized vRNA levels of 1 (log10(1) =0), while at Day 2, it is about 4 $\log_{10}$ higher (dark blue), and it keeps increasing until Day 10 post-infection (where it is more than 5 $\log_{10}$ higher than the inoculum). Moreover, we used a UV-inactivated TAHV (grey bars) to further highlight that vRNA does not increase in the case of replication-defective TAHV. Because we put the same volume, thus UV-inactivated virus can be considered as the input, which can barely be seen on the graph because it is so close to zero! Moreover, RG10b, an antiviral drug targeting viral replication, exhibits an antiviral activity in those settings, further demonstrating active TAHV replication. Next, we show that new infectious viral particles are produced in infected organoids, as plaque assays performed from the homogenates of infected-organoids reveal an increase in TAHV titer compared to the inoculum (more than 2 log increase when comparing day 2 post infection vs 5 hpi). Hence, we can unambiguously confirm that high TAHV replication occurs in brain organoids. We do not believe that this can be considered "low", although this remains a relative statement. Our lab routinely infects brain organoids with numerous viruses from different families (including SARS-CoV-2, Zika Virus, West Nile Virus, HIV), some being published, and TAHV is the far most infectious virus of the list. Anyway, according to the reviewer's suggestion, and thanks to a kind gift of an anti-bunyavirus antibody by Dr. Karin Peterson, we could now perform the requested experiment and show that TAHV infects brain organoids by immunofluorescence (**new Figure 1D**).

2. It's better to co-staining TAHV with markers for various cell types (e.g., neural stem cells and neurons), which is essential to clarify TAHV tropism toward specific neural cell types.

TAHV neuronal tropism was previously shown, and because we did not have appropriate antibody we did not assess this relevant question at the time, although we discussed this point in the previous version of the manuscript. However, thanks to the newly obtained TAHV antibody, we were able to confirm TAHV infection of brain organoids (new **Figure 1D**). Unfortunately, we were unable to accurately identify tropism because of the loss of our usual cell type markers GFAP and MAP2. Although we could not co-stain TAHV and neuronal markers, our data strongly support TAHV neuroinvasion in brain organoids. These data are now included in new **Figure 1D and Supp Figure S1A**.

3. The staining pattern seems inaccurate (false positive) for Bassoon and Homer1 proteins in Figure 2F-H. The authors need to provide high-resolution confocal images to enhance

reliability. Additionally, I recommend the authors to perform WB of these two proteins to characterize changes in synaptic protein expression following TAHV infection. The authors can confirm this argument with PSD95 and synaptophysin staining.

We thank the reviewer for this rightful comment. Although these images presented in former **Figure 2F-H** are from confocal microscopy, the low magnification and the loss of resolution from the pdf conversion, made the staining very difficult to interpret. Therefore, we are now providing new pictures of the various synaptic stainings as well as controls (**Figure 2C** and **Supp Figure S2B**). As requested by the reviewer, we also performed western blot analysis of Synaptopodin, PSD-95 and Homer1 and confirmed that TAHV decreases the expression of various synaptic components to different extents, and the data are now shown in new **Figure 2D**.

4. The authors need to improve the image resolution quality of Figure 2B.

We would like to apologize for the low quality of the images. These images were of high resolution but the different formatting processes shrunk image quality of the figures. We have now improved overall Figure resolution and hope that the submission process and reformatting will not be too deleterious.

5. It's better to provide proteome analysis of differentially expressed proteins in volcano plots or Venn plots.

As suggested, we are now providing a Volcano plot of the differentially expressed proteins (**new Figure 1G** and provide the complete list of up- and downregulated proteins in **Table S1**.

6. In the PCA analysis (Figure 4E, S3B), the cumulative variance contribution of the first two main components was ≤ 20%, which means the identified factor is not significant enough. The authors need to discuss these results.

We thank the reviewer for this comment. Given the high signal heterogeneity of OPAB local field potential, it is hard to expect a high cumulative variance of PC1 and PC2, which would actually be suspicious and unrealistic. Also, guidelines on PC analyses do not determine what is a significant enough threshold for PC1/PC2. Nevertheless, we agree with the reviewer that PCA data from fast Fourier transformed LFP may be confusing and difficult to interpret, thus we removed the PCA from our manuscript, as TAHV impact on neuronal activity and the benefit of GBP are evaluated by different functional analyses.

7. The authors only demonstrated the statistical charts of the electrophysiological data. The authors also need to provide representative neurophysiological recordings for each group and illustrate the amplitude and frequency of electrical signals. Additionally, neural organoids usually generate robust neuronal spike after three-month culture and bursts after six-month culture.

As suggested by the reviewer, we have now added representative electrophysiological recordings of each conditions in **new Figure 3E**.

We thank the reviewer for the rightful comment about brain organoids exhibiting low levels of activity in early cultures. The local field potential generated by organoids is nevertheless above background (5 SD), and sufficient to perform differential analyses. However, this is true that

we rarely observed robust bursts at this stage, although others have been able to show burst activity as soon as 5.5 weeks post-differentiation (Osaki et al, Nature Comm, 2024). Hence, we believe that we have observed some electrical parameters that were reliable in our system, but that further research could highlight other interesting aspects, in particular in the context of bursting neurons. As OPABs are 3D primary mature human-based neural models, we focused on OPABs functional analysis in the new version of this manuscript and removed electrophysiological hCO data.

8. The authors are encouraged to conduct animal experiments to validate the reparative effects of GBP on TAHV-induced electrophysiological and motor behavior disturbances.

We understand the point raised by the reviewers, and have now performed animal experiments to evaluate TAHV neuropathogenesis *in vivo* (**Figure 4**). We validated that following intranasal infection, TAHV invades mouse brains, where GBP has no antiviral effect (**Figure 4C**), as observed *in vitro*. As TAHV infection failed to induce clinical neurological symptoms in our mouse model, potentially due to age and strain specificities, we could evaluate the clinical benefit of GBP but we show in **new Figure 4** and **Suppl. Figure S4** that TAHV is neuroinvasive, but did not significantly impact neuroinflammatory-related mRNA expression (new **Figure 4D-H**). In contrast however, we observed that the mRNA of the metabotropic glutamate receptor 1 (grm1), a major player of neurotransmission contributing to fine-tuning of synaptic plasticity, was significantly upregulated upon TAHV infection, a phenotype that was potently reverted upon GBP treatment (new **Figure 4I**). Of note however, electrophysiological analyses in mice are very complex methodologies to be implemented in a virology lab like ours, and we were thus unable to reach this level of investigation, which we believe are beyond the scope of this work.

