nature portfolio

Peer Review File

Stimulation modulates gene-linked cell assemblies in the human brain

Corresponding Author: Dr Genevieve Konopka

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

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Editorial Note: To help resolve the divergence of opinion, another referee was consulted and found the authors' response satisfactory.

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
Manuscript by Moore et al investigates molecular and physiological consequences of brain stimulation in the human brain, taking advantage of direct access to human brain tissue from surgical specimens. The authors generate a remarkably important dataset outlining gene expression and enhancers activated or suppressed by neural activity in the human brian, which extends our knowledge beyond the limited set of activity dependent genes such as FOS and JUN or BDNF. A major strength of the paper is the physiologically relevant experimental model, as well as unique findings that some neuronal subtypes, particularly glutamatergic neurons, seem to initiate transcriptomic changes in response to stimulation much more readily than inhibitory neurons. This finding is crucial and opens new avenues for investigation. A weakness of the analysis is that it is very difficult to understand the connection between the first part of the paper where the authors investigate patterns of network propagation upon electrical stimulation with the molecular interrogation of dysregulated genes and chromatin state. In the end, I thing the authors should attempt to reconcile these results and if cannot, they should focus on the unique strengths of this study, which is the detail characterization of molecular changes within the brain after stimulation.
- The introduction of the paper provides an excellent background and framing to the key questions being pursued by the investigators, providing a compelling rationale for the study. One discussion angle that could factor into this introduction, however, would be the introduction of prior work on activity dependent gene expression, including the pioneering work by Michael Greenberg. What key immediate early genes and activity dependent molecular processes have been discovered so far and what remains to be discovered. I think this kind of discussion could help strengthen the rationale.
- The authors apply stimulation to human organotypic brain slices, which is a powerful approach to functionally interrogating functional connectivity with their tissue preparations. Their current analysis focuses overwhelmingly on co-variation of neural activity as a mechanism for inferring neuronal assembles, however this seems fairly limited and leaves opportunities for more sophisticated analyses unexplored, including measurements of oscillatory behaviors, recurrence, or inference of connectivity from not just measurements of co-firing dynamics but also latency in spike timing. The analysis could also better leverage the spatial distribution of units on the planar HD-MEA in the cortical tissue, with reference to the known patterns of cortical connectivity. Currently, there is only one Supplemental figure panel 2D that offers a very limited insight into this type of analysis. I hope the authors can elaborate on this point.
- Molecular analysis of gene expression changes after stimulation is a particularly novel and interest aspect of the manuscript. I find the lack of a strong signal in GABAergic interneurons to be particularly fascinating, and would encourage the authors to include this contrast to excitatory neurons as part of the main text figure. It would also be interested to investigate nominally significant DEGs in interneurons to see if they might correlate with FDR-significant DEGs from glutamatergic neuron analysis.
- Analysis of the epigenetic data leaves some additional room for improvement. Currently, it is unclear how many open chromatin regions are differentially accessible upon stimulation, including in which cell types, and whether specific TF motifs are enriched among those altered open chromatin regions. Prior work by the Greenberg lab also identified human specific activity regulated gene

- The lack of concordance between in vivo and in vitro stimulation seems particularly striking. Methodological differences in how the stimulus might have been applied through an MEA versus the Ojemann stimulator might contribute to some of the differences, but this does not seem to be extensively explored. The authors also do not comment on any potential effects in interneurons after in vivo stimulation. To extend the findings more broadly and provide a resource for the community, the authors could consider generating some stimulation and gene expression data for stem cell derived neurons or neural organoids. While these are highly distinct models to what is being studied here, a detailed comparison could be of broad interest to the community and elevate the impact of the paper.
- Although the authors do not comment on this, they also appear to detect changes in non-neuronal cells. Because the analysis is rather isolated, it is difficult to understand which transcriptomic changes are specific to non-neuronal cells versus neurons. The analysis, currently hidden in Figure S5, could be elevated and discussed in greater depth. In fact, what may be most striking is that transcriptional chance in glia are perhaps more numerous than in neurons.

(Remarks on code availability)

Referee #2

(Remarks to the Author)
The study by Moore et al. reports analysis of an exceedingly rare data set of the electrophysiologic and transcriptomic response to electrical stimulation of ex vivo human brain samples acquired following temporal lobectomy and then organotypic slice culture preparation. The ex vivo results were then confirmed with samples taking following in vivo stimulation of the prefrontal cortex, comparing stimulated vs control sites excised in this region during surgical treatment of epilepsy.
They find that with stimulation over 15 min, they tend to see greater spiking activity in the following 10-15 min block of time, with this effect driven by principally excitatory neurons based on spiking waveform features. They show that the recorded electrical activity could be reliably clustered into co-activating 'assemblies' and that across their recordings, of 19 defined assemblies, 11 showed stronger co-activation following stimulation, 5 showed weaker co-activation following stimulation, and 3 showed no change. They then delve into the rich data set of gene expression changes focusing principally on the changes in neurons seen following stimulation in their preparations. Both ex vivo and in vivo they see a wide panoply of changes of known activity-dependent genes and genes related to synaptic structure and maintenance, with different changes seen across cortical layers with the most concordant changes between the preparations seen in layers 3 and 5.

Overall, the principal advance in this study is the generation of these gene expression datasets from human brain tissues, which the authors are nicely making available to the community. The results reported in this analysis are incredibly interesting but fundamentally descriptive in a way that unfortunately falls short of yielding the 'clear mechanistic explanation of how exogenous stimulation reshapes circuit dynamics to produce…behavioral effects' to which the authors allude in the introduction. There appears to be some missed opportunities to provide further potential mechanistic insight in these datasets and similar experimental preparations, if it were logistically feasible to complete.

