nature portfolio

Peer Review File

Visual gamma stimulation induces 40 Hz neural oscillations in the hippocampus and alters phase synchrony and lag

Corresponding Author: Ms Tjasa Mlinaric

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)
The authors tested whether gamma entrainment using a 40Hz flickering LED stimulus altered hippocampal activity and connectivity to other brain areas in 11 patients undergoing seizure monitoring for epilepsy. This manuscript is timely as there is accumulating evidence of the efficacy of this stimulation protocol in animal models of Alzheimer's disease. Patients performed an attention task during regular rhythmic or arrhythmic stimuli were shown, and the authors measured phase synchrony in the hippocampus and other cortical areas. The authors observed increased 40Hz activity measured as SNR from the average power spectrum, alongside increased phase locking values (PLV), in occipital contacts, demonstrating that the flashing stimulus was indeed modulating visual activity. In hippocampus, a large >60% number of contacts showed increase in SNR. Overall, I am positive on this manuscript and find the methods, results, and conclusions to be clear and straightforward but could use some more unpacking. I believe this will be of interest to the intracranial recordings community alongside the increasing interest in non-invasive methods for targeting cognitive decline in AD. Specific comments below:

1. The authors could do more to "unpack" their findings by showing spectrograms or representative raw traces which illustrate the purported entrainment phenomenon.
2. The connectivity results are under-developed compared to the rest of the manuscript, and the authors only provide electrode-pair averaged data (Figure 3). I believe this result needs more unpacking. What proportion of electrode pairs show significant changes in PLV or PSI? How many patients show these effects?
3. How long does entrainment last in the hippocampus? Does the degree of entrainment depend on the electrophysiological / oscillatory "state" of the hippocampus? One would expect that entrainment may not be as strong in situations in which the hippocampus is actively engaged in other sorts of tasks.
4. I applaud the use of a simple attention task, but again, this aspect of the manuscript didn't come across particularly strongly. The authors should more clearly justify what they were hoping to achieve by including an attention task.
5. I understand the justification for focusing on the hippocampus based on the AD literature, but is this the region where the authors expected the strongest entrainment effects? I was left wondering whether the hippocampus particularly susceptible to entrainment, or is entrainment equally strong throughout the brain. It is also worth considering MTL is more frequently targeted in this patient population.
6. Because the authors swept across frequencies from 30-50 Hz in the irregular stimulation condition, it may be possible (and certainly of interest) to know if there is entrainment also at other gamma band frequencies. In other words, is 40Hz a particularly "special" frequency for entrainment, or can the HPC be entrained at 35 or 45 Hz? If this cannot be assessed in the current dataset this should be mentioned.

Reviewer #2

(Remarks to the Author)
Summary:
This manuscript examines the effects of gamma-frequency non-invasive stimulation (GENUS) in epileptic patients equipped with both subdural and depth electrodes, providing a valuable dataset that builds on prior animal studies and seeks to translate key findings to humans. The topic is timely, given the growing interest in gamma stimulation and its possible therapeutic benefits. While the study offers intriguing insights into how neural oscillatory mechanisms may be modulated in a clinical population, there are substantial concerns regarding the experimental design, statistical rigor, and interpretation of the results. Given the practical challenges of collecting additional data from similar patient group, comprehensive additional

analysis and clearer justification of the findings are essential. At present, the evidence presented does not convincingly support the authors' claims regarding the therapeutic potential of GENUS in humans

Major Concerns:
1. Lack of Behavioral Evidence for Cognitive Enhancement: While the study demonstrates hippocampal entrainment at 40 Hz, it does not assess whether GENUS leads to improved cognitive function. As authors know as well, prior animal studies examining GENUS effects have included either behavioral assessments or molecular level evidence to demonstrate the effectivity of such a visual stimulation paradigm. The presence of hippocampal effect alone does not necessarily imply an enhancement in memory or cognition (neither support the therapeutic effect for Alzheimer's disease cases, which has been stated in introduction and abstract!). Without such evidence, the claims regarding GENUS as a potential therapy remain speculative. It would be beneficial for the authors to either include behavioral tasks or explicitly acknowledge this limitation in their discussion.
2. Control Conditions and Stimulus Equivalence: The study relies on comparisons with an irregular flickering stimulus as a control condition, but it is unclear whether the effective stimulus magnitude was equivalent across conditions. Since connectivity measures can be influenced by stimulus intensity, it is necessary to confirm that the total stimulus power was comparable between conditions. At a minimum, reporting the root mean square (RMS) of the stimulus signal and its spectral properties would help clarify this point. There is a concern if non-prediodic visual stimulation cause a 1/f type of activity that won't be revealed with the limited analysis that is provided on the neural signals in this study.
2.1 Presentation and Organization: The experimental methodology is difficult to track due to its placement in the manuscript. Figure 4, which describes the experimental paradigm, should be introduced earlier, potentially integrated with Figure 1, to improve clarity. Furthermore, the methods section should be reorganized to align with the journal's standard format. Careful revisions to improve the manuscript's readability and logical flow are required.
3. Lack of Raw LFP Visualizations: The manuscript does not include visualizations of raw signals across stimulus conditions, making it difficult to assess the robustness of the reported effects. Providing representative raw traces (both filtered and unfiltered) for different stimulus condition/ regions would greatly enhance transparency. Even if these figures are included as supplementary materials, they are necessary for evaluating the strength of the effect and quality of the recorded signals.
4. Specificity of the 40 Hz Effect: The authors do not examine whether the observed effects are exclusive to 40 Hz stimulation. Would a different frequency (e.g., 80 Hz) yield similar connectivity changes? A stronger justification for selecting this frequency is needed, particularly for readers unfamiliar with animal research in this domain. At a minimum, the authors should include a discussion of prior findings that support the specificity of the 40 Hz frequency in modulating hippocampal function.
5. Spectral Analysis and Reporting: Figure 2B presents a limited spectral window, which does not provide a full picture of stimulation effects. A complete power spectrum should be shown to illustrate how the stimulation influences other frequency bands. Furthermore, the observed effects appear to be narrowly confined to the 40 Hz band, and additional discussion is required to interpret this finding in the context of broader oscillatory dynamics.
6. Temporal Continuity of Stimulation Effects: The manuscript does not clearly investigate whether the effects of stimulation persist beyond the stimulation period. If behavioral relevance is not being tested, at the very least, an analysis of how connectivity and spectral changes evolve prior, during and, post-stimulation should be included. This would provide insights into whether GENUS has lasting effects or whether its impact is transient.
7. Comprehensive spectral analysis: Overall the level of spectral analysis in the paper needs to be improved. While phase locking value (PLV) is an appropriate measure, its use here does not fully leverage its analytical potential. Given that gamma power differences exist across conditions, PLV increases are expected. The authors should investigate whether stimulation impacts the phase relationships independently of power differences and also look for potential non-oscillatory effects in the data.
8. Statistical Reporting and Directionality Claims: The connectivity analysis in Figure 3 lacks clarity regarding statistical significance. The authors should present differences in PLV values across conditions rather than just absolute values. Additionally, there is no information about standard deviations for PLV comparisons, making it difficult to assess the reliability of results. Given that connectivity is a key claim of this study, improved statistical transparency is essential.
9. Inter-Subject Variability: The manuscript does not sufficiently address whether effects were consistent across subjects. Were the reported findings observed in all individuals, or were they driven by a subset of participants? If variability exists, it should be explicitly discussed, and subject-level results should be provided (e.g., in supplementary figures). Figures 2 and 3 should also indicate individual subject contributions to highlight the robustness of the reported effects.
10. Limited Supplementary Data: The supplementary materials are minimal and do not adequately summarize patient-level data. Even if full datasets cannot be provided, a summary figure displaying patient-specific responses for the main analyses (Figures 2 and 3) would be highly beneficial. Since the study relies solely on neural measures to claim GENUS efficacy, more detailed patient-specific data are necessary.
11. Figure Legends and Data Representation: The figure legends lack details regarding what data points represent (e.g., whether they correspond to individual patients or session averages). This ambiguity must be addressed to ensure clarity in data interpretation.

Minor Issues:
• The excessive use of abbreviations (RN, RO, IN) makes the results harder to follow. It would improve readability to use clearer labels such as "With Attention" and "Without Attention."
• Figure 3 uses inconsistent terminology—while the text refers to "regular without attention," Figure 2 uses "RN," and Figure 3 states "Regular - No Task." The authors should standardize their terminology.
• The introduction does not clearly articulate the research gap being addressed. It currently lists related studies without a clear motivation for the present work. A revision emphasizing the unresolved questions and how this study addresses them would strengthen the manuscript's rationale.

• The paper moves abruptly from discussing Alzheimer's disease to testing hippocampal responses to gamma stimulation without adequately bridging these topics. While this connection seems implicit, a clearer rationale should be provided in the introduction. The authors should explicitly justify why hippocampal gamma entrainment is relevant to Alzheimer's pathology and temper their claims regarding its therapeutic potential.

Final Evaluation and recommendation:
While the dataset and objectives are valuable, the analysis and interpretation require further development to provide a more robust and well-substantiated contribution. Substantial revisions—particularly in experimental controls and behavioral effect assessment (if not feasible, advanced analyses, eg. the stimulation's temporal effects), clarity of statistics, and clarity of interpretation—are necessary before the manuscript can be considered for publication.