Minor issues:

1. *In Figure 1E, the MAP2-specific expression is unclear. The authors should provide high-magnification confocal images to improve clarity.*

As requested, we are now providing high-resolution images of infected and non-infected neurons in brain organoids in **new Figure 1E**.

2. Certain figures, such as Figures 2D–2E, 3A, and S2, lack clarity. The authors should enhance their overall quality to improve data readability.

We apologize for the low quality of the figures and we have now enhanced them all in the new version of the manuscript.

3. The authors should specify whether Figure 2H was stained in the Mock group or in TAHV-infected organoids.

The former **Figure 2H** show images acquired on Mock-infected condition. This information has now been added to the figure legend.

4. The authors should describe Figure S2 in the results section, as it is currently not mentioned in the manuscript.

The paper has been reorganized and this issue is now addressed.

References:
[1] Winkler, Clayton W., et al. "Neuronal maturation reduces the type I IFN response to orthobunyavirus infection and leads to increased apoptosis of human neurons." Journal of Neuroinflammation 16 (2019): 1-16.

[2] Ojha, Durbadal, et al. "Rottlerin inhibits La Crosse virus-induced encephalitis in mice and blocks release of replicating virus from the Golgi body in neurons." Nature Microbiology 6.11 (2021)1398-1409.

[3] Partiot, Emma, et al. "Organotypic culture of human brain explants as a preclinical model for AI-driven antiviral studies." EMBO Molecular Medicine 16.4 (2024): 1004-1026.

Reviewer #2 (Remarks to the Author):

This study focuses on demonstrating the potential of network science in drug repurposing, in the case of viral neuroinfection. The case study they choose is significant, as it is under-studied, and it lacks comprehensive, vast datasets. The study is based on a versatile combination of experimental and computational methods (network models and machine learning). I focused in my review on the computational part of the paper. Overall, I found some major problems in that part of the study, especially a lack of explanations and details that hindered my evaluation of the significance and of the solidity of the computational results. I list my comment below.

We thank the reviewer for pointing out the significance of our work and versatility of our combined approaches.

Major comments

- The data used for training the machine learning models is not described. There is a reference to it on page 22, line 562 ('available data for machine learning analysis'), but that part of the text is missing from the manuscript.

We apologize for this. Indeed, part of the data analysis description was missing in the previous version of the manuscript. We have now added a paragraph "Data availability" in which we mention the GitHub link of the RFC-based analysis pipeline.

- The random forest classifier seemed to work well on 2 donor models, and less well on one. Absent a larger validation set of donor data, some discussion on what this means is needed. It seems that a good classifier may need more features than those in the current data, I wonder if the authors can speculate on what they may be.

We thank the reviewer for this rightful comment. Indeed, the RFC did not work well on one donor. To be able to better interpret what this could mean, we analysed an additional donor and rerun the whole analysis at the donor level. As suggested by the reviewer, we tried to identify why the RFC was not always working, and based on our new data, we could show that TAHV was causing an increased firing rate in donors 1,2 and 3, but not in donor 4 (**Suppl. Figure S3C**). Hence, because significant perturbations were observed in these three donors, our RFC

algorithm would work very well. In contrast, the electrical activity of donor 4 was not very impacted by TAHV perturbations, and thus RFC not successful. This makes a lot of sense and we thank the reviewer for his/her comments as we are now able to better explain the observed phenotypes. As such, we have rearranged the manuscript in **Figure 2-3** to show these differences, and discuss it in the new version of the manuscript.

- Line 148: "Comprehrensive network analysis of dysregulated pathways reveals…" Please add details on how this analysis was done and what were its concrete conclusions.

We apologize to the reviewer as this information was not clearly detailed. In the previous manuscript, we used STRING-db for proteomic analysis. In the new manuscript, we analysed the data using SynGo, GSEA analysis and GO analysis. The information regarding these methods has now been added in the Material & Method section of the new manuscript.

- Line 157: "Quantitative analysis revealed that …" Please explain the details of this quantitative analysis.

This was a quantification based on microscopy images. We have added this information and further described in the Material & Methods.

- It is unclear to me what "Mock", "Mock-infected" and "Mock/TAHV-infected" are. This is important to clarify because Mock plays a key role in the design and training of the machine learning models. I assumed in my reading that it means the control, non-infected samples.

"Mock" corresponds to non-infected hCO exposed to a volume of supernatant from non-infected cells equal to the one used for TAHV infection to avoid media composition bias. This has been clearly stated in the new version of the manuscript.

- Figure 2D: the color code is unclear. Clarifications are needed on whether it applies both to node coloring, as well as to interaction coloring, on how to interpret multi-color coloring applied to some of the nodes, on how interactions should be interpreted, and on how the network was generated. This is better explained in Figure S2. There is a mention in the caption of Figure 2 to "String network analysis" that I am not sure how to read, did you mean that you used the STRING database? If yes, how?

We thank the reviewer for this comment. The network was generated with the STRING database. As new bioinformatic analyses were conducted, this STRING analysis was removed.

Minor comments

- There is a strange change of font size in the middle of the Introduction.

This has been corrected.

- Introduce the abbreviation FC.

This has been added.

- Are the network models and the machine learning models available? What about the dataset used to generate/train them?

We have now added a paragraph "Data availability" in which we mention the GitHub link of the RFC-based analysis pipeline. As mentioned in the dedicated method section "***Machine learning algorithm for LFP analysis***": each donor gave rise to one dataset, each whole dataset was separated into a training dataset containing 70% of the data to fit an RFC model, and a testing part containing the remaining 30%, which served as unseen data to test the previously established model.

Reviewer #3 (Remarks to the Author):

In this manuscript, Gorda, Bouget et al describe the infection of human cortical organoids with bunyavirus Tahyna (TAHV) in order to identify drugs potentially able to alleviate symptomatic manifestations of the brain infection in humans. Although the concept is interesting, the presentation of the topic confuses different concepts (e.g. antiviral drugs vs symptomatic drugs, neurological manifestations due to direct neuroinfection or indirectly in post-infectious status (post-COVID syndromes), etc) and exaggerate the hazard of TAHV, especially as compared to other viruses from the same serogroup. The efforts pulled by the authors culminate with the identification of gabapentin, an anticonvulsant drug.