Specific comments:
• The term 'plasticity' in describing the assembly level changes (Fig. 2) is used a bit loosely and are reported in response to a single stimulation protocol. Long-term plasticity is usually reported over the course of ~1 hour, not the 10-15 min over which these recordings were completed. Further, it is not clear how long these changes may have persisted or not over the 10-15 min recording period in these data. Finally, it is already known that electrical stimulation can yield changes in functional brain activities in humans in the off period following brain stimulation – for instance, Parkinson's symptoms recur on a somewhat stereotyped time course following turning off of DBS, with different delays in different symptoms with tremor recurring within 10s of seconds (as Fig. 2F suggests), then bradykinesia/rigidity coming back later, and so on. As is the electrical recording results themselves are limited in terms of novel mechanistic insights.
• To gain further mechanistic insight into the electrical recordings, the response to differing stimulus paradigms, and how those correlate to gene expression data, would be of great interest given the known differential response of human neural circuits to DBS or TMS of different stimulation frequencies, which is thought to be mediated by different neuronal and perhaps non-neuronal cell types.
• What correlated analysis could be completed between the electrical and gene expression datasets? Could we understand which gene expression profiles correlate to increasing neuronal assembly strength following stimulation vs the decreased neuronal assembly strength?
• Looking at Fig. S6, it appears the greatest DEG level changes are seen, among neurons, in layer 2/3 and layer 4 – even though these neurons show anti-correlated or minimally correlated changes between the MEA and IVS results (Fig. 6). In contrast, it appears relatively greater overall changes are seen in the non-neuronal cells, across astrocytes, microglia/PVMs, and oligodendrocytes (both precursor and mature). From this vantage, it appears that the greater signal is in the non-neuronal cells although the authors focused principally on the neuronal changes in this analysis. How do the neuronal and non-neuronal changes correlate to each other to mediate the observed changes in electrical spiking behavior? Did the non-neuronal gene changes correlate as well with the electrical recording findings as the neuronal gene changes? How concordant are the non-neuronal changes between the MEA and IVS preparations?

Minor comments
• How do the authors interpret that the MEA vs IVS changes are anti-correlated in layer 2/3 cells (Fig. 6)? Does this reflect that the recordings were completed in different brain regions? Or is the cell culture process altering the layer 2/3 cells in some way?

• More for curiosity, were the temporal and prefrontal cortical recordings typically from a particular gyrus (STG vs parahippocampal, SFG vs IFG, etc) or was it from any frontal or temporal region that may be readily included in the surgical field?
• In the Fig 1 legend, I believe the comment re log(FR+1) presentation is about panels D and F, not D and G
• Were these trials registered on ClinicalTrials.gov? If so, the registration number should be included.

(Remarks on code availability)
I believe the code is of use to the community, and definitely the code with the GEO database repository is a great addition. At a cursory glance the code looks appropriately constructed.

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)
The reviewers have addressed my concerns and the manuscript represents an interesting an novel contribution to the literature.

(Remarks on code availability)

Referee #2

(Remarks to the Author)
In this revised manuscript the authors have admirably added clarifying discussion, methodological detail, and new analysis compared to the initial submission. In this study the investigators took human post-surgical brain samples (from the anterior temporal lobe) and cultured them in a slice preparation. Then with multielectrode arrays (MEAs) they stimulated patterns of activity in the ex vivo brain tissue, measured the spiking response of neurons in response to the stimulation, and analyzed the changes in gene expression associated with this stimulation. They then compared similar metrics to prefrontal cortical (PFC) tissue also indicated for neurosurgical resection. They stimulated the target PFC region and measured gene expression changes resultant from the stimulus following excision, and compared the results of in vivo stimulation (IVS) to MEA stimulation post culture. The authors observed that stimulation is associated with a short-term plasticity in the form of increasing neuronal co-firing in assemblies in the 10-15 min following stimulation. They further observed that neuronal assemblies – defined as co-activating/firing sets of single units – generally show an increase of co-activiation, with >half of assemblies showing strengthened co-activation following stimulation. In the genetic analysis, the authors find that stimulation is associated with a general upregulation of activity dependent and synapse associated genes. Curiously, the authors also find that simulation yields substantial changes in non-neuronal cells like microglia and oligodendrocytes. However, the genetic response to stimulation was largely anti-correlated between the in vivo prefrontal cortical stimulation case and the anterior temporal cultures MEA preparation.

Altogether, the study remains an interesting one, as it confirms that assembly architecture in response to stimulation is seen in human brain tissue like it has been observed in animal models, and is conserved between in vivo and ex vivo preparations. The added hdWGNA analysis and new discussion all help to clarify pending questions from the original submission. However, the fundamental conclusions of this study continue to fall short of revealing mechanistic insight into how neuronal assemblies of co-activating neurons form or contribute to functional effects. That the gene expression data are so different from the cultured MEA/anterior temporal and the IVS/prefrontal cortical stimulation studies indicates that the generalizability of these findings may be limited. For instance, from the MEA results, layer 4 cells are principally implicated as the mediators of the response to simulation, but after comparing to the IVS results it seems the focus should more be on layer 3 and 5 cells. To fully understand which results here would truly generalize would require a greater number of samples across different stimulation protocols and preparations that are likely impractical to implement as the authors note in their rebuttal. Finally, the more surprising and curious results in this analysis – namely that the non-neuronal cells show a similar level of gene expression changes in response to stimulation as neurons – continues to be under-explored in this study.

Overall, the revised study remains an interesting contribution to the literature, but one whose conclusions may be more appropriate for a more specialized journal and audience.