Reviewer #3

(Remarks to the Author)
The goal of this study was to build on work previously published by the same group showing that the inclusion of a cognitive task during visual 40 Hz stimulation increased strength and propagation of response. In this paper, intracranial recordings were collected in 11 individuals with epilepsy during 5 minutes of visual 40 Hz stimulation with or without a visual oddball task.
Data showed strong entrainment to stimulation in terms of signal-to-noise ratio (40 vs 38-42 Hz) and phase-lag value in the hippocampal leads. While both conditions created significant response in frontal, insular, and temporobasal regions, further analysis using phase slope index to determine direction of information flow showed that the oddball tasked reversed the activity in gamma, alpha, and beta frequency bands from hippocampus to frontal areas to a more "top-down" structure of flow from the frontal area to hippocampus.
Overall, this work builds to further elucidate the influence of attention and cognitive load on response to 40 Hz sensory stimulation, confirms the entrainment of deep brain structures in human patients using intracranial recordings in a larger cohort than previously shown, and promotes future work to examine the clinical impact of these differences in neural entrainment.
We would support the publication of this manuscript. In terms of suggestions for revisions:
1. The authors mention the small sample size in limitations of the study. Was any of the data analyzed in a way that accounts for patient-to-patient differences? We see that SNR and PLV are correlated. Are any of the results driven by a subset of high-responders or low-responders rather than cortical region differences?
2. Previous work has highlighted the importance of multisensory stimulation. Is there a reason that this study focused only on visual?
3. Was the presentation of stimulation conditions randomly ordered? If not, the authors should mention this within the methods.
4. The interaction between hippocampal integrity assessed by MRI and the results presented is not clear.
5. Finally, accuracy of oddball response was also collected. It would be interesting and useful to see if neural entrainment values are reflected or correlated with task accuracy or other finer metrics of cognitive load.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have done a good job addressing my comments; I am ready to sign off on this manuscript for publication.

Reviewer #2

(Remarks to the Author)
I appreciate the authors' comprehensive responses to my comments and concerns.
The revision substantially improved the manuscript, particularly regarding statistical transparency, visualization of raw and processed data, and clarification of methodological and interpretive points. The inclusion of individual-level data, aperiodic exponent analysis, and careful attention to possible confounds (power vs. phase-locking) in the revision letter strengthen the analysis clarity and support the manuscripts' conclusions.

I also appreciate that the authors have now properly tempered claims about the therapeutic relevance of GENUS and clarified that their study is not designed to assess cognitive enhancement. Their justification of 40 Hz as a target frequency are convincing and well-contextualized.

While the absence of behavioral outcomes limits the interpretive scope, this is now clearly acknowledged, and the manuscript appropriately reframes its contribution as physiological evidence of neural entrainment.

Minor suggestions:

Fig3.B. the y-axis labels for p7 and p8 are misaligned.
Fig1.C. Consider overlaying the LFP traces for different conditions within each region. Plotting them on the same axis (with color-coded lines) would make comparisons across conditions more intuitive and easier to interpret.

Reviewer #3

(Remarks to the Author)
The authors have addressed all of our concerns satisfactorily and thoroughly. This study elucidate the influence of attention and cognitive load on response to 40 Hz sensory stimulation. The authors show the entrainment of deep brain structures such as the hippocampus in human patients using intracranial recordings in a larger cohort than previously shown. This work will likely promote future work to examine the clinical impact of these differences in neural entrainment. I therefore support the publication of this manuscript in Communications Biology.

Response to Reviewers

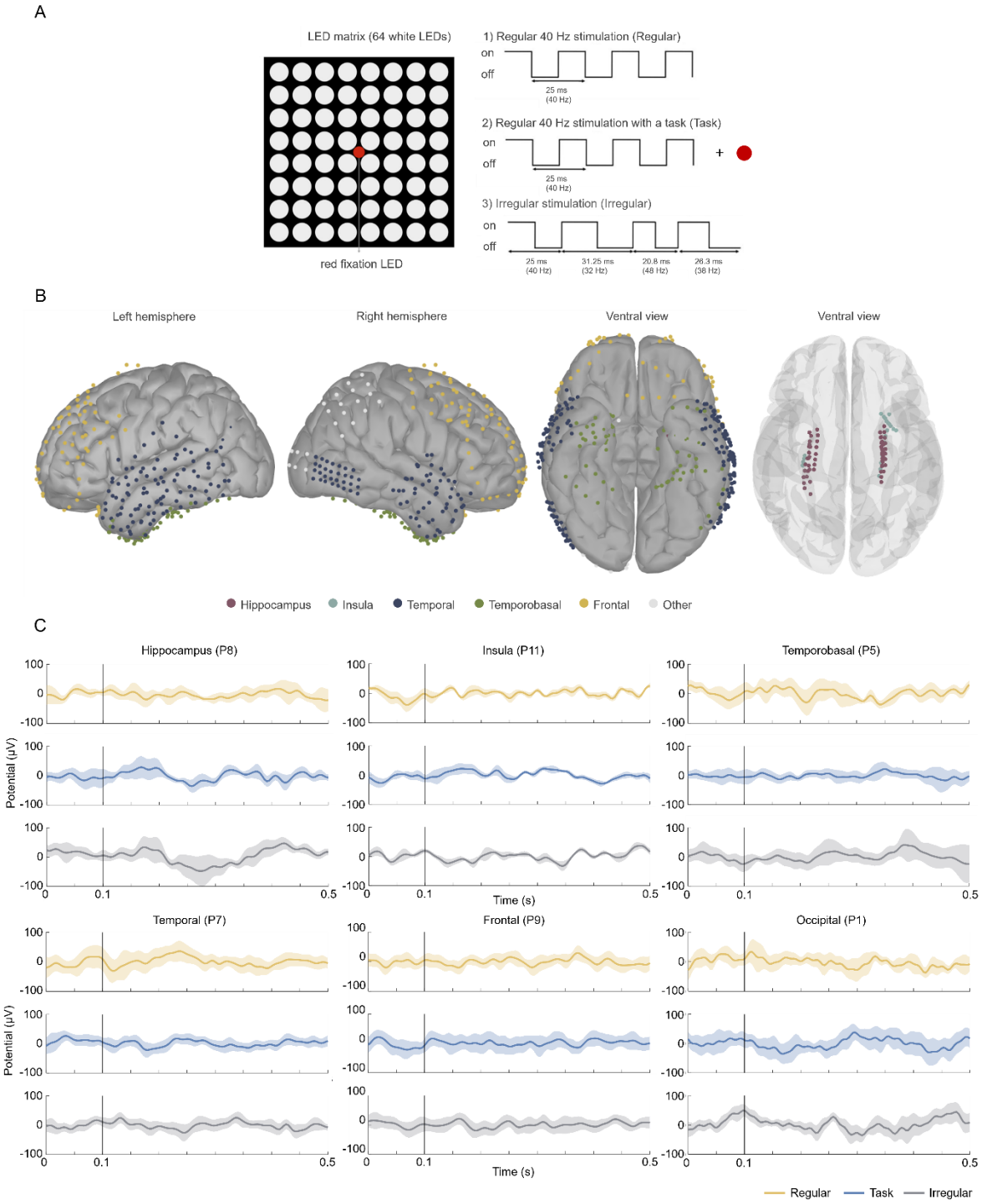
We thank all three reviewers for their insightful comments and constructive feedback. In response to their suggestions, we have made significant modifications to the manuscript and the supplementary materials. Changes are highlighted in the revised text. Please find below our replies to the comments point by point.

Reviewer #1 (Remarks to the Author):

The authors tested whether gamma entrainment using a 40Hz flickering LED stimulus altered hippocampal activity and connectivity to other brain areas in 11 patients undergoing seizure monitoring for epilepsy. This manuscript is timely as there is accumulating evidence of the efficacy of this stimulation protocol in animal models of Alzheimer's disease. Patients performed an attention task during regular rhythmic or arrhythmic stimuli were shown, and the authors measured phase synchrony in the hippocampus and other cortical areas. The authors observed increased 40Hz activity measured as SNR from the average power spectrum, alongside increased phase locking values (PLV), in occipital contacts, demonstrating that the flashing stimulus was indeed modulating visual activity. In hippocampus, a large >60% number of contacts showed increase in SNR. Overall, I am positive on this manuscript and find the methods, results, and conclusions to be clear and straightforward but could use some more unpacking. I believe this will be of interest to the intracranial recordings community alongside the increasing interest in non-invasive methods for targeting cognitive decline in AD. Specific comments below:

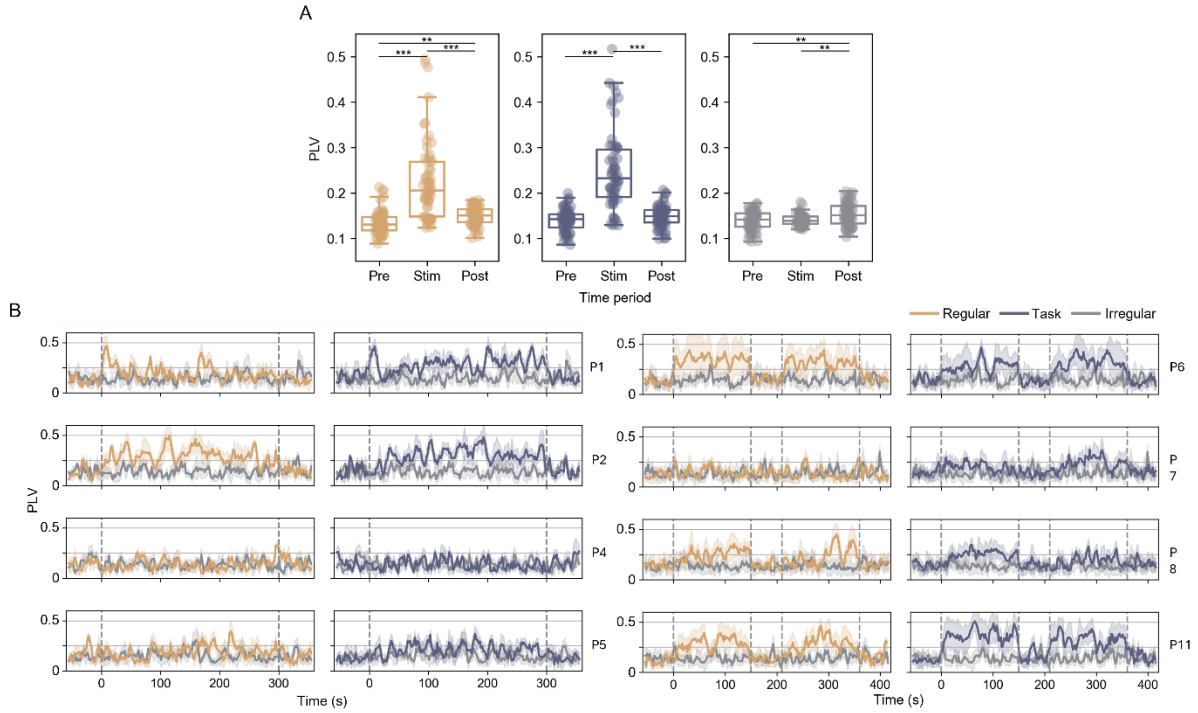
1. The authors could do more to "unpack" their findings by showing spectrograms or representative raw traces which illustrate the purported entrainment phenomenon.