We thank the reviewer for pointing out the interesting nature of the concept presented in our article. However, it is unclear to us what the reviewer means by "presentation of the topics confuses different concepts". Indeed, the reviewer rightfully points out that we explained the difference between drugs that target the virus versus targeting the symptoms (as specifically stated in the introduction of our former manuscript "Current strategies for antiviral development mostly rely on the targeting of viral or host factors. While essential, this viro-centric approach needs to be complemented with symptom-oriented development of small molecules aiming at restoring or counter-balancing the functionality of infected tissues.". However, as these are opinions rather than factual introductory statements, we reshaped the introduction section to make it more traditional and provide general information for readers to contextualize our work.

The reviewer also mentions direct versus indirect cause of neurological manifestations. We indeed included in our introduction examples of neurocognitive disorders such a long COVID, for which there is abundant literature, but we acknowledge that this might be confusing, because in this case, it remains controversial whether this is due to a peripheral infection of direct persistence of the virus in the brain as previously proposed (Stein SR et al, Nature, 2022). As this is not the main focus of our work, we should not enter such debate. We have now rephrased to "viral exposure" and rather point to the general COVID-19 infection associated with neurocognitive symptoms.

We apologize if our wording on TAHV hazard sounded exaggerated. We presented current literature on various orthobunyaviruses, and then underlined the very few studies published on TAHV in mice and humans. We believe that we remained factual ("shown to induce neuromotor dysfunctions in mice, and associated with febrile illness in patients"), but we understand that it lacks some context, highlighting in particular that this is rare and not well documented. We have now changed this in the new version of the manuscript.

General comments:

This study is clearly interesting in the sense that it uses human cerebral organoids (HCO) to assess the effect of TAHV infection of neurons and test the potential of drugs. HCO represent indeed a novel and promising avenue for this type of studies.

We thank the reviewer for pointing to the novelty and promising nature of our study.

Nevertheless, the reviewer has several concerns as some conclusions are not really supported by data. First, given that other viruses from the same serogroup (California, La Crosse) as the TAHV are known to induce seizure and that anticonvulsant drugs are already used for treating those patients, the potential usefulness of gabapentine in TAHV infection as revealed by HCO does not come as a surprise.

To our knowledge, there is no report of the use of anticonvulsant to treat La Crosse virus infection to our knowledge, and pubmed retrieves no results when prompting "gabapentin la crosse virus" or even "gabapentin bunyavirus". Furthermore, the CDC indicates that there is currently no recommended treatment against La Crosse virus infection. As supportive treatment, CDC recommends "Patients with severe meningeal symptoms often require pain control for headaches and antiemetic therapy and rehydration for associated nausea and vomiting.". Hence, we truly believe that our work provides important information that can help orientate therapeutic strategies.

One may assume that the potential usefulness of Gabapentin is obvious for clinicians, but we believe that this is a relatively hazardous statement, if it has never been experimentally demonstrated. Here, we did not "confirm" existing data, we literally point towards the potential efficacy of a therapeutic strategy to treat a disease. Of course, this needs to be confirmed in patients, but this represents in our opinion an important first study with key experimental validation. Moreover, beyond the potential clinical importance, this finding also validates our methodological pipeline, using hCO and network medicine approaches (bioinformatics on perturbomes) to propose novel supportive care strategies. According to comments by another reviewer, we added additional information regarding the rescue of neurotransmitter receptor expression in mice upon gabapentin treatment of infected mice. This is probably still not enough to demoinstrate clinical efficacy, but this is in our opinion an important step forward. Finally, we reformulated our manuscript so that the main message that we convey is that TAHV is able to invade neural models in which it causes functional disturbances that can be pharmacologically targeted.

Second, important control data are lacking to support a strong effect of gabapentine on TAHV infection and its symptoms. For instance, it would be crucial to determine what the effect of gabapentine on non-infected neurons is.

There is a heavy literature on Gabapentin in non-infected neurons. Specifically, it has been shown to have a high affinity for binding sites throughout the brain corresponding to the presence of the voltage-gated calcium channels, especially α-2-δ-1, which seems to inhibit the release of excitatory neurotransmitters in the presynaptic area that participate in epileptogenesis. In the previous version of the manuscript, we presented in the former Figure 4I, that GBP has no significant effect on the firing rate of non-infected brain explants, but GBP dampens the increase in firing activity induced by TAHV infection (new **Figure 3E**). The new data performed in mice also include all along a condition with gabapentin in the absence of infection in **Figure 4** and **Suppl. Figure S4**. We hope that this new data and clarifications will address adequately the reviewer's concern.

Another point of concern is that it is unclear how the authors came to pinpoint GBP. To correctly sustain that their proteomic-based approach led them to identify GBP, the authors should also assess the potential of other hits in their candidate list and demonstrate that most of the top hits do present some effect on the proxy of symptoms used in the in vitro model. Without this demonstration, the identification of BGP may appear as the result of chance rather than really due to this approach.

We apologize for the lack of details on how we came up to identify and pick GBP among our hits. Because this is a global bioinformatic analysis, we ended up with many hits that cannot be used as potent antiviral therapy, such as amino acids, copper, NADH (now presented in **Figure 3B** and the full anlaysis is provided in Suppl. Table S2). These compounds might still be of interest to understand TAHV perturbations, but are unlikely to have major effects on infection, might even be toxic, and may exhibit limited bioavailability in the brain.

Among actual drugs identified, drugs that have no known neurological effects such as dexlansoprazole (gastric) and terbinafine (skin), were not investigated further as it was more logical to focus on GBP, because of its known neurological potential and safety, as well as the fact that two other GBP derivatives were also identified among the hits. The chances that 3 drugs with the same properties come out of an unbiased bioinformatic analysis are extremely low. Specifically, each of them has a significant p value < 0.05; they represent 3/74 identified drugs out of a total of 1875 possible drugs. Moreover, we also tested a well-known antiviral molecule, ivermectin, which did not result in promising data (shown in **Figure 3C** and **Suppl. Figure S3B**).

Finally, we would like to stress that the type of analyses that we performed to validate the drug efficacy, using four brain donors and multiple slices for four conditions, are very heavy, costly, and time-consuming. Because the readout is so demanding, it would have been unrealistic to test so many drug treatments and we rather sorted them out based on likelihood to have an effect. In this regard, Ivermectin and Gabapentin were picked, and we proved the importance of GBP as a symptomatic TAHV treatment. Nevertheless, we fully agree that other drugs of the list might be of interest. As this strategy might not have been detailed well enough, it has now been clarified in the new version of the manuscript.

Major comments regarding the data and interpretations:

Figure 1

- The antiviral effect of RG10b can't be classified of moderate given that only one out of the four timepoints demonstrated a significant effect. The antiviral potential is thus low.