Few additional questions/comments:
• Could the non-neuronal cellular response to stimulation have implications for the gliosis observed in response to DBS? How may we interpret the microglial and oligodendroglia gene networks being recruited in terms of the plasticity that is observed or expected in the firing rate results? Do the genes induced in microglia and astrocytes in response to stimulation cohere more with a disease-associated phenotype or the homeostatic phenotype of each cell type?
• The current analysis comparing the MEA and IVS results focuses on the DRGs upregulated in concert between the paradigms - what more can be understood by looking also at the commonly downregulated genes between the MEA and IVS preparations?

(Remarks on code availability)

Please find our responses to the reviewers below in blue text.

Referee #1 (Remarks to the Author):

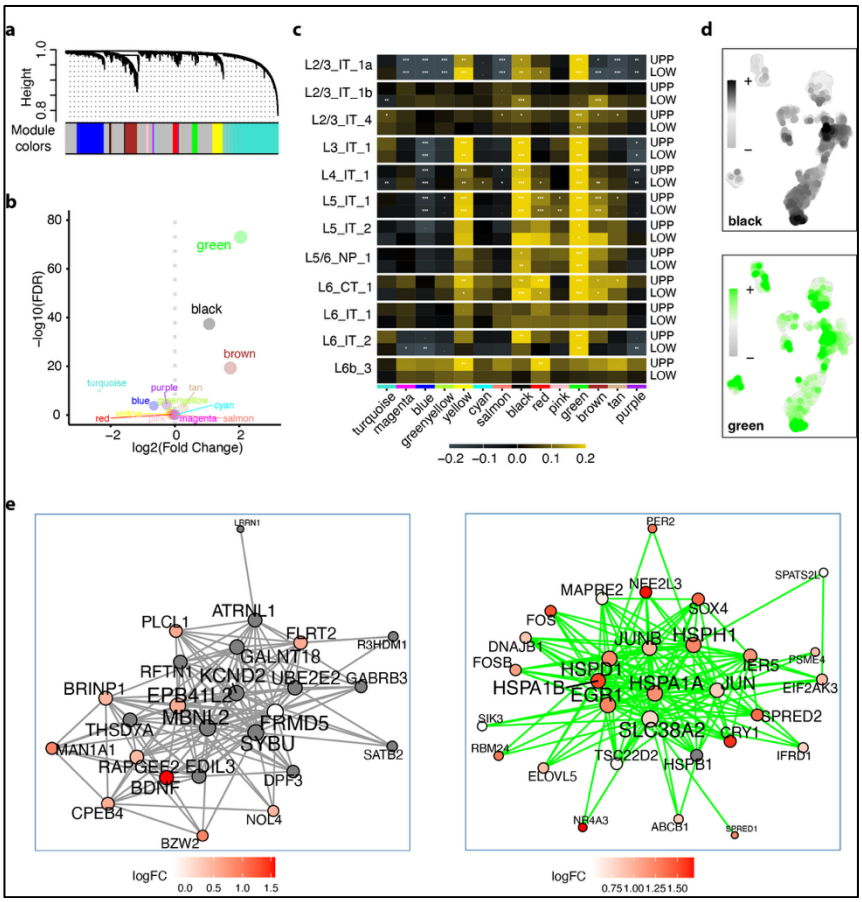
Manuscript by Moore et al investigates molecular and physiological consequences of brain stimulation in the human brain, taking advantage of direct access to human brain tissue from surgical specimens. The authors generate a remarkably important dataset outlining gene expression and enhancers activated or suppressed by neural activity in the human brian, which extends our knowledge beyond the limited set of activity dependent genes such as FOS and JUN or BDNF. A major strength of the paper is the physiologically relevant experimental model, as well as unique findings that some neuronal subtypes, particularly glutamatergic neurons, seem to initiate transcriptomic changes in response to stimulation much more readily than inhibitory neurons. This finding is crucial and opens new avenues for investigation. A weakness of the analysis is that it is very difficult to understand the connection between the first part of the paper where the authors investigate patterns of network propagation upon electrical stimulation with the molecular interrogation of dysregulated genes and chromatin state. In the end, I thing the authors should attempt to reconcile these results and if cannot, they should focus on the unique strengths of this study, which is the detail characterization of molecular changes within the brain after stimulation.

We thank the reviewer for this positive summary of our manuscript that highlights the strength of our approach, as well as the reasonable critique about integrating the two distinct datasets we have generated. The challenge, as the reviewer notes, is reconciling the dynamic nature of the electrophysiology measurements in the first half of the paper compared to the static “snapshot” of gene expression data in the second half. Integrating these two data types is a limitation in the field due to the absence of a method that measures gene expression longitudinally in the same cells over time. We note that we cannot carry out a correlation analysis similar to those we previously conducted for oscillatory measures in human brain (Berto et al., 2018 and 2021), given the sample size in this current study, as such analyses would be underpowered and may not result in statistically robust results. We plan to now further clarify such limitations and further discuss them in a revised manuscript.

In addition, in lieu of carrying out an underpowered one-to-one correlation between individual genes and electrophysiological metrics, we implemented a robust and sufficiently powered analysis that can optimize integration of the available datasets. **We will now include new single cell/high dimensional weighted gene co-expression network analysis (hdWGCNA) results that directly link transcriptional programs to firing rate metrics.** As an example of what we propose to present in a revised manuscript, here we show the preliminary results from excitatory neurons only. We generate hdWGCNA networks from single-cell gene expression data and identify co-expression modules that are differentially weighted between stimulated and control conditions. We then correlate module eigengenes with MEA-derived firing rate metrics, thereby directly connecting stimulation-induced network activity to coordinated transcriptional responses rather than single genes. As an example of this integrative approach, we present preliminary results focused on excitatory neurons. Here, we found two modules (“green” and “black”) that are both differentially weighted following stimulation and significantly correlated with excitatory neuronal firing rates (see Response Figure 1 below). These modules are most highly expressed in L2/3 and L4/5 excitatory neurons and are enriched for canonical activity-dependent transcriptional regulators, including *FOS*, *JUN*, and *BDNF*, which are hub genes within these networks.