We included plots with filtered data for all assessed bran regions to the main text (Figure 1C) and examples of raw traces for same regions were included in the Supplementary materials (Supplementary Figure 1). Additionally, to illustrate the entrainment phenomenon, we expanded our analysis of PLV to the stimulation to assess its temporal dynamics. An additional figure was included in the main text (Figure 3), which shows the time course of PLV to the stimulation for hippocampal contacts on the group and patient level.



*"**Figure 1. Stimulation conditions, electrode placements for all patients, and LFP traces at the beginning of stimulation**. **(A)** LED panel comprising an 8 × 8 LED matrix with central red LED used as a fixation point and to elicit a visual oddball (left panel) and three stimulation conditions used in the experiment (right panel). **(B)** A total of 490 contacts were included in the analysis, which were grouped by anatomical region. Parietal and occipital contacts were excluded from the connectivity analyses, as only one patient was implanted at those locations. **(C)** Example of LFP traces at the beginning of each stimulation for each of the anatomical regions included, averaged across contacts*

corresponding to a specific location for one patient. Lines and shaded areas indicate mean ± standard deviation. Vertical gray lines indicate the beginning of the stimulation. Data was filtered between 2 and 45 Hz for visualization purposes. Examples of raw traces are shown in the Supplementary Fig. 1."



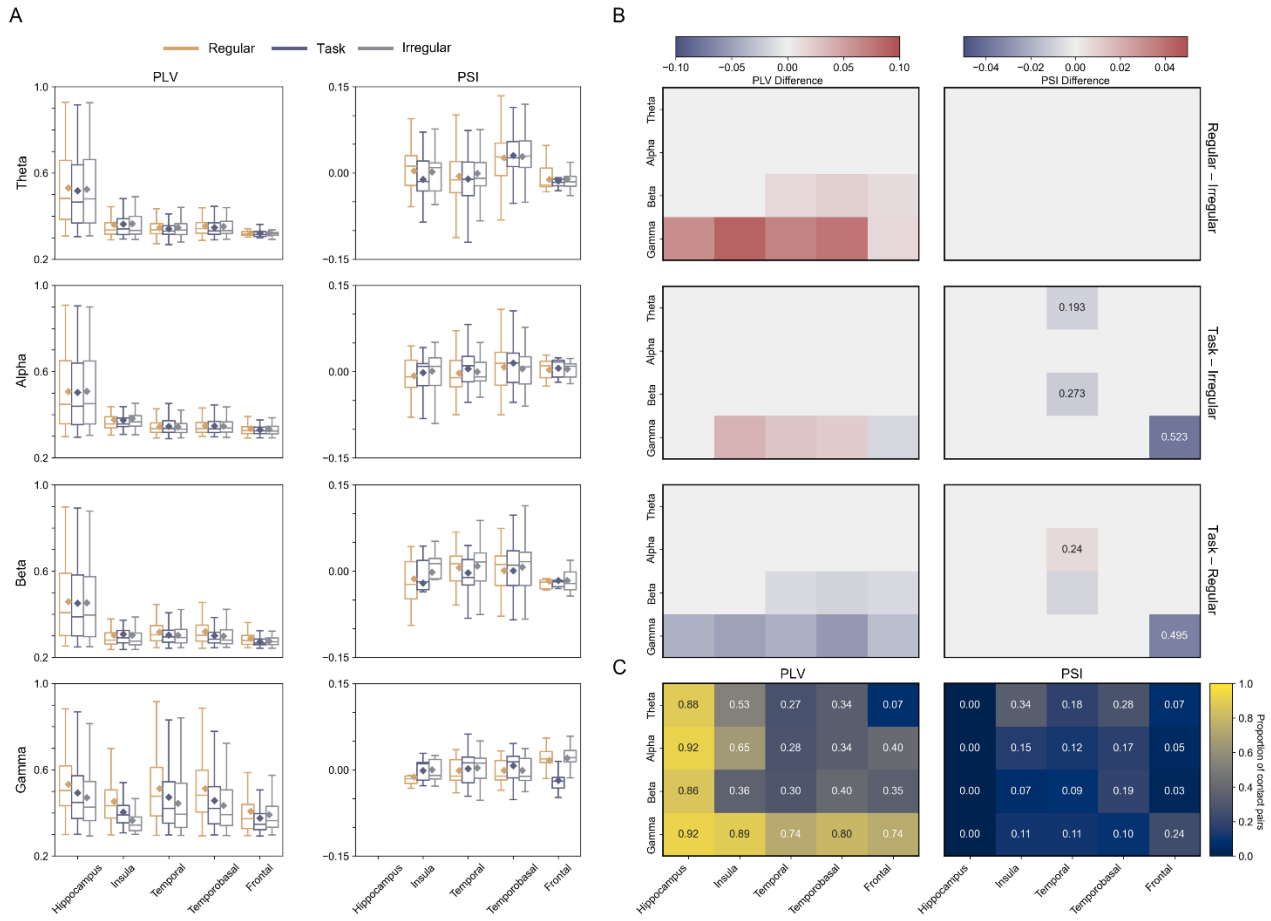
***"Figure 3. Time course of PLV to the stimulation for hippocampal contacts. (A)** For each patient, average PLV to the stimulation was estimated during the stimulation period (Stim), as well as during one-minute pre- (Pre) and one-minute post-stimulation (Post) periods for each hippocampal contact across patients. Each dot represents one hippocampal contact. Box plots follow the same convention as in Fig. 2, with the median marked by the central line and the box indicating IQR, and statistical significance indicated by black lines with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. **(B)** Each row shows the time course of PLV to the stimulation averaged across hippocampal contacts for each patient implanted with depth electrode targeting the hippocampus. Solid lines represent the mean, and shaded areas indicate ± 1 standard deviation. Gray vertical lines mark the onset and offset of stimulation. Stimulation lasted 5 minutes for all patients but was divided into two 2.5-minute trials for patients P6–P11 to minimize visual strain."*

2. The connectivity results are under-developed compared to the rest of the manuscript, and the authors only provide electrode-pair averaged data (Figure 3). I believe this result needs more unpacking. What proportion of electrode pairs show significant changes in PLV or PSI? How many patients show these effects?

Due to the variability in electrode placement across patients, which is a limitation of clinical intracranial recordings, we performed a group-level analysis. As not all patients had electrodes in every region of interest, patient-wise comparisons within identical

region-pairs are limited. To address this, we aggregated data across patients (only including region pairs that at least 2 subjects contributed electrodes to), which allowed us to identify connectivity patterns on the group level, despite the heterogeneity of electrode placements.

We acknowledge the limitation of our current presentation and have extended our analysis to account for between- and within-subject variability and included additional information in the main text and supplementary figures in response to the reviewer's concern. We thereby retained only contact pairs showing a significant interaction for all conditions (determined by a threshold value estimated from surrogate data), followed by linear mixed models. Figure 5 (previous Figure 3) now includes annotations that indicate the number of electrode pairs showing significant interactions (Figure 5C). Additional to showing mean values (Figure 5A), we also include significant differences in PLV and PSI between region pairs (Figure 5B). Furthermore, we have included boxplots showing PLV and PSI between each region pair for each frequency band and stimulation condition as well as matrices showing mean data and mean differences in the Supplementary materials (Supplementary Figures 8-10).



*"**Figure 5. PLV and PSI between hippocampus and other regions for different frequency bands. (A)** Box plots for PLV (left column) and PSI (right column) per frequency band (rows) for all contact pairs between hippocampus and other regions showing*

significant interactions in all stimulation conditions. Positive PSI value indicates an information flow from the hippocampus to regions of interest and negative PSI value indicates a reversed flow. Box plots follow the same convention as in Fig. 2, with the median marked by the central line and the box indicating IQR. The dot corresponds to the mean value. ***(B)*** *Mean difference in PLV (left column) and PSI (right column) between conditions for contact pairs showing significant interactions in all conditions (linear mixed effects model with FDR correction for multiple comparisons; $p < 0.05$). Rows in each matrix correspond to the four frequency bands (theta, alpha, beta, and gamma around the stimulation frequency (38–42 Hz)), columns to brain regions. Colors indicate the magnitude of the change. Annotations in the PSI matrices indicate the proportion of contacts for which a switch in the direction of the information flow occurred. (C) Proportion of contact pairs per region pair showing significant interactions in all three stimulation conditions for PLV (left) and PSI (right) for different frequency bands. Rows in each matrix correspond to the four frequency bands, columns to brain regions."*

3. How long does entrainment last in the hippocampus? Does the degree of entrainment depend on the electrophysiological / oscillatory "state" of the hippocampus? One would expect that entrainment may not be as strong in situations in which the hippocampus is actively engaged in other sorts of tasks.

We appreciate the reviewer's insightful question regarding the temporal duration of hippocampal entrainment and its potential dependency on the electrophysiological state of the hippocampus. While this is a highly relevant and interesting avenue for future research, we believe it falls beyond the scope of the present study. In the current study, we examined neural response in contacts targeting the hippocampus to visual 40 Hz stimulation, accompanied with or without a simple task. Our results suggest that a simple attention task may induce a stronger response, however, looking at the patient-level data, the effect was not consistent across patients.

We agree with the reviewer that the effect might be diminished by ongoing hippocampal engagement in unrelated tasks, but evaluating this hypothesis would require a different experimental design, such as presenting stimulation during concurrent, hippocampus-dependent tasks. We consider this an important direction for future work and added the following to the discussion (line 380):

"While our findings suggest efficient modulation of hippocampal activity by visual 40 Hz stimulation, its potential modulation by ongoing cognitive processes remains an open question. In our design, each stimulation condition was preceded by a rest period to standardize pre-stimulation activity. However, it is plausible that the strengths of entrainment could be diminished when the hippocampus is engaged in other cognitive tasks, particularly those related to memory encoding or retrieval. Investigating this would require a paradigm specifically designed to assess state-dependent modulation. Future

studies should explore how concurrent hippocampal engagement impacts the strength and temporal variability of entrainment."