We recomputed statistical comparisons for those data, using $\log_{10}$FC transformed values, two-way ANOVA followed by Sidak multiple comparisons revealing an effect of RG10b at reducing the viral load at 10 dpi. We agree that the antiviral potential remains low and emphasized less on this small molecule as it is not the main message of this work.

- The authors also claim that treatment with RG10b induce little toxicity but the graph shows a higher toxicity with RG10b than TAHV alone, thus in contradiction with their conclusion of virus-induced toxicity. In addition, treatment with RG10b alone is missing to draw the proper conclusion on toxicity.

To clarify this point, we rephrased our analysis for this figure panel, and show that TAHV does not induce significant cytotoxicity (measured by LDH release assay), which remains true when organoids are infected with TAHV and treated with RG10b. The comparison between TAHV versus TAHV+RG10b showing no statistical difference and is highly similar at 7 and 10 dpi. There is no reason to believe that RG10b alone would be more toxic than TAHV+RG10b and due to time and cost constrains, we did not perform additional experiments on this occasion.

- LDH assay is used as a proxy for toxicity, yet it can be influenced by different parameters like metabolism or proliferation which are not controlled in this experimental setting. Thus the author can't present their data as cytotoxicity data. In addition, the differences are not significant thus the data do not support the sentence "some viro-induced cellular toxicity".

The measurement of LDH levels using the "LDH-Glo™ Cytotoxicity Assay" from Promega is a traditional proxy to assess for neural cytotoxicity. Actually, the increased activity of Lactate DeHydrogenase is used as a predictive prognostic of brain damage for many decades. Moreover, LDH assay is not dependent on cell proliferation as it is released by dying cells and not by live/proliferative cells. Hence, measuring LDH in the supernatant allows to directly measure the relative number of cells at different times post-infection. Other methods, such as CellTiter Glo, are in contrast dependent on cell proliferation and metabolism, and are not recommended for this type of study. To make this point clearer, we have better described the LDH cytotoxicity assay in the new version of the manuscript.

- The suppl figure 1 does not provide correlation between nuclei morphology and toxicity, not even for the control GA thus it is unclear what additional information this suppl figure 1 provides.

We understand the reviewer's concern here, and apologize for the vague statements we made. The reviewer is right, we could not see significant difference of toxicity, but based on the shape of the nuclei in former Suppl Figure S1 and the loss of MAP2 staining later on, we wanted to fairly mention that the organoids seemed damaged upon TAHV infection. This is true that the images of nuclei morphology do not very well illustrate our point and thus, according to the reviewer's comment, we removed this data in the new version of the manuscript. Furthermore, we toned-down our claims based on the LDH assay overall to avoid overinterpretation of the data.

- MAP2 assessment is not enough to claim profound alterations of hCO integrity. Other neuronal and non-neuronal markers should be assessed. In addition, out of several thousands proteins tested, the relatively low number of dysregulated proteins (50 up and 87 down) and the limited toxicity (LDH readout) does not speak for profound alterations.

We thank the reviewer for his/her comment, as this is a crucial point raised here. By providing a volcano plot, we can better justify the important proteomic remodelling observed following the infection (**new Figure 1G**). Notably, more than 590 proteins are differentially expressed following the infection (FDR<2.65%). The 50 and 87 proteins detailed relates to the ones with highest fold change. There is a profound alteration of the organoids based on the many stainings we did, and we apologize to not have documented them well enough in the previous version of the manuscript. Therefore, as suggested by the reviewer, we have now included MAP2, GFAP, synaptopodin, synaptophsyin, bassoon and PSD-95 immunostainings to further illustrate the TAHV-induced alterations we were mentioning (**new Figure 1E, 2C, Suppl. Figure S1A and S2B**). We also provide WB analysis confirming the decrease in synaptopodin, PSD-95 and Homer1 abundance upon infection (**new Figure 2D**).

FISH or other RNA visualization method should be used to assess the extent of infection (ratio of infected cells over total cells). Finally, since the authors use hCO, it would be interesting to have an idea of the impact of TAHV infection not only on neurons, but also on glial cells.

We could not make the FISH work in organoids so far, probably because of the thickness of the samples and the difficulty for the probes to penetrate deep enough. Moreover, there is no commercially available antibodies. Following the reviewers' comments, we obtained an anti-orthobunyavirus serum that allowed us to address this point. We confirmed TAHV neuroinvasion in hCO, however could not discriminate between astrocytic of neuronal tropism, mainly because of the loss of protein expression following the infection (**Figure 1E** and **Suppl. Figure S1A**).

- The authors conclude that hCO are very permissive to TAHV infection but the total absence of infectious particle production as seen with the plaque assay speaks against the conclusion which should be revised.

It must be noted that in 3D models, most viruses are not secreted much in the supernatant as they are mostly produced within the organoid and remain stuck within the dense extracellular matrix, or uptaken by adjacent cells. To better assess TAHV release, we are now showing in new **Figure 1B** the infectivity potential of TAHV released within the organoid through the adaptation of a homogenization protocol for virus retrieval from tissues (rather than supernatant) previously published (*Partiot et al., Nature Microbiology, 2024*). We show that infected organoids produce new infectious viral particles, as plaque assays performed on organoid homogenates reveal an increase in TAHV titers compared to the inoculum, with more than a 2-log increase observed at day 2 post-infection. We do not believe that this can be considered "low", although this remains a relative statement.

Figure 2

- The authors claim that "hCO showed that Bassoon signal was mostly lost at 10 dpi, while Homer-1 remained relatively unchanged" but the pictures presented do not support this sentence and there is no attempt to provide an unbiased quantification of these altreations. There is only one picture for Mock, thus one timepoint is missing and there is no information of the timpoine presented. From the pictures, Homer seems to be incresed by infection, both 4 dpi and 10 dpi compared to Mock (not unchanged), Basson seems higher in 4 dpi than in the 10 dpi and mock thus it can't be classified as "lost".

We now provide confocal images of different neuronal, glial and synaptic markers over the course of the infection, including MAP2, GFAP, synaptopodin, synaptophysin, bassoon, PSD-95, and indicated clearly the corresponding timepoints for each condition in **new Figure 1E, 2C, Suppl. Figure S1A and S2B**. We also provide WB analysis confirming the decrease in synaptopodin, PSD-95 and Homer1 abundance upon infection (**new Figure 2D**).