Together, this integrative analysis linked physiological and molecular components of the study by showing that stimulation-induced changes in network firing are supported by coordinated activation of activity-dependent gene regulatory programs. These results are consistent with a model in which electrical stimulation preferentially engages excitatory neurons in upper and middle cortical layers and activates transcriptional networks that may contribute to circuit-level plasticity. In line with the reviewer’s recommendation, we have also refined the focus

of the manuscript to emphasize its central strength: a detailed molecular characterization of human brain responses to physiologically relevant stimulation, now directly connected to electrophysiological measurements.



Response Figure 1. New hdWGCNA data using snRNA-seq and firing rate data from excitatory neurons. A) Network dendrogram. B) Significance of each module correlated with firing rate fold change. C) Correlation of each module with cell type and upper or lower layer neuron firing. D) Feature plot of expression of genes in the black or green module in the original multiomic UMAP space. E) Visualization of the top 25 hub genes in the black and green modules. Red color indicates logFC of gene expression in stimulated vs control cells.

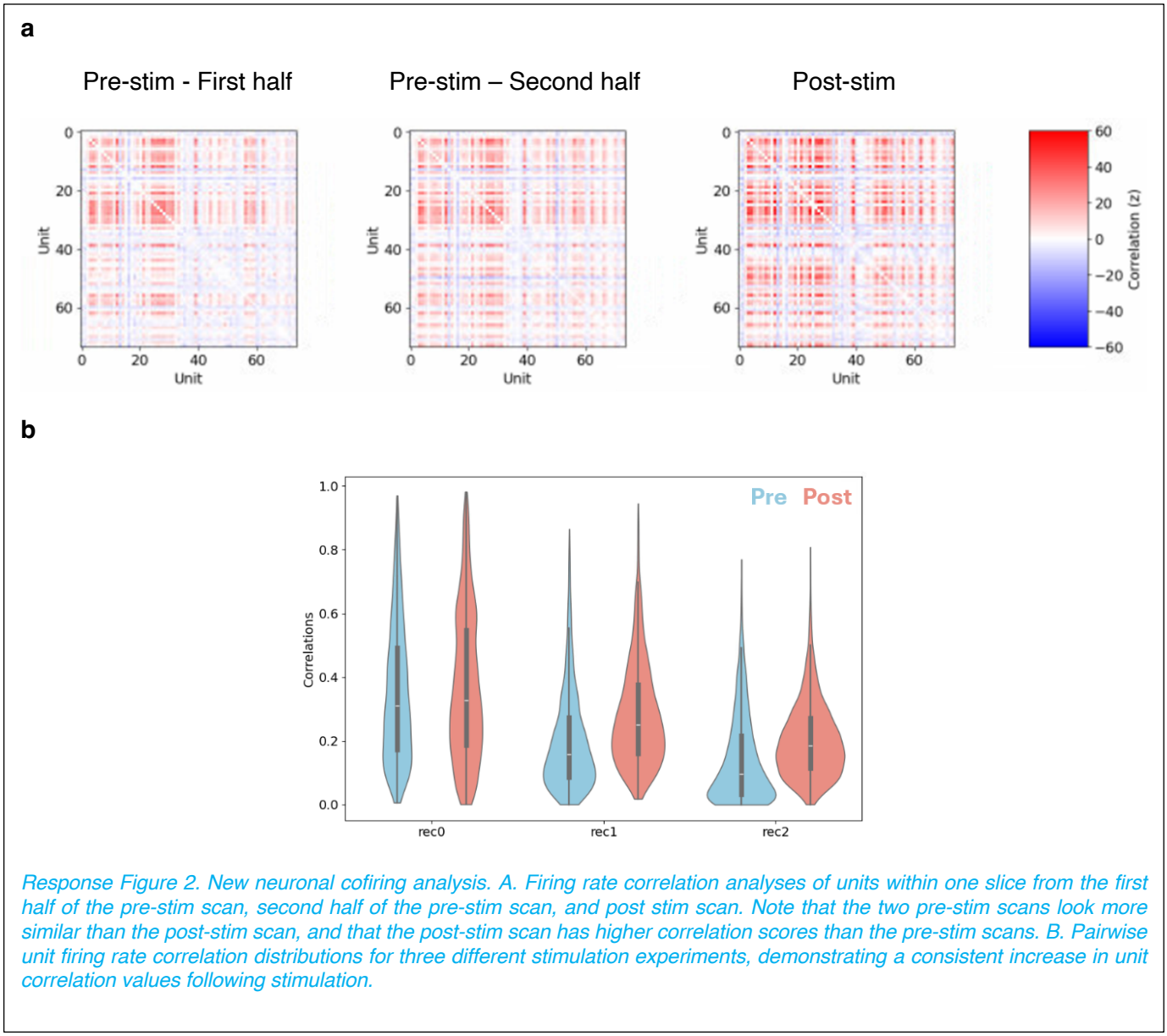
- The introduction of the paper provides an excellent background and framing to the key questions being pursued by the investigators, providing a compelling rationale for the study. One discussion angle that could factor into this introduction, however, would be the introduction of prior work on activity dependent gene expression, including the pioneering work by Michael Greenberg. What key immediate early genes and activity dependent molecular processes have been discovered so far and what remains to be discovered. I think this kind of discussion could help strengthen the rationale.