Regarding the temporal duration of the entrainment, we expanded our PLV to the stimulation analysis and assessed it with sliding windows to estimate temporal dynamics of the PLV, starting from a one-minute period before the stimulation and ending one-minute after the stimulation. A figure was included to the main text (Figure 3, shown in response to comment 1) showing a time course of PLV in response to the stimulation for hippocampal contacts per patient to illustrate the time evolution of entrainment in hippocampus. Additionally, we assessed any potential differences in PLV to the stimulation before, during, and after the stimulation, which was added to the results section (line 173): *"Fig. 3B shows a clear increase in PLV for most of the patients after the onset of stimulation for Regular and Task conditions, which is not present for the Irregular condition. After the offset of stimulation, PLVs return to their pre-stimulation level, suggesting a transient effect of entrainment."*

The following information was added to the methods section (line 494): *"To estimate the temporal dynamics of PLV, a sliding window approach was used (10-second windows with 2-second steps), starting from the 1-minute rest period before stimulation onset to the 1-minute rest period after stimulation offset. For each window, the phase difference between the sinusoid and the filtered data was computed. The mean PLV was then calculated for each contact and condition across three time periods: pre-, during, and post-stimulation."*

4. I applaud the use of a simple attention task, but again, this aspect of the manuscript didn't come across particularly strongly. The authors should more clearly justify what they were hoping to achieve by including an attention task.

The inclusion of the attention task builds on our previous EEG study (Khachatryan et al., 2022) where we showed that this can enhance and propagate neural entrainment further into the brain in healthy young adults, which was confirmed in a single patient implanted with intracranial electrodes. In the current study, we aimed to verify these findings using a larger sample of patients with intracranial electrodes in different brain regions, but with a particular focus on the hippocampus as it is one of the earliest and most affected regions in Alzheimer's disease. We included following explanations in the introduction (line 89):

"Despite these promising findings, the factors driving entrainment propagation beyond early visual areas remain poorly understood. In animal models, combining visual 40 Hz stimulation with physical exercise has led to the most effective reduction of Aβ and tau levels, potentially improving cognitive function 10. However, in humans, the impact of simultaneous engagement during GENUS remains unclear, as many studies have either not recorded EEG responses or have not examined the effects of concurrent tasks

13,16,18,19. Building on these findings and our previous EEG study 20, where we showed that including a cognitive task during stimulation enhances the strength and extent of the neural response, we aim to provide further evidence that task engagement facilitates stronger and more widespread entrainment. Since steady-state visual responses are typically restricted to early visual areas 21, it is important to evaluate potential benefits of cognitive engagement, as gamma-based interventions primarily target deeper structures such as the hippocampus, one of the earliest and most affected regions in AD."

5. I understand the justification for focusing on the hippocampus based on the AD literature, but is this the region where the authors expected the strongest entrainment effects? I was left wondering whether the hippocampus particularly susceptible to entrainment, or is entrainment equally strong throughout the brain. It is also worth considering MTL is more frequently targeted in this patient population.

Our focus on the hippocampus was motivated by both its central role in memory, its early vulnerability in AD, and from the association between hippocampal atrophy and both early AD and drug-resistant temporal lobe epilepsy (Moran et al., 2001). Temporal lobe epilepsy, being one of the more common focal epilepsies, provide a unique opportunity to record neural activity directly from the hippocampus. That said, we did not specifically expect the hippocampus to exhibit the strongest effects, as prior research has shown that steady-state visual responses are typically restricted to early visual areas (Wittevrongel et al., 2018), suggesting that primary sensory regions may be more susceptible to entrainment using sensory stimulation. Notably, our results show that amongst the assessed regions, insula showed the strongest effect, stronger than the hippocampus, as measured by both SNR and PLV to the stimulation.

Nonetheless, we believe it is of particular interest that hippocampal region can engage in gamma entrainment, given their location, function, and potential relevance for therapeutic interventions in AD.

6. Because the authors swept across frequencies from 30-50 Hz in the irregular stimulation condition, it may be possible (and certainly of interest) to know if there is entrainment also at other gamma band frequencies. In other words, is 40Hz a particularly "special" frequency for entrainment, or can the HPC be entrained at 35 or 45 Hz? If this cannot be assessed in the current dataset this should be mentioned.

We agree with the reviewer that exploring stimulation at other frequencies would be of certain interest as well as the comparison of the effect between these different frequencies in the gamma band. In our current study, only the regular stimulation condition involved sustained stimulation at 40 Hz. The irregular condition was designed as a nonrhythmic control, and as such consisted of single square-wave periods at random integer frequencies between 30 and 50 Hz, each lasting for only one cycle. Because the frequency changed every cycle, this condition does not allow for frequency

specific entrainment or phase-locking to any individual frequency. While our data cannot address whether hippocampus can be entrained at other gamma frequencies, results of previous studies show the specificity of the 40 Hz stimulation (Blanpain et al., 2024; Iaccarino et al., 2016) but with possible shifts in optimal stimulation frequency with age (Lee et al., 2021; Park et al., 2022).

We have expanded the introduction (line 50) to highlight the motivation for using 40 Hz stimulation based on prior findings:

"Iaccarino et al. 7 reported that both short- and long-term 40 Hz visual stimulation, but not using 20 Hz, 80 Hz, or random frequencies, significantly reduce Aβ levels in the visual cortex of an AD mouse model as well as in deeper brain regions, including the hippocampus. This specificity aligns with neurophysiological studies which show that slow gamma oscillations (~40 Hz) are involved in memory retrieval processes in the hippocampus 12. Furthermore, a recent study using intracranial recordings showed that 40 Hz stimulation led to a neural response in significantly more contacts compared to 5.5 Hz or 80 Hz stimulation 13"

We added the following to the discussion (line 335):

"Although our results are limited to stimulation at 40 Hz and show a narrow spectral response, it would be valuable to investigate whether other frequencies within the low gamma band can induce similar effects and their impact on intrinsic gamma oscillations. A recent EEG study in younger adults showed that visual stimulation at 34-38 Hz elicited stronger and more widespread neural response compared to stimulation at 40-50 Hz 28. Similarly, in older adults, visual stimulation at 32 or 34 Hz generated stronger response than stimulation at 36 Hz, 38 Hz, or 40 Hz 29. These results suggest that optimal stimulation frequency may shift with age. However, 40 Hz has previously been associated with resonance phenomena in the human visual system, suggesting that stimulation at this frequency induces stronger steady-state responses compared to adjacent frequencies, despite identical stimulus parameters 30. This implies that neural populations exhibit intrinsic frequency preferences, possibly due to recurrent circuitry and conduction delays within oscillatory networks."

Reviewer #2 (Remarks to the Author):

Summary:

This manuscript examines the effects of gamma-frequency non-invasive stimulation (GENUS) in epileptic patients equipped with both subdural and depth electrodes, providing a valuable dataset that builds on prior animal studies and seeks to translate key findings to humans. The topic is timely, given the growing interest in gamma stimulation and its possible therapeutic benefits. While the study offers intriguing insights into how neural oscillatory mechanisms may be modulated in a clinical population, there are substantial concerns regarding the experimental design, statistical rigor, and interpretation of the results. Given the practical challenges of collecting additional data from similar patient group, comprehensive additional analysis and clearer justification of the findings are essential. At present, the evidence presented does not convincingly support the authors' claims regarding the therapeutic potential of GENUS in humans

Major Concerns:

1. Lack of Behavioral Evidence for Cognitive Enhancement: While the study demonstrates hippocampal entrainment at 40 Hz, it does not assess whether GENUS leads to improved cognitive function. As authors know as well, prior animal studies examining GENUS effects have included either behavioral assessments or molecular level evidence to demonstrate the effectivity of such a visual stimulation paradigm. The presence of hippocampal effect alone does not necessarily imply an enhancement in memory or cognition (neither support the therapeutic effect for Alzheimer's disease cases, which has been stated in introduction and abstract!). Without such evidence, the claims regarding GENUS as a potential therapy remain speculative. It would be beneficial for the authors to either include behavioral tasks or explicitly acknowledge this limitation in their discussion.

We thank the reviewer for raising this important concern. We acknowledge that evaluating potential therapeutic effects of the GENUS approach was indeed not the focus of our study. Our single-trial experiments were conducted in younger epilepsy patients implanted with intracranial EEG, rather than in a longitudinal cohort of AD patients, which would be necessary to assess any therapeutic effects. In response to the reviewer's concerns, we have removed the final sentence of the abstract and revised several related statements throughout the manuscript accordingly.