- Fig 2H should provide pictures of higer resolutions for mock as well given that the low resolution picutres from 2G do not allow differentiating TAHV from Mock

We apologize for the low quality of the figures (which are in part due to the reformatting and compression during submission of the manuscript) and we have now enhanced them all in the new version of the manuscript, and hope that it will retain acceptable quality.

Figure 3

- The table B should present at least the top 10 drugs as per the results obtained, not just gabapentin, assimilates of gabapentine, and ivermectin in order to really display the potential of the approach used here.

We have now added a table with the top 10 drugs for clarity, complemented with neuromodulatory drugs of interest in our case, with the corresponding rank (**Figure 3B**).

- A dose-response should be performed to correctly assess the antiviral effect of both ivermectin and GBP to support the conclusions written.

We used two concentrations of Ivermectin, and the highest dose was toxic to the organoids while showing no significant antiviral activity. Regarding GBP we used a concentration of 100 µM, which is on the high end. Moreover, this concentration was used in subsequent experiment, proving that GBP functionally rescues TAHV-induced electrical perturbation without affecting virus replication. Nevertheless, we did the experiment in human neurons showing that ivermectin is highly toxic at antiviral concentration, and that GBP does not limit viral replication at 3 different concentrations (**Figure 3D** and **Suppl. Figure S3B**).

Figure 4

- The author mention that GBP results in misclassification of infection status by their algorithm but the data displayed still present with more than 70% of correct classification in the treated infected group thus showing at best a moderate effect of GBP in this model (about 15% of reduction, not more). In addition, there is no statistical test to assess if TAHV and TAHV+GBP results are significantly different.

As suggested, we now present the data from 4 different brain donors (new **Figure 3E** and **Suppl. Figure 3C**). In 2 donors, gabapentin was able to nearly fully rescue a physiological phenotype following TAHV infection (classification as Mock > 90%). In one other donor, gabapentin had no detectable effect, while in the fourth donor, TAHV infection produced no electrophysiological signature consistently detectable by our machine learning algorithm. Thus, we present gabapentin's action primarily as a proof-of-concept that modulating neuronal activity during viral infection may alleviate neurological symptoms.

As requested by the reviewer, we show in **Figure 3E** the presence of a statistically significant difference (non-parametric Wilcoxon test; p value < 0.01 (**)) between TAHV and TAHV+GBP, as well as between TAHV and Mock.

Importantly, LFP data should be shown, not just the results of the classification algorithm. Algorithm predictions are not enough to have an idea of the extend on perturbations triggered by TAHV infection.

We have now exemplified LFP data for each condition, and provide firing rate quantification and statistical significance (**Figure 3E**).

- The PCA indeed shows a good overlap between TAHV GBP-treated and non-treated while the divergent points with GBP do not go cluster with the mock data points, thus the authors can't claim that GBP is protective. It is changing the LFP profile, but not getting it back to the

non-infected (normal) system. As mentioned in the general remarks section, GBP alone is missing to really have all the control conditions to have a proper idea of its effectiveness.

We agree with the reviewer that PCA data from fast Fourier transformed-LFP data may be slightly artificial and ambiguous to interpret and we thus removed them from our manuscript as key conclusions from our work are supported by other more compelling data.

- All donors should be presented regarding the OPAB model, there is no reason that donors 1 and 3 are presented only in suppl figure 3 just because donor 2 looks the best one. This display is somewhat partial and does not reflect the data generated. At the minimum, statistics including all 3 donors should be done to support a significant effect of BGP. The variability due to the use of different donors can't be just resolved in stating that the infection had a different effect on donor 3. The algorithm still shows a correct classification score of more than 70% in the TAHV infected condition. This experiment should ideally be repeated to include at least 5 donors in order to assess whether donor 3 is a true outlier or whether GBP does not work for an important proportion of donors which would reduce its interest as a drug in this context.

According to the reviewer's comment, we have now added another donor. Moreover, in the new version of the manuscript we relied less on PCA and machine learning classifications to support our conclusions. Importantly, we have conducted mouse experiments that support GBP action on TAHV-induced modulation of gene expression related to neuronal communication (**Figure 4I-K**).

Minor corrections:

Regarding the manuscript itself, some statements are not adapted or over-stretched:
- SARS-CoV-2 can't be considered neurotropic with the level of scientific evidence available at the moment. The jury is still out.

We would like to respectfully disagree with the reviewer's opinion. It has been well established by us and others (Partiot et al Nature Microbiol, 2024; Stein SR et al, Nature, 2022) that SARS-CoV-2 can infect neurons and thus has neurotropic potential. This is true however that the extent of SARS-CoV-2 neuroinvasion is relatively low and uneven. Moreover, we never stated that SARS-CoV-2 is neurotropic, but only that COVID-19 is associated with neurological disorders, which is very well documented by numerous labs and high-quality clinical studies.

- Virus-induced and not viro-induced; virus-centered and not viro-centric

This has been corrected.

- Precise the meaning of "in cellulo", it is not clear if it is used as a synonym of in vitro or also encompass ex vivo cellular analysis;

This expression has been removed.

- Neurohealth is not a validated term in clinic and claiming that organoids have the potential to serve as a surrogate for global neurological health is an overstatement, the authors should stick to the potential of organoids for electrophysiological functions

As requested by the reviewer, we have removed the mention that organoids can be used as surrogates.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Point by point response to reviewers' comments

Reviewer #1 (Remarks to the Author):

The authors have addressed most of the technical and data presentation issues raised in the initial review. However, two critical scientific concerns remain unresolved, and these directly affect the robustness and experimental validity of the study's main conclusions.

Major Issues:

1. While the authors have done a reasonable job contextualizing the novelty of their viability findings and acknowledging prior work on bunyavirus cytotoxicity, a fundamental issue regarding the validity of their synaptic immunostaining data—central to their claim of "loss of neuronal and synaptic markers"—has not been addressed. This undermines the study's key conclusions. In the control (Mock) samples, the staining patterns for PSD-95 and Synaptophysin do not match what is typically seen in the literature. PSD-95, a well-known postsynaptic density marker, appears as a non-physiological, continuous reticular or mesh-like pattern, rather than the discrete punctate labeling characteristic of mature synapses. Similarly, Synaptophysin, a presynaptic vesicle protein, shows a diffuse, sheet-like signal instead of the expected clustered axonal puncta. These unusual patterns raise concerns about antibody specificity, technical artifacts (such as over-permeabilization or non-optimal antibody concentration), or insufficient neuronal maturation. Although Synaptopodin staining looks superficially consistent with dendritic spine localization, the fact that both pre- and postsynaptic markers fail to show canonical synaptic puncta means the authors' quantification of "synaptic loss" (Figures 2C–E, Suppl. Figure S2B) is unreliable and cannot be taken as evidence of TAHV-induced synaptic damage. To address this, the authors need to validate their PSD-95 and Synaptophysin antibodies using fully matured primary neuronal cultures (e.g., hippocampal neurons) to confirm that they can label characteristic synaptic puncta. Without this validation, the data do not support the conclusions regarding disrupted neural integrity and synaptic composition.