We had previously referenced work from a protégé of Dr. Greenberg (Dr. Jesse Gray; PMID 29681534; Reference #21 in the manuscript) and Dr. Greenberg himself (PMID 20393465; Reference #22 in the manuscript) in our description of activity related genes. We don't use the term "immediate early genes" but adopt the updated nomenclature of activity regulated genes (ARGs) separated by rapid primary response genes (rPRGs) delayed primary response genes (dPRGs) and secondary response genes (SRGs). However, we will now include additional references from Dr. Greenberg.

- The authors apply stimulation to human organotypic brain slices, which is a powerful approach to functionally interrogating functional connectivity with their tissue preparations. Their current analysis focuses overwhelmingly on co-variation of neural activity as a mechanism for inferring neuronal assembles, however this seems fairly limited and leaves opportunities for more sophisticated analyses unexplored, including measurements of oscillatory behaviors, recurrence, or inference of connectivity from not just measurements of co-firing dynamics but also latency in spike timing. The analysis could also better leverage the spatial distribution of units on the planar HD-MEA in the cortical tissue, with reference to the known patterns of cortical

connectivity. Currently, there is only one Supplemental figure panel 2D that offers a very limited insight into this type of analysis. I hope the authors can elaborate on this point.

We agree with the reviewer that several additional electrophysiological analyses are feasible with these data, although we note that the reviewer does not recommend a specific approach that examines the effects of stimulation differently. We chose cofiring in key 25 msec time windows due to the importance of neuronal assemblies to in vivo physiology in both humans and animal models. However, we performed a novel analysis in which we mapped the cofiring structure across all neurons, identifying 1) stability within the pre-stimulation baseline and 2) increased cofiring across several time scales, consistent with our previous reports. Below, we share a preliminary look at these new results. We will update Figure 2 to reflect these new data, including the impact of stimulation. We will further execute a novel analysis in which we describe cofiring in short time scales across neuronal layers (3 msec), thought to be characteristic of monosynaptic connectivity especially for pyramidal/interneuron dyads. More broadly, the number of additional analyses one can perform with this dataset is extensive, and future work can incorporate local field potentials and interventions such as optogenetic activation of neuronal assemblies.



Response Figure 2. New neuronal cofiring analysis. A. Firing rate correlation analyses of units within one slice from the first half of the pre-stim scan, second half of the pre-stim scan, and post stim scan. Note that the two pre-stim scans look more similar than the post-stim scan, and that the post-stim scan has higher correlation scores than the pre-stim scans. B. Pairwise unit firing rate correlation distributions for three different stimulation experiments, demonstrating a consistent increase in unit correlation values following stimulation.

- Molecular analysis of gene expression changes after stimulation is a particularly novel and interest aspect of the manuscript. I find the lack of a strong signal in GABAergic interneurons to be particularly fascinating, and

would encourage the authors to include this contrast to excitatory neurons as part of the main text figure. It would also be interested to investigate nominally significant DEGs in interneurons to see if they might correlate with FDR-significant DEGs from glutamatergic neuron analysis.

We agree that the gene expression in interneurons is certainly of interest. We focused on excitatory neurons because that is where we observed the most change physiologically in the MEA data, and we were striving to link these two distinct datasets as much as possible. To address the reviewer's point though, we have **now carried out a RRHO analysis of the excitatory and inhibitory gene expression and found both overlapping and distinct gene expression programs.** Here, we outline these new results that would be included in a revised manuscript.

- L2/3-IT-1a upregulated genes exhibit high overlap with upregulated *and* downregulated genes in several types of interneurons, including cell clusters marked by *LAMP5*, *PVALB*, or *VIP* expression. As described in the manuscript, activity regulated genes, including IEGs, were not differentially regulated in L2/3-IT-1a cells or the interneuron subtypes. However, L2/3-IT-1a cells were flagged as the third highest priority in the Augur analysis (Fig 3F), had the second highest increase in peak-to-gene linkages after stimulation (Fig 3G), and showed activation of IEG-regulated GRNs (Fig 4A). The convergence seen in the upregulated genes of L2/3-IT-1a cells and many interneuron cell types may represent a non-traditional activity regulated gene program that is commonly activated during stimulation in an otherwise diverse group of cells.
- L3, L4, and L5 IT neurons showed similar gene regulation patterns in our original analysis. As such, we see similar degrees of convergence with different interneuron subtypes. For example, both upregulated and especially downregulated genes in these three excitatory neuron types converge well with PVALB-5, SST-11, and SST-24 interneurons. Interestingly, we also identified these three interneuron subtypes as the most highly responsive in terms of differential gene expression and differential gene regulatory network activation in Fig S4.

This preliminary analysis can be expanded upon in the revised manuscript to allow for a more in-depth characterization of the gene regulation occurring after stimulation in interneurons and pyramidal cells in our study.

- Analysis of the epigenetic data leaves some additional room for improvement. Currently, it is unclear how many open chromatin regions are differentially accessible upon stimulation, including in which cell types, and whether specific TF motifs are enriched among those altered open chromatin regions. Prior work by the Greenberg lab also identified human specific activity regulated gene

We agree with the reviewer that our SCENIC+ analysis presented in Figure 4 does not directly address the reviewer's specific comments. To address this, we have already performed the requested analysis and now include a new Supplementary Figure that explicitly visualizes: the number of stimulation-associated DARs in each cell type and the enrichment of transcription factor motifs within these regions. As an example, we find that DARs associated with stimulation in excitatory cell class are significantly enriched for motifs corresponding to canonical activity-dependent transcription factors, including FOS, JUN, and EGR1, consistent with well-established activity-regulated gene programs. We also now cite the Carter et al. preprint by the Greenberg lab but believe that their human-chimpanzee iPSC-derived tetraploid system is too different from our human brain tissue to directly compare.