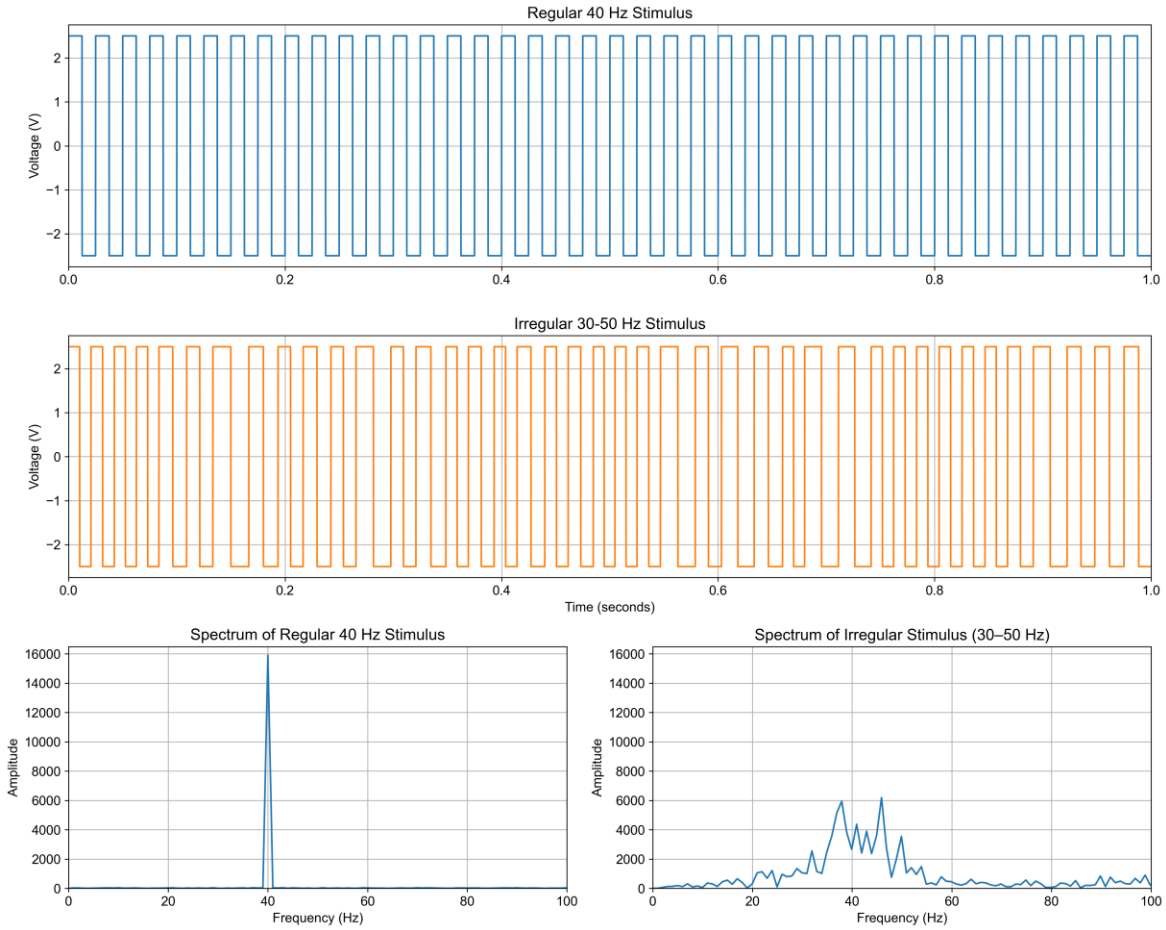
The concluding sentence in the discussion now reads as (line 411): *"Overall, our study provides valuable insights into the neurophysiological effects of visual gamma entrainment, encouraging further research on the use of GENUS in the scope of AD and related neurodegenerative disorders."*

We have acknowledged the limitation in the discussion (line 389): *"We acknowledge that although our results show successful modulation of neural activity in response to visual 40 Hz stimulation, they do not necessarily imply any clinical or cognitive benefit."*

2. Control Conditions and Stimulus Equivalence: The study relies on comparisons with an irregular flickering stimulus as a control condition, but it is unclear whether the effective stimulus magnitude was equivalent across conditions. Since connectivity measures can be influenced by stimulus intensity, it is necessary to confirm that the total stimulus power was comparable between conditions. At a minimum, reporting the root mean square (RMS) of the stimulus signal and its spectral properties would help clarify this point. There is a concern if non-prediodic visual stimulation cause a 1/f type of activity that won't be revealed with the limited analysis that is provided on the neural signals in this study.

In our setup, both regular and irregular stimulations were delivered using the Arduino Uno microcontroller with a 5V output and a 50 % duty cycle. These parameters, along with a controlled LED brightness (225 and 250 lumen across participants), ensured consistent stimulation across conditions. Based on that, we believe that RMS was similar for all stimulus conditions as it is not primarily determined by frequency but rather by peak voltage and duty cycle, which were kept constant across all stimulation conditions.

To illustrate our point, we have simulated both regular (40 Hz) and irregular (30 – 50 Hz) stimulus waveforms, using the above-mentioned parameters, and calculated the RMS, which was equal across conditions (~ 3.54 V). Figure below shows simulated stimulation conditions, normalized to 0 mean. Based on this, we believe that the results in our current study are not likely to be driven by differences in stimulus intensity.



RMS without centering (Regular 40 Hz): 3.53 V, RMS without centering (Irregular 30–50 Hz): 3.54 V

Regarding the concern that irregular stimulation may induce a 1/f type of activity, we recognize that this could influence spectral power analyses. However, as our connectivity measures rely on phase rather than amplitude, the impact of 1/f activity should be minimal. Furthermore, the SNR at 40Hz was computed by dividing the amplitude at 40 Hz by the average amplitude of the surrounding frequencies (38 – 42, excluding 40 Hz), which also corrects for 1/f type of activity. Nonetheless, as an additional control, we included and analyzed a one-minute rest period before the first stimulation condition. We computed both SNR and PLV to the stimulation for this period, as well as the aperiodic exponent (further explained in our response to comment 7).

2.1 Presentation and Organization: The experimental methodology is difficult to track due to its placement in the manuscript. Figure 4, which describes the experimental paradigm, should be introduced earlier, potentially integrated with Figure 1, to improve clarity. Furthermore, the methods section should be reorganized to align with the journal's standard format. Careful revisions to improve the manuscript's readability and logical flow are required.

We moved our previous Figure 4 to now Figure 1A (shown in response to reviewer 1, comment 1), to improve readability. The current structure follows the journal's standard format (abstract, introduction, results, discussion, methods) as described in the journal's style and formatting guide (https://www.nature.com/documents/commsj-life-style-formatting-guide-accept.pdf).

3. Lack of Raw LFP Visualizations: The manuscript does not include visualizations of raw signals across stimulus conditions, making it difficult to assess the robustness of the reported effects. Providing representative raw traces (both filtered and unfiltered) for different stimulus condition/ regions would greatly enhance transparency. Even if these figures are included as supplementary materials, they are necessary for evaluating the strength of the effect and quality of the recorded signals.

We agree with the reviewer and have now included examples of raw traces for each assessed brain region and each condition in Supplementary Figure 1. Additionally, we also include a figure with filtered data for each condition and the same locations into the main text (Figure 1C, shown in response to reviewer 1, comment 1).

4. Specificity of the 40 Hz Effect: The authors do not examine whether the observed effects are exclusive to 40 Hz stimulation. Would a different frequency (e.g., 80 Hz) yield similar connectivity changes? A stronger justification for selecting this frequency is needed, particularly for readers unfamiliar with animal research in this domain. At a minimum, the authors should include a discussion of prior findings that support the specificity of the 40 Hz frequency in modulating hippocampal function.

We have expanded the introduction to highlight the motivation for using 40 Hz stimulation based on prior findings (line 50):

"Iaccarino et al. 7 reported that both short- and long-term 40 Hz visual stimulation, but not using 20 Hz, 80 Hz, or random frequencies, significantly reduce Aβ levels in the visual cortex of an AD mouse model as well as in deeper brain regions, including the hippocampus. This specificity aligns with neurophysiological studies which show that slow gamma oscillations (~40 Hz) are involved in memory retrieval processes in the hippocampus 12. Furthermore, a recent study using intracranial recordings showed that 40 Hz stimulation led to a neural response in significantly more contacts compared to 5.5 Hz or 80 Hz stimulation 13."

We added the following to the discussion (line 335):

"Although our results are limited to stimulation at 40 Hz and show a narrow spectral response, it would be valuable to investigate whether other frequencies within the low gamma band can induce similar effects and their impact on intrinsic gamma oscillations. A recent EEG study in younger adults showed that visual stimulation at 34-38 Hz elicited

stronger and more widespread neural response compared to stimulation at 40-50 Hz 28. Similarly, in older adults, visual stimulation at 32 or 34 Hz generated stronger response than stimulation at 36 Hz, 38 Hz, or 40 Hz 29. These results suggest that optimal stimulation frequency may shift with age. However, 40 Hz has previously been associated with resonance phenomena in the human visual system, suggesting that stimulation at this frequency induces stronger steady-state responses compared to adjacent frequencies, despite identical stimulus parameters 30. This implies that neural populations exhibit intrinsic frequency preferences, possibly due to recurrent circuitry and conduction delays within oscillatory networks."

5. Spectral Analysis and Reporting: Figure 2B presents a limited spectral window, which does not provide a full picture of stimulation effects. A complete power spectrum should be shown to illustrate how the stimulation influences other frequency bands. Furthermore, the observed effects appear to be narrowly confined to the 40 Hz band, and additional discussion is required to interpret this finding in the context of broader oscillatory dynamics.

We limited the frequency range in previous Figure 2B to 35–45 Hz to highlight the stimulation-related effect around 40 Hz. To address the reviewer's comment, we have now included the full power spectrum (1–120 Hz) for the hippocampal contacts in the Supplementary materials (Supplementary Figure 2 on a group level and Supplementary Figure 3 on an individual level).

The narrow increase at 40 Hz shown in our current study aligns with known characteristics of rhythmic sensory stimulation, inducing frequency-specific response (Herrmann, 2001). Furthermore, the same study showed that certain frequencies, including 40 Hz, are associated with resonance phenomena in the human visual system, eliciting stronger steady-state responses compared to neighbouring frequencies. Moreover, it was suggested that such resonant responses might stem from non-linearly coupled neural systems that naturally favour specific frequencies. In this sense, a narrow 40 Hz response is a marker of entrainment, whereas a broader frequency band modulation might be expected from a random, non-periodic stimulation, as it was partially shown in Blanpain et al. (2024) and by the figure above in response to comment 2. Based on this and our own results, we can confirm that in our current study 40 Hz stimulation evoked a frequency-selective neural resonance rather than a broader modulation. However, even though we primarily show a narrow response, we acknowledge that changes in the aperiodic exponent could reflect broader spectral modulations, potentially indicating altered excitation/inhibition balance.

The following was added to the discussion (line 335) to interpret a narrow increase in the context of broader oscillatory dynamics: *"Although our results are limited to stimulation at 40 Hz and show a narrow spectral response, it would be valuable to investigate whether other frequencies within the low gamma band can induce similar effects and their impact*

on intrinsic gamma oscillations. […] However, 40 Hz has previously been associated with resonance phenomena in the human visual system, suggesting that stimulation at this frequency induces stronger steady-state responses compared to adjacent frequencies, despite identical stimulus parameters 30. This implies that neural populations exhibit intrinsic frequency preferences, possibly due to recurrent circuitry and conduction delays within oscillatory networks. Rather than indicating a lack of broader modulation, the narrow spectral response may point to a precise and targeted engagement of frequency-specific networks."

6. Temporal Continuity of Stimulation Effects: The manuscript does not clearly investigate whether the effects of stimulation persist beyond the stimulation period. If behavioral relevance is not being tested, at the very least, an analysis of how connectivity and spectral changes evolve prior, during and, post-stimulation should be included. This would provide insights into whether GENUS has lasting effects or whether its impact is transient.