We thank the reviewer for this important point made on a technical aspect of our work. From the comments, we understand two main issues: 1. The antibody staining is not reflecting the typical pre/post-synaptic distribution of PSD95 and SYN; and 2. as a consequence we cannot conclude on the disrupted synaptic composition induced by TAHV. Both points are addressed below:

1. We have performed new immunofluorescence staining if organoids using two new antibodies: PSD-95 (Synaptic System #124 008) and Syn1 (Synaptic System #101 308) antibodies that are widely used, and have been validated in knockout experiments by the company. Using these antibodies, we performed new experiments in brain organoids, and indeed obtained more typical punctuated labeling. Hence, the images from the former antibody stainings were removed and the new data obtained with the new antibodies are shown in new Figure 2G. The manuscript has been updated with this new information. For the other figures mentioned in the reviewer's comment, there might be a confusion as the former Figure 2D was not an IF (it was a western blot); Former Figure 2E and Suppl S2B were not showing PSD95 nor Syn1 staining but Bassoon and Homer1 stainings. For former Figure 2E (new Figure 2H), the magnified snapshots highlight punctuated patterns of Bassoon and Homer1. Some diffuse staining can also be observed in the background,

although punctae can readily be thresholded. The former Suppl Figure S2B is a typical Bassoon punctuated pattern. Both Bassoon (mAb clone SAP7F407) and Homer-1 (Synaptic System #160 003) antibodies used in our study are widely used and reported for their ability to identify synapses by IF. In conclusion, our additional stainings improved the quality of the presented images and confirm the previous interpretations, as we still observe a robust loss of staining upon TAHV infection.

2. Besides these immunostainings clearly showing the loss of synaptic puncta, we were also presenting western blot analyses of synaptic components in the former version of the manuscript (former Figure 2D, new Figure 2F). Specifically, PSD-95 and Synaptopodin expressions, as assessed by WB, were strongly lost at 10 dpi, and a milder decrease of Homer-1 was also seen.

Hence, by combining immunofluorescence and WB analyses, we believe that the reviewer's concerns were addressed, which ultimately reinforced our statement about synaptic loss upon TAHV infection.

2. The authors attribute the failed TAHV–cell type co-staining to virus-induced loss of MAP2/GFAP signals, but they do not provide validation that this loss is indeed due to true protein degradation rather than staining artifacts. They also have not attempted to use alternative neural markers (e.g., NeuN, S100β, SOX2) or optimize their protocols, leaving the specific target cell types of TAHV in hCOs undefined.

In the former version of the manuscript, we showed that MAP2 (former Figure 1D), and GFAP (former Suppl S1A) stainings were lost upon TAHV infection, using reliable antibodies that we have validated repeatedly (for instance there is absence of GFAP staining at early time points of organoid developments (<45 days), as one should expect for astrocyte differentiation kinetics, which is clearly seen using this anti-GFAP antibody). To reinforce this observation, we performed a WB analysis to confirm the loss of MAP2 and GFAP in organoids (new Figure 1F). Therefore, while we are confident about these observed phenotypes, we agree with the reviewer that this is frustrating to be unable to determine the tropism of TAHV in our organoid model. Thus, as suggested, we used alternative antibodies to stain neurons and astrocytes. *In fine*, we managed to obtained co-distribution of TAHV and CTIP2, a neuronal marker, while TAHV staining poorly colocalizes with the astrocytic marker S100B (New Figure 1G-H). To further assess the number of infected neuronal cells, we performed flow cytometry using the surface markers NCAM1 and S100B and showed that >97% of the infected cells are from neuronal lineage (new Figure 1I-J). These data are consistent with previously reported in other neural systems by us and others.

Minor Issue:

Throughout the statistical figures, there is no clear distinction between biological and technical replicates, and the ambiguous "n" values (e.g., "4 organoids from 2–3 productions") compromise reproducibility. For each statistical figure in both the main text and supplementary materials, please specify in the legend whether the replicate is biological or technical, provide exact counts (n_bio, n_tech) per group, and clarify how technical replicates were incorporated into the

analysis. A consistent definition of "biological replicate" should also be provided for each experimental system (hCO, mouse, OPAB) across all figures and in the Methods section.

We apologize for the lack of clarity. We have now defined in the figure legends the type of replicate and counts.

Reviewer #2 (Remarks to the Author):

This manuscript combines proteomics, human brain organoids, electrophysiology, and computational analyses to investigate the impact of TAHV infection and to identify potential therapeutic strategies. The experimental component is extensive and, in several respects, compelling, and the link between proteomic perturbations and functional neuronal effects is of clear interest. My comments below focus specifically on the computational aspects of the study, where I have substantial concerns regarding both methodology and interpretation.

We thank the reviewer for pointing the clear interest of this work and his/her thoughtful comments regarding its compelling and extensive nature. The concerns regarding the computational aspects are addressed below.

The machine learning analysis of LFP signals is presented as an "unbiased machine learning framework" capable of discriminating infection status with high accuracy. However, based on the description in the Methods, this characterization is not justified.

We agree with the reviewer that this wording is not fully accurate, and we have removed the term "unbiased" in the new version of the manuscript.

The construction of the dataset relies on splitting each 1-minute recording into multiple 2-second segments, which are then treated as independent samples and randomly divided into training and test sets (70/30 split). Because these segments originate from the same underlying recording, they are strongly correlated. As a result, the random split likely introduces substantial dependence between training and test data, leading to data leakage and an inflation of the reported performance. Under such conditions, classification accuracy above 90% cannot be interpreted as evidence of genuine predictive ability.