- The lack of concordance between in vivo and in vitro stimulation seems particularly striking. Methodological differences in how the stimulus might have been applied through an MEA versus the Ojemann stimulator might contribute to some of the differences, but this does not seem to be extensively explored. The authors also do not comment on any potential effects in interneurons after in vivo stimulation. To extend the findings more broadly and provide a resource for the community, the authors could consider generating some stimulation and gene expression data for stem cell derived neurons or neural organoids. While these are highly distinct models to what is being studied here, a detailed comparison could be of broad interest to the community and elevate the impact of the paper.

We thank the reviewer for this comment. In original Figure S8, we showed some overlap in the gene regulation between IVS and MEA inhibitory neurons, just not to the extent that we see in excitatory neurons. We now plan to include additional comparisons of the in vivo interneuron data with that from the MEA data. Preliminarily, we have found that in vivo, *EGR1* is upregulated in several SST subtypes. Additionally, several SST subtypes have DEGs with synaptic gene enrichment. SST23 cells appear to be the most significantly engaged in this regard. This is interesting because one SST subtype (SST11) was more engaged than any other interneuron type in the MEA SCENIC+ analysis. RRHO2 between MEA SST11 versus the IVS SST subtypes shows convergence across differentially regulated genes, suggesting that SST interneurons may play common, subtle roles during in vivo and ex vivo stimulation.

We would also like to note that our gene expression changes were in line with a preprint from Ted Abel's lab that carried out in vivo stimulation, and we had discussed this in our original submission. Therefore, we assert that these expression changes in human brain tissue are reproducible across two independent studies in different patient populations and brain regions that were carried out using distinct in vivo stimulation parameters. And while we would have hypothesized that the MEA vs Ojemann stimulator should lead to different effects on the tissue, we show robust overlap of a proportion of the gene expression programs, highlighting the similarities of the techniques and potential genes ripe for further characterization that we believe will be of keen interest to readers.

Finally, while we agree that gene expression changes in iPSC-derived neurons could be informative to determine the extent of reproducibility of our results across models, we emphasize that our system of intact human brain tissue, which preserves native cellular diversity as well as spatial and circuit-level organization, offers substantial advantages over current iPSC-derived systems. While we appreciate the suggestion that any overlaps in cell systems would be valuable, our primary objective is to establish the physiology and genomic changes most relevant to *in vivo* neuromodulation rather than shifting toward more simplified model systems that are further from this goal.

- Although the authors do not comment on this, they also appear to detect changes in non-neuronal cells. Because the analysis is rather isolated, it is difficult to understand which transcriptomic changes are specific to non-neuronal cells versus neurons. The analysis, currently hidden in Figure S5, could be elevated and discussed in greater depth. In fact, what may be most striking is that transcriptional chance in glia are perhaps more numerous than in neurons.

We agree there are striking changes in non-neurons and thank the reviewer for pointing this out. We again did not focus on these data as it did directly link to the first half of the paper that focuses on physiology in neurons. We rather provided these data for completeness for the field. We will now provide additional text about these data in the Discussion.

Referee #2 (Remarks to the Author):

The study by Moore et al. reports analysis of an exceedingly rare data set of the electrophysiologic and transcriptomic response to electrical stimulation of ex vivo human brain samples acquired following temporal lobectomy and then organotypic slice culture preparation. The ex vivo results were then confirmed with samples taking following in vivo stimulation of the prefrontal cortex, comparing stimulated vs control sites excised in this region during surgical treatment of epilepsy.
They find that with stimulation over 15 min, they tend to see greater spiking activity in the following 10-15 min block of time, with this effect driven by principally excitatory neurons based on spiking waveform features. They show that the recorded electrical activity could be reliably clustered into co-activating 'assemblies' and that across their recordings, of 19 defined assemblies, 11 showed stronger co-activation following stimulation, 5 showed weaker co-activation following stimulation, and 3 showed no change. They then delve into the rich data set of gene expression changes focusing principally on the changes in neurons seen following stimulation in their preparations. Both ex vivo and in vivo they see a wide panoply of changes of known activity-dependent

genes and genes related to synaptic structure and maintenance, with different changes seen across cortical layers with the most concordant changes between the preparations seen in layers 3 and 5.

Overall, the principal advance in this study is the generation of these gene expression datasets from human brain tissues, which the authors are nicely making available to the community. The results reported in this analysis are incredibly interesting but fundamentally descriptive in a way that unfortunately falls short of yielding the 'clear mechanistic explanation of how exogenous stimulation reshapes circuit dynamics to produce…behavioral effects' to which the authors allude in the introduction. There appears to be some missed opportunities to provide further potential mechanistic insight in these datasets and similar experimental preparations, if it were logistically feasible to complete.

We thank the reviewer for appreciating the rarity, value, and challenges associated with compiling the data in this manuscript. We agree that we can provide further context in the text of the manuscript to link our data more thoroughly to circuits and behavior. We would like to emphasize though that we are limited ethically by our ability to directly manipulate circuits or behavior in living humans and are therefore only able to measure certain features in tissue removed from the patients.