To address this suggestion, we extended our PLV to the stimulation analysis to additionally assess the temporal dynamics of neural entrainment. Specifically, we applied a sliding window approach to track the changes in PLV over time for each stimulation condition. A figure was included to the main text (Figure 3, shown in response to reviewer 1, comment 1) showing a time course of PLV in response to the stimulation for hippocampal contacts per patient to illustrate the time evolution of entrainment in hippocampus. Additionally, we assessed any potential differences in PLV to the stimulation before, during, and after the stimulation, which was added to the results section (line 173): *"Fig. 3B shows a clear increase in PLV for most of the patients after the onset of stimulation for Regular and Task conditions, which is not present for the Irregular condition. After the offset of stimulation, PLVs return to their pre-stimulation level, suggesting a transient effect of entrainment."*

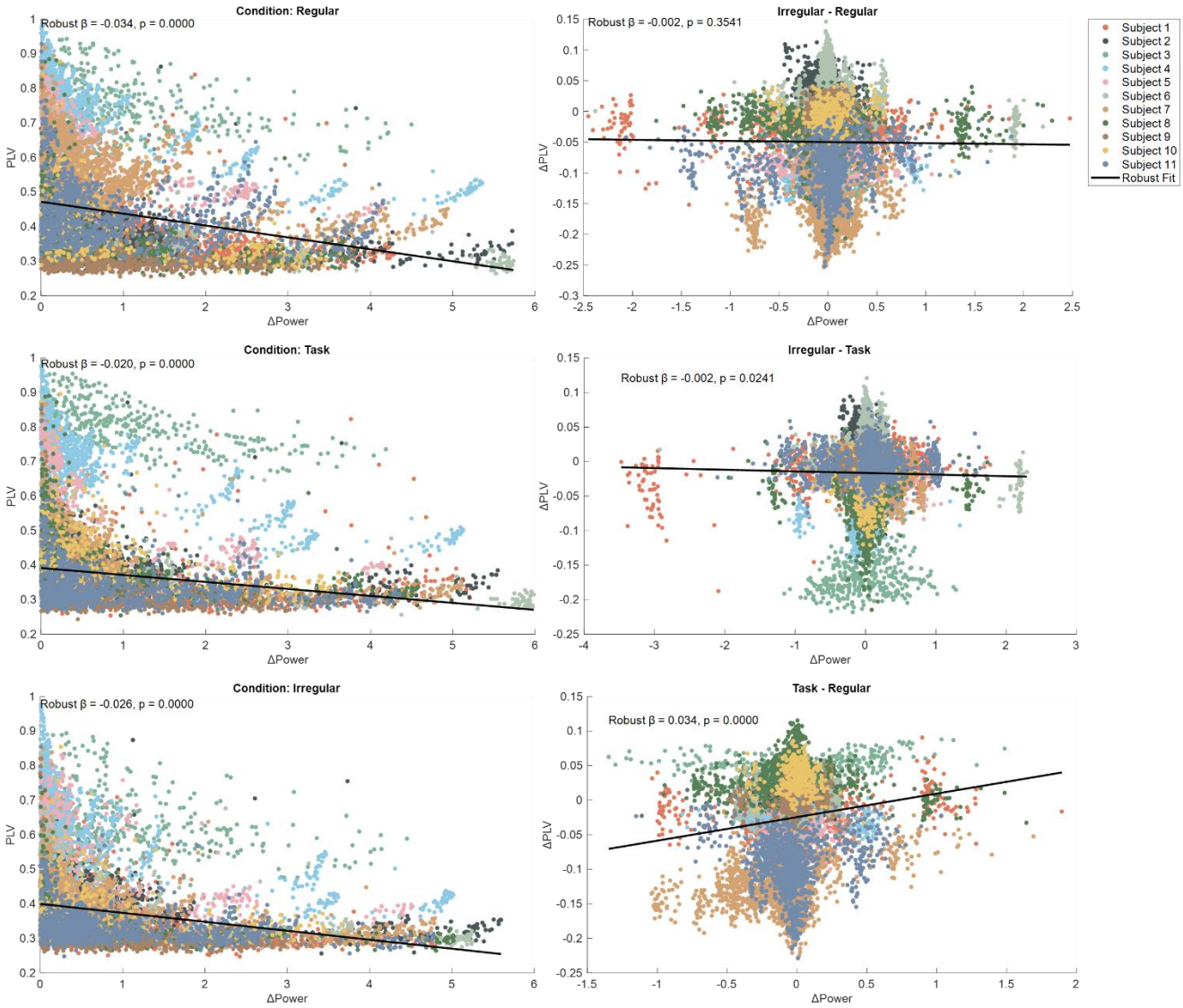
The following information was added to the methods section (line 494): *"To estimate the temporal dynamics of PLV, a sliding window approach was used (10-second windows with 2-second steps), starting from the 1-minute rest period before stimulation onset to the 1-minute rest period after stimulation offset. For each window, the phase difference between the sinusoid and the filtered data was computed. The mean PLV was then calculated for each contact and condition across three time periods: pre-, during, and post-stimulation."*

Given the apparent transient effects of the stimulation, which are in line with previous studies (Lee et al., 2021), we believe this analysis sufficiently addresses the reviewer's concern regarding the duration of the stimulation effect. Therefore, we have not included separate connectivity analyses across the same periods, as they are unlikely to reveal additional information beyond what is already captured by the PLV time course.

7. Comprehensive spectral analysis: Overall the level of spectral analysis in the paper needs to be improved. While phase locking value (PLV) is an appropriate measure, its use here does not fully leverage its analytical potential. Given that gamma power differences exist across conditions, PLV increases are expected. The authors should investigate whether stimulation impacts the phase relationships independently of power differences and also look for potential non-oscillatory effects in the data.

In order to evaluate whether stimulation impacts the phase relationships independently of power differences, we performed additional follow-up analyses. First, we examined whether the **PLV between contact pairs** is driven by the power difference within each condition. Using robust regression, we found a significant but small negative relationship for each condition (*see* Figure below, left column), suggesting that PLV decreases as power differences increase.

Next, we assessed whether the **PLV difference between conditions** is driven by power differences. We computed the difference in absolute power between contact pairs and then calculated the difference between conditions. We then computed the difference in PLV for same the contact pairs between conditions. Robust regression showed that for most comparisons, there is either no relationship (Irregular-Regular) or a very small relationship (Irregular-Task) between power difference and PLV difference (see Figure below, right panel). Importantly, the lack of significant association between the Regular and Irregular conditions suggests that our main PLV findings are not explained by power differences across conditions.



In order to assess possible non-oscillatory effects of stimulation, we included an additional analysis, focusing on the aperiodic component of the signal. For this, we applied the FOOOF algorithm (Donoghue et al., 2020) and assessed the aperiodic exponent for each stimulation condition as well as for the one-minute rest period prior to the first stimulation condition. Our results suggest a region- and condition-specific modulation of the aperiodic exponent by visual stimulation. Notably, while the aperiodic exponent was higher for all three stimulation conditions compared to the baseline period in the hippocampus, temporal, and temporobasal region, the opposite was true for the insular region.

The analysis was accordingly described in the methods section (line 483):

"Additionally, power spectra densities (PSDs) were calculated for each contact and condition using Welch's method 42 (2-second windows, 50 % overlap) for 5-minute stimulation epochs and 1-minute rest period. Power spectra for each contact and condition were parameterized into periodic and aperiodic components using the FOOOF

algorithm 43 with the following settings: peak width limits (1, 12), aperiodic mode (fixed), minimum peak height (0.1), and maximum number of peaks (6). Goodness-of-fit was estimated for each fit and the contacts with the $R^2 < 0.80$ were excluded from subsequent analyses."

We included an additional paragraph in the Results section (line 226):

"Aperiodic exponent

In order to evaluate whether visual stimulation modifies the neuronal excitation-inhibition balance, the aperiodic exponent of the power spectrum was estimated over the gamma band (30–120 Hz), for each contact and condition. Due to a bad fit ($R^2 < 0.80$), data from all contacts of one subject (P11) was excluded for Rest condition.

There was a significant difference in aperiodic exponent between conditions for the hippocampal contacts ($X^2 = 21.90$, $p < 0.001$, Fig. 2C). All three stimulation conditions differed from the baseline rest activity ($Z = -3.55$, $p < 0.001$, $Z = -4,21$, $p < 0.001$, and $Z = -3.41$, $p = 0.001$ for Regular, Task, and Irregular conditions, respectively). The aperiodic exponent was significantly higher for the Task condition compared to the Irregular condition ($Z = -2.85$, $p = 0.004$). There was no significant difference between Regular condition and the Irregular condition ($Z = -0.69$, $p = 0.49$) or Regular and Task conditions ($Z = -1.52$, $p = 0.129$). We additionally observed modulation of aperiodic exponents in response to stimulation in other brain regions (Fig. 4C), where all three stimulation conditions resulted in an increased exponent in the temporal lobe and the basal surface of the temporal lobe. Interestingly, for contacts in the frontal lobe, the aperiodic exponent was higher only in the Regular condition. The pattern was the opposite in the insular region, where the exponent was the lowest for the Regular condition. All median values for other brain regions are presented in Fig. 4C. Results on individual level are presented in Supplementary Fig. 6 and Supplementary Table 2."

We address these results in discussion (line 350):

"In addition to narrowband changes at 40 Hz, we assessed changes in the aperiodic exponent, the 1/f-like slope of the power spectrum, over the gamma range, which was previously suggested to reflect the neuronal excitation-inhibition (E/I) balance 31. We observed a significant increase in the aperiodic exponent across hippocampal contacts for all stimulation conditions compared to rest, with the strongest effect for the Task condition. This suggests that visual gamma stimulation may shift the E/I balance towards inhibition, especially when combined with a simple attention task. These changes are particularly relevant in light of recent studies reporting distrupted E/I balance in AD 32,33. Interestingly, our resutls suggest a region-specific effect of visual gamma stimulation on the aperiodic exponent, with contact in insular region showing the opposite effect compared to other regions, with the lowest exponent in the Regular condition."