We thank the reviewer for this rightful comment. Our aim here was to highlight the presence of unique features in the infected samples for which we thought splitting in time would be sufficient. However, we do agree with the reviewer that this is not sufficient to claim generalization across slices or donors and now acknowledge this issue explicitly in the results section. To address the issue raised by the reviewer, we came back to the initial electrophysiological recordings and split

them prior to any processing in a train and a test batch. As such the training batch used per donor now corresponds to two recordings out of three per OPAB, and the remaining third recording is used for validation purpose thus being a 2/3 to 1/3 split ratio. With this analysis, presented in new Figure 3A), we confirmed that test accuracy was high for donor 1 and 2 (0.88-0.92), less efficient but still good for donor 3 (0.8), and unreliable for donor 4 (0.67). These results are consistent with the data presented in the previous version of the manuscript, although it is not as high as before. As suggested however, we are unable to run accurate tests across donor due to the lack of sufficient number of donors, and we are also acknowledging this limitation in the new version of the manuscript.

More fundamentally, the effective number of independent biological units in this analysis is the number of donors, which is four. With such a limited sample size, it is not possible to design a robust machine learning analysis that supports generalizable conclusions across individuals. The current approach, in which models are trained and evaluated separately within each donor, does not address cross-donor generalization and therefore cannot substantiate claims about consistent infection-related signatures. The variability observed across donors (e.g., lower performance in one donor) is attributed to biological heterogeneity, but the evaluation protocol itself does not allow such an interpretation. In my view, the machine learning component should be regarded as exploratory at best, and the use of the term "unbiased" is inappropriate in this context. I do not see how a robust machine learning design can be done with only 4 donors.

As mentioned in the answer to the previous question, we agree with the reviewer that there is not enough data for accurate generalization across individuals. In this work, we focused on the use of machine learning as a useful tool to better comprehend the influence of TAHV infection in OPAB, and we were not aiming at providing a generalized model for infection-related LFP analysis. We have now mentioned this explicitly. Moreover, we added the extracted feature importance of all four donors in new Figure 3B to underline the donor-dependent heterogeneity.

The implementation details of the classifier further exacerbate these concerns. The Random Forest model is used with default parameters from scikit-learn, notably with max_depth=None, which allows each decision tree to grow until leaves are pure or contain a single sample. This effectively removes depth-based regularization and permits very deep trees, increasing model capacity. While the default max_features="auto" (i.e., sqrt(p) features considered at each split) introduces some randomness and decorrelation between trees, it does not prevent individual trees from fitting highly specific patterns in the data. In the present setting, where the number of independent biological units is extremely small and many samples are highly correlated due to segmentation, such unconstrained trees can easily memorize recording-specific structure rather than learn generalizable features of infection status. This risk is further amplified by the data splitting strategy, which likely places correlated segments from the same recording in both training and test sets. The absence of constraints on tree depth or leaf size (e.g., min_samples_leaf), and the lack of any sensitivity analysis, make it difficult to assess whether the reported performance reflects a robust signal or simply model capacity interacting with non-independent data.

The final RFC models chosen in this work were in fact the ones with best performance after comparison between multiple parameters combination performances. We apologize that this was not clearly mentioned in the previous manuscript. It was by coincidence that the default parameters worked best on our data. Nevertheless, we have now used an exhaustive grid search method across multiple selected parameters. The searched parameters were the following (according to scikit-learn python library version 1.3.1 of the RandomForestClassifier class) : n_estimator (values: 100, 200, 350), criterion (values: gini, entropy, log_loss), max_features (values:sqrt, log2, None), max_depth (5, 10, 15) and min_sample_split (2, 4, 6). The other parameters were kept as default. All of the 108 possible combination of parameters were tested and only the best performing one was kept as final model. This exhaustive grid search method was applied separately for each donor, hence giving different optimization for each donor. The tuned parameters for each donor have been added in new Suppl Table S4 and further details added in the Method section.

The evaluation is also limited to accuracy and a non-standard "confidence upon prediction" metric, without calibration analysis, baseline comparisons, or statistical validation. Altogether, the machine learning analysis, as currently designed and reported, does not provide reliable evidence supporting the claims made in the manuscript.

The confidence upon prediction metric is indeed not a standard method, but we found that it was a relevant information that provides useful information for interpretation. However, we agree that more standards methods should be used to evaluate our model's performance. Hence, we have now reported all results using testing accuracy, 10-fold cross validation accuracy, f1-score, precision, and recall. We added the information in new Figure 3A, and all tests show highly similar trends, strengthening our interpretation of the data.

In contrast, the proteome-based network medicine analysis is conceptually sound and follows a standard pipeline, linking differential proteomics to functional enrichment, phenotype association, and drug repurposing. The identification of gabapentin and its subsequent experimental validation is a strength of the study. However, the methodological description remains insufficiently transparent. The manuscript does not clearly specify the underlying interactome and annotation databases used within the SAveRUNNER framework, nor their versions, which limits reproducibility. The analysis appears to rely primarily on existing tools rather than a fully specified network model, and no assessment of the robustness of the drug ranking is provided. The data availability statement reflects this imbalance. The proteomics dataset is appropriately deposited in ProteomeXchange (PXD058349) and made accessible to reviewers, which is commendable and ensures reproducibility at the data level.

We thank the reviewer for this comment. SAveRUNNER is a previously published and publicly available R-based framework (https://github.com/sportingCode/SAveRUNNER/tree/main), developed by another lab. In the revised manuscript, we now specify the databases used within the SAveRUNNER framework, together with their corresponding versions, when available, in order to improve transparency and reproducibility.

Our study aimed to apply the already published SAveRUNNER pipeline to our dataset rather than develop a new network medicine framework or modify the underlying algorithm. Accordingly, we relied on the metrics provided by the original pipeline. SAveRUNNER primarily evaluates drug–disease associations based on network proximity and provides an associated p-value estimating whether the observed association is greater than expected by chance (significance threshold: $p \leq 0.05$). However, the framework does not provide additional robustness metrics for drug ranking.

In our analysis, "gabapentin enacarbil" showed a strong association with the TAHV disease module ($p = 6.17 \times 10^{-6}$; third lowest p-value among identified compounds), while "gabapentin" and "pregabalin" were also significantly associated ($p = 1.05 \times 10^{-2}$). The chances of identifying these three closely related gabapentinoids is $< 6.9 \times 10^{-11}$. Moreover, these drugs are all known to reduce neuronal excitability, and our observation that TAHV significantly increases firing activity in human brain explants motivated us to experimentally evaluate gabapentin in our infection models. We agree that additional functional validation of the ranking robustness would further strengthen the analysis, but given the complexity and experimental workload associated with the functional assays, systematic testing of multiple additional compounds, including lower-ranked or mechanistically unrelated candidates, is beyond the scope of the present study. To address the reviewer's concern, we have now expanded the Methods section to provide a clearer description of the SAveRUNNER workflow and its implementation in this study.