Specific comments:
• The term 'plasticity' in describing the assembly level changes (Fig. 2) is used a bit loosely and are reported in response to a single stimulation protocol. Long-term plasticity is usually reported over the course of ~1 hour, not the 10-15 min over which these recordings were completed. Further, it is not clear how long these changes may have persisted or not over the 10-15 min recording period in these data.

We agree that improved precision in the use of these terms is warranted. We have updated our Discussion to include the points raised by the reviewer in terms of the timing of plasticity. We now describe the cofiring changes we observed as "short term plasticity" to differentiate from sustained changes elicited by LTP. However, we also note that the cofiring on the 25msec time scale is thought to be critical for fostering LTP among the assembly member neurons, and that future experimentation can examine these recordings over longer time scales with alternative stimulation paradigms or optogenetics.

Finally, it is already known that electrical stimulation can yield changes in functional brain activities in humans in the off period following brain stimulation – for instance, Parkinson's symptoms recur on a somewhat stereotyped time course following turning off of DBS, with different delays in different symptoms with tremor recurring within 10s of seconds (as Fig. 2F suggests), then bradykinesia/rigidity coming back later, and so on. As is the electrical recording results themselves are limited in terms of novel mechanistic insights.

The reviewer points out that the neurophysiological responses we observed have some echo in known results of in vivo deep brain stimulation. We think this is absolutely a strength of our experimental paradigm, in that we observe convergence with existing clinical literature, which supports the choice of stimulation parameters we utilized. However, we respectfully disagree that such correspondence indicates that our results are not novel insights, especially as they are directly linked with specific gene expression programs in humans as well as layer specific neurophysiology, which have not otherwise been reported. We also note that examining these physiological effects in our OSC system complements work done in rodent models of motor—related circuits. We do think this comment indicates an opportunity to clarify one of the principal motivations we had in executing our electrophysiological analysis, which is to validate that gene expression programs including ARGs are reflected in expected circuit changes, and to begin describing these changes neurophysiologically.

• To gain further mechanistic insight into the electrical recordings, the response to differing stimulus paradigms, and how those correlate to gene expression data, would be of great interest given the known differential response of human neural circuits to DBS or TMS of different stimulation frequencies, which is thought to be mediated by different neuronal and perhaps non-neuronal cell types.

The reviewer offers an excellent suggestion for future experimentation. For this initial, groundbreaking work we selected previously validated MEA stimulation parameters. The availability of these tissue specimens is rare; as

such, we made the decision to focus on a single stimulation protocol for the current manuscript. But we have updated the Discussion to include explicit mention of these alternative stimulation parameters. We would like to emphasize that it would take years to collect enough tissue to study different stimulation parameters, though we do acknowledge this future program in the updated Discussion. Therefore, we view our manuscript as a primer to further exploration.

• What correlated analysis could be completed between the electrical and gene expression datasets? Could we understand which gene expression profiles correlate to increasing neuronal assembly strength following stimulation vs the decreased neuronal assembly strength?

Please see our response to reviewer #1, where we detail our new hdWGCNA preliminary data that directly integrates single cell gene expression with the firing rate parameters from the MEA.

• Looking at Fig. S6, it appears the greatest DEG level changes are seen, among neurons, in layer 2/3 and layer 4 – even though these neurons show anti-correlated or minimally correlated changes between the MEA and IVS results (Fig. 6). In contrast, it appears relatively greater overall changes are seen in the non-neuronal cells, across astrocytes, microglia/PVMs, and oligodendrocytes (both precursor and mature). From this vantage, it appears that the greater signal is in the non-neuronal cells although the authors focused principally on the neuronal changes in this analysis. How do the neuronal and non-neuronal changes correlate to each other to mediate the observed changes in electrical spiking behavior? Did the non-neuronal gene changes correlate as well with the electrical recording findings as the neuronal gene changes? How concordant are the non-neuronal changes between the MEA and IVS preparations?

These are excellent questions and we provided some context to similar questions by Reviewer #1 where we emphasized our attempt to link gene expression in excitatory neurons with the physiology observed in the first half of the manuscript. **We now carry out a weighted gene co-expression network analysis that directly compares the gene co-expression with MEA values**. Furthermore, we are not surprised at the relatively small correlation between MEA and IVS. We wanted to emphasize the features that do correlate while discuss the reasons why many things do not correlate (different brain region, different stimulator, etc.). We hope the reviewer can appreciate the first-of-its-kind data we have generated and that there are many future studies that can be carried out to address the reviewer's specific questions.

With respect to the question about how the non-neuronal changes correlate to changes in electrical spiking behavior, we can only provide some speculation. MEA recordings capture network-level spiking activity that is directly generated by neurons, whereas transcriptional responses in non-neuronal cells are expected to reflect secondary or modulatory processes. Thus, strong transcriptional responses in non-neuronal cells do not necessarily translate into acute correlations with spiking metrics. This is an extremely understudied aspect of modern neuroscience and beyond the scope of our study. For instance, astrocytes and oligodendrocytes do not generate action potentials and therefore cannot be directly assessed using MEA-based electrophysiological recordings. Consistent with this, stimulation-dependent DEGs in astrocytes and oligodendrocytes are enriched for functions related to energy metabolism, kinase activity, chromatin organization, and axonal/synapse-associated processes. These findings suggest that glial cells, such as astrocytes and oligodendrocytes, respond indirectly to neuronal activity through transcriptional and homeostatic mechanisms that do not necessarily correlate with MEA electrical readouts. In the revised manuscript, we will discuss that the expression changes in non-neurons are likely secondary to those elicited in neurons. We will also carry out an RRHO approach for non-neurons in MEA vs IVS.