8. Statistical Reporting and Directionality Claims: The connectivity analysis in Figure 3 lacks clarity regarding statistical significance. The authors should present differences in PLV values across conditions rather than just absolute values. Additionally, there is no information about standard deviations for PLV comparisons, making it difficult to assess the reliability of results. Given that connectivity is a key claim of this study, improved statistical transparency is essential.

We acknowledge the limitation of our current presentation and have adapted our analysis to account for between- and within-subject variability and included additional information in the main text (Figure 1C (shown in response to reviewer 1, comment 1), Figure 2 (shown in response to comment 9), Figure 3B (shown in response to reviewer 1, comment 1), Figure 5 (shown in response to reviewer 1, comment 2)) and the supplementary figures in response to the reviewer's concern. Furthermore, we adapted our analysis of connectivity measures and retained only contact pairs showing significant interaction in all three conditions, followed by linear mixed models to account for between- and within-subject variability. In Figure 5 (previous Figure 3) we now include annotations that indicate the number of electrode pairs showing significant interactions (Figure 5C). Mean values are now shown using box plots (Figure 5A), and we additionally include differences in PLV and PSI between significantly different region pairs (Figure 5B). Furthermore, we have included boxplots showing mean PLV and PSI between each region pair for each frequency band and stimulation as well as the difference matrices for all frequency bands in the Supplementary materials (Supplementary Figures 8-10).

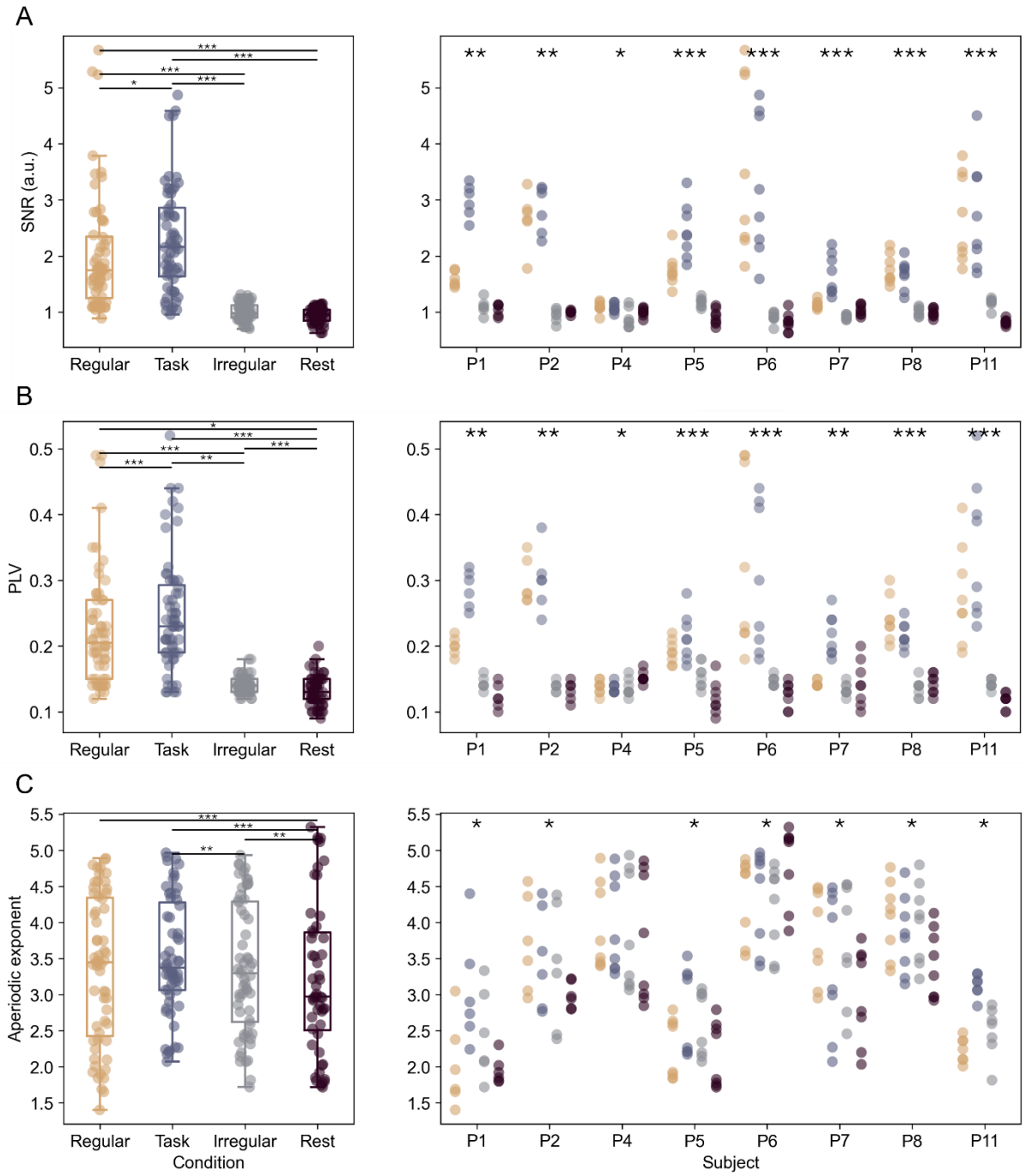
9. Inter-Subject Variability: The manuscript does not sufficiently address whether effects were consistent across subjects. Were the reported findings observed in all individuals, or were they driven by a subset of participants? If variability exists, it should be explicitly discussed, and subject-level results should be provided (e.g., in supplementary figures). Figures 2 and 3 should also indicate individual subject contributions to highlight the robustness of the reported effects.

While there was some variability in the findings, our results are robust in showing that visual 40 Hz stimulation modulates neural activity in the hippocampus in all but one patient. The following was added to the discussion (line 314):

"Our results show that visual stimulation effectively modulates neural activity in the hippocampal region. All patients, except for one (P4), showed a significant increase in SNR in response to regular conditions. In the same patient, gliotic transformation in the parahippocampal gyrus was observed, which has been previously linked to altered inhibitory synaptic transmission in hippocampal neurons 26."

Additionally, we modified Figure 2 in the main text, showing results for the hippocampus, so that both results on group- and individual level are presented. Results on individual

level for other brain regions were added to the Supplementary materials (Supplementary Figures 4–6).



*"**Figure 2. Results for hippocampal contacts.** **(A)** SNR, **(B)** PLV to the stimulation, and **(C)** aperiodic exponent boxplots for each hippocampal contact on group- (left) and patient level (right), for each condition. Each dot represents one hippocampal contact. Color convention: yellow – Regular, blue – Task, gray – Irregular, purple – Rest. Box plots: The central line on each box marks the median and the bottom and top edges mark the lower (Q1, 25th percentile) and upper quartile (Q3, 75th percentile), the whiskers mark the range from Q1 – 1.5 * IQR to Q3 + 1.5 * IQR, where the inter-quartile range (IQR) is*

*defined as Q3 – Q1. The horizontal black lines denote the presence of a statistically significant difference between conditions, the stars the significance level (nonparametric Friedman test for within-subject comparisons and Wilcoxon signed-rank test for pairwise comparisons on a group level) with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$."*

10. Limited Supplementary Data: The supplementary materials are minimal and do not adequately summarize patient-level data. Even if full datasets cannot be provided, a summary figure displaying patient-specific responses for the main analyses (Figures 2 and 3) would be highly beneficial. Since the study relies solely on neural measures to claim GENUS efficacy, more detailed patient-specific data are necessary.

In response to this concern, we have expanded the Supplementary materials and the figures in the main text to improve the interpretability of our findings and to better capture individual-level data across our outcome variables.

Figure 2 (shown in response to comment 9) in the main text now displays both group-level and subject-level results for hippocampal contacts. To further address inter-subject variability in terms of synchrony measures, we include a subject-level time series plot of PLV to the stimulation in the main text (Figure 3, shown in response to reviewer 1, comment 1).

Supplementary Figures 4-6 now provide group- and subject-level results for SNR, PLV, and the aperiodic exponent for other brain regions. Additionally, we included examples of raw data traces for each condition, and full-spectrum plotted on a group and subject-level (Supplementary Figures 1-3).

Together, these figures offer a more comprehensive overview of between-subject variability and patient-specific responses. We hope this addresses the reviewer's concern and strengthens the transparency and interpretability of our findings.

11. Figure Legends and Data Representation: The figure legends lack details regarding what data points represent (e.g., whether they correspond to individual patients or session averages). This ambiguity must be addressed to ensure clarity in data interpretation.

Both the SNR and PLV to the stimulation were estimated as a single value per contact. In the box plots, each dot represents one contact (for all subjects on a group level and for one subject on an individual level). This has been clarified in the corresponding figure captions.

Minor Issues:

• The excessive use of abbreviations (RN, RO, IN) makes the results harder to follow. It would improve readability to use clearer labels such as "With Attention" and "Without Attention."

We appreciate for pointing this out. To improve readability, we decided to use the following labels for the conditions and changed them in the text: (Regular 40 Hz stimulation - Regular, Regular 40 Hz stimulation combined with task - Task, and Irregular stimulation - Irregular).

• Figure 3 uses inconsistent terminology—while the text refers to "regular without attention," Figure 2 uses "RN," and Figure 3 states "Regular - No Task." The authors should standardize their terminology.

We thank the reviewer for this observation. We corrected the inconsistencies and use only the terms listed above.

• The introduction does not clearly articulate the research gap being addressed. It currently lists related studies without a clear motivation for the present work. A revision emphasizing the unresolved questions and how this study addresses them would strengthen the manuscript's rationale.

One of the unresolved questions from previous research remains how entrainment propagates beyond early visual areas. While it was shown in mice that combining stimulation with physical exercise may enhance its effect (Park et al., 2020), studies in humans are lacking. Building on our previous study (Khachatryan et al., 2022), we aimed to provide additional insight into the effects of task engagement on stronger and more widespread entrainment. This is now clearly motivated in the introduction (line 89).