In contrast, the dataset underlying the machine learning analysis is not made available; only a GitHub repository for the analysis pipeline is provided, and it is described as still being developed. Given the concerns about data splitting and sample independence, access to the underlying LFP data is essential to evaluate the validity of the analysis. As it stands, the machine learning results cannot be independently reproduced or verified.

We apologize for not providing the dataset used for the machine learning analysis. We have now added a Suppl File S1 to allow fully transparency.

Regarding code availability, we indeed keep improving the FireLearn software being used and thus annotate it as still under development. This software is fully open access on GitHub. We now provide the version used for our analysis in the revised manuscript.

Overall, while the experimental and proteomics components of the study are strong, the computational analyses, particularly the machine learning component, are methodologically weak and over-interpreted. I recommend that the authors substantially revise this part of the manuscript, either by redesigning the analysis with appropriate evaluation protocols (if feasible), or by clearly reframing it as exploratory and tempering the associated claims. Clarification of the network analysis pipeline and improved transparency regarding computational methods would further strengthen the work.

We thank the reviewer for his/her constructive comments. As previously stated and corrected, we have now toned-down our claims in the new version of the manuscript to avoid over-interpretation of the results of our RFCs. We also reanalyzed our data and provide more methodological details

as well as the datasets used in this study. We believe that with these changes, the new manuscript retains the same main message as before, but featuring more robust analyses, while providing balanced conclusions that acknowledge strengths and limitations of the approach.

Reviewer #3 (Remarks to the Author):

Gorda, Bouget et al have made a remarkable effort to address most of the concerns regarding their work. In particular, it is very appreciable to see that the study emphazises much more on the potential of gabapentin as a symptomatic drug for TAHV infections and their conclusions strictly stick to this effect without over interpretation. The novel presentation of the work together with the additional data (huge amount of work really appreciated, congrats) is now really convincing regarding the authors claims. I don't have further concerns.

We thank the reviewer for his/her thoughtful comments.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We thank the reviewer for his/her time in supervising the reviewing of our work by an early career researcher.

Point-by-point response to the reviewers

Reviewer #1 (Remarks to the Author):

The revised manuscript has been substantially improved, and the authors have addressed many of the previous technical concerns. The new data, including direct viral staining, improved tropism analysis, synaptic marker validation, proteomic visualization, representative OPAB electrophysiological traces, clearer replicate information, and an in vivo mouse experiment, all strengthen the study.

We thank the reviewer for his/her thoughtful comments.

However, I still have a major conceptual concern regarding the coherence of the evidence across the three experimental systems. At present, the authors use different readouts in different models: in human cerebral organoids, they show viral infection and changes in synaptic protein expression; in human adult brain explants, they show electrophysiological changes; and in mice, they show transcriptional changes in neurotransmission-related genes. Although these findings are broadly consistent, they are not linked by a common functional endpoint. As a result, the central conclusion that TAHV induces synaptic dysfunction and neuronal hyperexcitability, and that gabapentin counterbalances this process, is supported by separate lines of evidence rather than by a unified cross-model demonstration.

We agree that our data are broadly consistent using orthogonal approaches, which leads to multiple lines of evidence that could be further strengthened by applying cross-model demonstrations. This limit has been acknowledged in the discussion of the revised manuscript.

To strengthen the manuscript, I recommend that the authors add electrophysiological characterization in both human cerebral organoids and mice. For the cerebral organoids, MEA recordings or another appropriate electrophysiological approach should be used to determine whether the observed loss of synaptic proteins is accompanied by functional changes in neuronal network activity, such as altered firing rate, burst frequency, network synchrony, oscillatory activity, or other relevant parameters. This would directly connect the proteomic and synaptic marker changes in hCOs with the hyperexcitability phenotype observed in OPABs. For the mouse model, in vivo electrophysiological assessment, such as EEG or LFP recordings, should be added to determine whether the transcriptional changes in grm1, gria1, or other neurotransmission-related genes correspond to measurable alterations in brain activity. This is particularly important because the infected mice do not show overt neurological symptoms, and the current mRNA data alone cannot establish functional neuronal dysregulation or functional rescue by gabapentin in vivo.

The proposed experiments, although highly relevant, are extremely complex to be performed as a revision article. In particular, in vivo electrophysiological assessment is a very technical approach that requires strong expertise and dedicated equipments that fall beyond our labs' specific skills, and actually, there are very few lab (if any) that successfully performed in vivo electrophysiology in the context of a viral infection. As per organoids.

A unified electrophysiological endpoint across models would substantially improve the logic of the study. It would allow the authors to test whether TAHV infection consistently produces neuronal

hyperexcitability or related network-level dysfunction in hCOs, OPABs, and mice, and whether gabapentin mitigates the same type of functional abnormality across these systems. Without such data, the manuscript should avoid presenting the hCO synaptic protein changes, OPAB electrophysiological alterations, and mouse transcriptional changes as a single continuous mechanistic chain. Therefore, I recommend major revision. The authors should either provide electrophysiological validation in hCOs and mice, or substantially temper the main claims to state that TAHV infection is associated with synaptic protein remodeling in hCOs, hyperexcitability in OPABs, and altered neurotransmission-related transcripts in mice, while the functional connection among these observations remains to be established.

As suggested, we have added in the discussion additional comments to acknowledge these limitations and specify that further work is needed to strengthen these observations.

Reviewer #2 (Remarks to the Author):

The authors have substantially improved the machine learning analysis and, importantly, have revised both the methodology and the interpretation of the results. The use of held-out recordings instead of randomly split time windows addresses my main concern regarding data leakage, and the manuscript now clearly acknowledges the donor-specific and exploratory nature of the models. The additional reporting of performance metrics and the release of the underlying dataset further improve transparency.

We thank the reviewer for his/her thoughtful comments.

One caveat remains. The manuscript reports 10-fold cross-validation metrics, but given the small number of recordings per donor it is unclear how these folds were constructed. If the cross-validation was performed on segmented windows rather than independent recordings, the resulting accuracy estimates should be interpreted with caution and should not be viewed as an independent assessment of generalization performance. In my view, the held-out recording results provide the more meaningful evaluation.

As suggested, we have added in the revised manuscript additional details about the use of the 10-fold cross-validation metrics in the Methods section to address the reviewer's concern.

Most importantly, the main conclusions of the manuscript are supported by multiple independent experimental approaches and do not rely critically on the machine learning analysis. I consider my major concerns to have been adequately addressed.

We thank the reviewer for approving our revisions guided by his/her suggestions, and for pointing out that our main conclusions remain supported by multiple independent approaches.