"Despite these promising findings, the factors driving entrainment propagation beyond early visual areas remain poorly understood. In animal models, combining visual 40 Hz stimulation with physical exercise has led to the most effective reduction of Aβ and tau levels, potentially improving cognitive function 10. However, in humans, the impact of simultaneous engagement during GENUS remains unclear, as many studies have either not recorded EEG responses or have not examined the effects of concurrent tasks 13,16,18,19. Building on these findings and our previous EEG study 20, where we showed that including a cognitive task during stimulation enhances the strength and extent of the neural response, we aim to provide further evidence that task engagement facilitates stronger and more widespread entrainment. Since steady-state visual responses are typically restricted to early visual areas 21, it is important to evaluate potential benefits of cognitive engagement, as gamma-based interventions primarily target deeper structures such as the hippocampus, one of the earliest and most affected regions in AD."

• The paper moves abruptly from discussing Alzheimer's disease to testing hippocampal responses to gamma stimulation without adequately bridging these

topics. While this connection seems implicit, a clearer rationale should be provided in the introduction. The authors should explicitly justify why hippocampal gamma entrainment is relevant to Alzheimer's pathology and temper their claims regarding its therapeutic potential.

We have added the following explanations to the introduction (line 41, line 53) to explain the rationale to focus on the hippocampus and gamma oscillations more clearly:

"The hippocampus is among the earliest and most severely affected regions in Alzheimer's disease, exhibiting pronounced atrophy and accumulation of amyloid and tau pathology 5,6. As a key structure for memory encoding and retrieval, its vulnerability to early degeneration makes it a central target for therapeutic interventions."

"This specificity aligns with neurophysiological studies which show that slow gamma oscillations (~40 Hz) are involved in memory retrieval processes in the hippocampus 12. Furthermore, a recent study using intracranial recordings showed that 40 Hz stimulation led to a neural response in significantly more contacts compared to 5.5 Hz or 80 Hz stimulation 13."

Furthermore, we have revised some sentences implying therapeutic potential of such stimulation (line 66, line 84):

"In addition to reducing amyloid and tau levels, GENUS has been associated with neuroprotective changes, including enhanced glymphatic clearance 11, reduced neuroinflammation 8,9, and modulation of glial cell activity 9 in AD mouse models."

"While studies of GENUS in humans remain limited, the reported findings suggest GENUS could offer a promising tool for probing neural mechanisms associated with AD."

Final Evaluation and recommendation:

While the dataset and objectives are valuable, the analysis and interpretation require further development to provide a more robust and well-substantiated contribution. Substantial revisions—particularly in experimental controls and behavioral effect assessment (if not feasible, advanced analyses, eg. the stimulation's temporal effects), clarity of statistics, and clarity of interpretation—are necessary before the manuscript can be considered for publication.

Reviewer #3 (Remarks to the Author):

The goal of this study was to build on work previously published by the same group showing that the inclusion of a cognitive task during visual 40 Hz stimulation increased strength and propagation of response. In this paper, intracranial recordings were collected in 11 individuals with epilepsy during 5 minutes of visual 40 Hz stimulation with or without a visual oddball task.

Data showed strong entrainment to stimulation in terms of signal-to-noise ratio (40 vs 38-42 Hz) and phase-lag value in the hippocampal leads. While both conditions created significant response in frontal, insular, and temporobasal regions, further analysis using phase slope index to determine direction of information flow showed that the oddball tasked reversed the activity in gamma, alpha, and beta frequency bands from hippocampus to frontal areas to a more "top-down" structure of flow from the frontal area to hippocampus.

Overall, this work builds to further elucidate the influence of attention and cognitive load on response to 40 Hz sensory stimulation, confirms the entrainment of deep brain structures in human patients using intracranial recordings in a larger cohort than previously shown, and promotes future work to examine the clinical impact of these differences in neural entrainment.

We would support the publication of this manuscript. In terms of suggestions for revisions:

1. The authors mention the small sample size in limitations of the study. Was any of the data analyzed in a way that accounts for patient-to-patient differences? We see that SNR and PLV are correlated. Are any of the results driven by a subset of high-responders or low-responders rather than cortical region differences?

While there was some variability in the findings, our results are robust in showing that visual 40 Hz stimulation modulates neural activity in the hippocampus in all but one patient. The following was added to the discussion (line 314):

"Our results show that visual stimulation effectively modulates neural activity in the hippocampal region. All patients, except for one (P4), showed a significant increase in SNR in response to regular conditions. In the same patient, gliotic transformation in the parahippocampal gyrus was observed, which has been previously linked to altered inhibitory synaptic transmission in hippocampal neurons 26."

Additionally, we modified Figure 2 (shown in response to reviewer 2 comment 9) in the main text, showing results for the hippocampus, so that results both on group and individual level are presented. Results on individual level for other brain regions were added to the Supplementary materials (Supplementary Figures 4–6 for SNR, PLV, and the aperiodic exponent).

2. Previous work has highlighted the importance of multisensory stimulation. Is there a reason that this study focused only on visual?

While previous research suggests that combining visual and auditory stimulation may enhance neural responses, most studies using audiovisual stimulation have been conducted in humans, with limited physiological data available from animal models to clarify its mechanisms at the neuronal level (Blanpain et al., 2024). Given this gap, our study aimed to isolate the effects of 40 Hz visual stimulation on neural entrainment across the brain. By focusing on a single sensory modality, we aimed to better characterize its independent contribution before exploring potential interactions with other modalities.

3. Was the presentation of stimulation conditions randomly ordered? If not, the authors should mention this within the methods.

The presentation of stimulation conditions was randomly ordered between participants. We added the following clarification to the Experimental procedure and data acquisition section (line 445):

"The order of stimulation conditions was randomized across participants."

4. The interaction between hippocampal integrity assessed by MRI and the results presented is not clear.

Our motivation for assessing hippocampal structural integrity stems from the association between hippocampal atrophy and both early AD and drug-resistant temporal lobe epilepsy (Moran et al., 2001). In the current study, we evaluated atrophy of the hippocampus with an *MTA*-score (medial temporal lobe atrophy score) to determine its potential effects on gamma entrainment. Importantly, we observed the effect of stimulation regardless of the presence of hippocampal atrophy (Figure 2 now includes both group- and patient-data, shown in response to reviewer 2, comment 9). Out of the eight patients with hippocampal depth electrodes, three of them showed signs of atrophy (P1, P2, P11), yet they all showed the effects of stimulation. This suggests that hippocampal atrophy, at least to the degree observed in our study, does not prevent a neural response to visual gamma stimulation.

We acknowledge that definitive conclusions about the relationship are difficult to make, given the limited number of patients with atrophy and the small sample size in our current study. However, our findings show that visual 40 Hz stimulation can elicit a neural response even in the presence of structural alterations of the hippocampus. We have revised the Discussion section (line 309) to articulate this point more clearly.

"Importantly, in this study, gamma entrainment was observed in both passive visual stimulation and stimulation combined with an attention task, despite the presence of atrophy in 3 out of 8 patients implanted with depth electrodes targeting the hippocampus.

While this suggests that visual gamma stimulation can elicit a neural response even in the presence of atrophy in the hippocampus, our small sample size limits drawing stronger conclusions about the interaction between hippocampal structural integrity and the effects of stimulation. Future studies on larger samples are needed to systematically examine this relationship."

The following explanation was added to the Results section (line 108):

"Given that hippocampal atrophy or sclerosis is frequently observed in patients with drug-resistant mesial temporal lobe epilepsy 22, the structural integrity of the hippocampi of the patients included in this study was assessed."

5. Finally, accuracy of oddball response was also collected. It would be interesting and useful to see if neural entrainment values are reflected or correlated with task accuracy or other finer metrics of cognitive load.

We appreciate the suggestion and agree with the reviewer. While it would indeed be valuable to examine the relationship between task accuracy and entrainment, the current task was designed primarily to maintain engagement rather than to assess cognitive load. Given its simplicity, (only 10 oddballs were presented), participants performed with an average accuracy of 94.6% (± 0.06). This near-ceiling performance limits the possibility of seeing meaningful correlations with cognitive load. However, we recognize its potential importance and believe that it would certainly be of interest to estimate this in the future work. We added the following to the discussion (line 368):

"While the current oddball task design effectively ensured participants' engagement, its simplicity limits the ability to assess a potential relation between entrainment and cognitive load given the small number of oddballs and the near-ceiling accuracy. Future studies could address this limitation by incorporating a more demanding cognitive task, which may be particularly relevant in patients with cognitive decline."

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Response to Reviewers

We thank again all reviewers for their constructive feedback and suggestions that allowed us to improve our manuscript.

Reviewer #1 (Remarks to the Author):

The authors have done a good job addressing my comments; I am ready to sign off on this manuscript for publication.

Reviewer #2 (Remarks to the Author):

I appreciate the authors' comprehensive responses to my comments and concerns.
The revision substantially improved the manuscript, particularly regarding statistical transparency, visualization of raw and processed data, and clarification of methodological and interpretive points. The inclusion of individual-level data, aperiodic exponent analysis, and careful attention to possible confounds (power vs. phase-locking) in the revision letter strengthen the analysis clarity and support the manuscripts' conclusions.
I also appreciate that the authors have now properly tempered claims about the therapeutic relevance of GENUS and clarified that their study is not designed to assess cognitive enhancement. Their justification of 40 Hz as a target frequency are convincing and well-contextualized.
While the absence of behavioral outcomes limits the interpretive scope, this is now clearly acknowledged, and the manuscript appropriately reframes its contribution as physiological evidence of neural entrainment.

Minor suggestions:
Fig3.B. the y-axis labels for p7 and p8 are misaligned.

We thank the reviewer for this observation and have now fixed the misalignment in the figure accordingly.

Fig1.C. Consider overlaying the LFP traces for different conditions within each region. Plotting them on the same axis (with color-coded lines) would make comparisons across conditions more intuitive and easier to interpret.

We adapted the Fig. 1C, which now shows LFP traces for the Regular and Irregular conditions on one subplot and the Task and Irregular conditions on the second one.

Reviewer #3 (Remarks to the Author):

The authors have addressed all of our concerns satisfactorily and thoroughly. This study elucidate the influence of attention and cognitive load on response to 40 Hz sensory stimulation. The authors show the entrainment of deep brain structures such as the hippocampus in human patients using intracranial recordings in a larger cohort than previously shown. This work will likely promote future work to examine the clinical impact of these differences in neural entrainment. I therefore support the publication of this manuscript in Communications Biology.