Minor comments

• How do the authors interpret that the MEA vs IVS changes are anti-correlated in layer 2/3 cells (Fig. 6)? Does this reflect that the recordings were completed in different brain regions? Or is the cell culture process altering the layer 2/3 cells in some way?

Either or both of these are possible, and we now discuss them both in detail.

• More for curiosity, were the temporal and prefrontal cortical recordings typically from a particular gyrus (STG vs parahippocampal, SFG vs IFG, etc) or was it from any frontal or temporal region that may be readily included in the surgical field?

The temporal samples were principally from the caudal extent of the STG. In the frontal cortex, stimulation included MFG and SFG near BA9.

• In the Fig 1 legend, I believe the comment re log(FR+1) presentation is about panels D and F, not D and G

Yes, thank you for catching that typo.

• Were these trials registered on ClinicalTrials.gov? If so, the registration number should be included.

No, we had no intervention seeking to observe a behavioral or health outcome, so this was not a clinical trial.

Referee #2 (Remarks on code availability):

I believe the code is of use to the community, and definitely the code with the GEO database repository is a great addition. At a cursory glance the code looks appropriately constructed.

Please find our responses to the reviewers below in blue text.

Referee #1 (Remarks to the Author):

Manuscript by Moore et al investigates molecular and physiological consequences of brain stimulation in the human brain, taking advantage of direct access to human brain tissue from surgical specimens. The authors generate a remarkably important dataset outlining gene expression and enhancers activated or suppressed by neural activity in the human brian, which extends our knowledge beyond the limited set of activity dependent genes such as FOS and JUN or BDNF. A major strength of the paper is the physiologically relevant experimental model, as well as unique findings that some neuronal subtypes, particularly glutamatergic neurons, seem to initiate transcriptomic changes in response to stimulation much more readily than inhibitory neurons. This finding is crucial and opens new avenues for investigation. A weakness of the analysis is that it is very difficult to understand the connection between the first part of the paper where the authors investigate patterns of network propagation upon electrical stimulation with the molecular interrogation of dysregulated genes and chromatin state. In the end, I thing the authors should attempt to reconcile these results and if cannot, they should focus on the unique strengths of this study, which is the detail characterization of molecular changes within the brain after stimulation.

We thank the reviewer for this positive summary of our manuscript that highlights the strength of our approach, as well as the reasonable request for expanded integration across gene expression and neurophysiological data. First, we have updated our framing to explain that the neurophysiological data provide a detailed understanding of the specific circuit level changes elicited by the differential gene expression programs characterized in the second part of the manuscript. We believe these data will add considerably to reader interest in our findings as they precisely link these gene programs to neurophysiology highly relevant for cognition (assembly formation) rather than non-specific firing changes alone. We further wish to emphasize that linking these gene expression changes to circuit dynamics described using assembly analysis highlights the relevance of different expression programs.

Integrating these two data types is a limitation of existing gene expression profiling techniques due to the absence of a method that measures gene expression longitudinally in the same cells over time. We note that we cannot carry out a correlation analysis similar to those we previously conducted for oscillatory measures in human brain (Berto et al., 2018 and 2021), given the sample size limitations for this type of work as such analyses would be underpowered and may not result in statistically robust results. However, to address the reviewer concerns and further link together our findings, **we now include new single cell/high dimensional weighted gene co-expression network analysis (hdWGCNA) results that directly link transcriptional programs to neurophysiological metrics.** The new results section and supplemental figures are copied below for the reviewers' convenience. In line with the reviewer's recommendation, we have focused on the central strength of our study: a detailed molecular characterization of human brain responses to physiologically relevant stimulation.

> **Linking neuronal activity with transcriptional programs**
>
> Separately, the MEA and multiomics results presented here outline cell type-specific alterations following stimulation. The multiomics data represent a snapshot of the period directly following MEA stimulation and recording. Using high dimensional weighted gene co-expression network analysis (hdWGCNA), we can link this transcriptional snapshot with electrophysiological changes at the level of individual cell types (see *Methods*). With this approach, we identified hdWGCNA co-expression modules (**Fig S10A**) that were differentially weighted between stimulation and control conditions (**Fig S10B**). Next, we correlated module eigengenes with MEA-derived firing rate metrics (see *Methods*) to identify networks of genes that are significantly correlated with stimulation-induced neuronal activity (**Fig S10C**). In excitatory neurons, two modules ("EM1" and "EM5") are positively correlated with pyramidal neuron firing rate and are most highly expressed in L2/3, L4, and L5 excitatory neurons (**Fig S10D**). These modules are enriched for canonical ARGs including *FOS, JUN,* and *BDNF,* which are also top network hub genes (**Fig S10E**). Within these modules, many genes are regulated by activity-dependent transcriptional regulators including EGR1 and NFIA (**Fig S10F**).

We repeated this analysis in inhibitory neurons by integrating gene networks with interneuron firing rate (**Fig S11A**). As we did in excitatory neurons, we identified several gene modules that were differentially associated with stimulation (**Fig S11B**). Modules associated with upper layer interneuron firing rate were especially highly expressed in SST-11 inhibitory neurons (**Fig S11C**). The IM5 module exhibited significant covariation with firing rate in several cell types (**Fig S11C-D**). The IM5 module was enriched for FOS binding activity (**Fig S11E**) and contained many activity-regulated hub genes (**Fig S11F**) similar to those seen in the EM5 module in excitatory neurons.

Together, this integrative analysis links physiological and molecular components of stimulation *ex vivo* by showing that stimulation-induced changes in network firing are supported by coordinated activation of activity-dependent gene regulatory programs. These results are consistent with a model in which electrical stimulation preferentially engages specific neuron types and activates transcriptional networks that may contribute to circuit-level short-term plasticity.

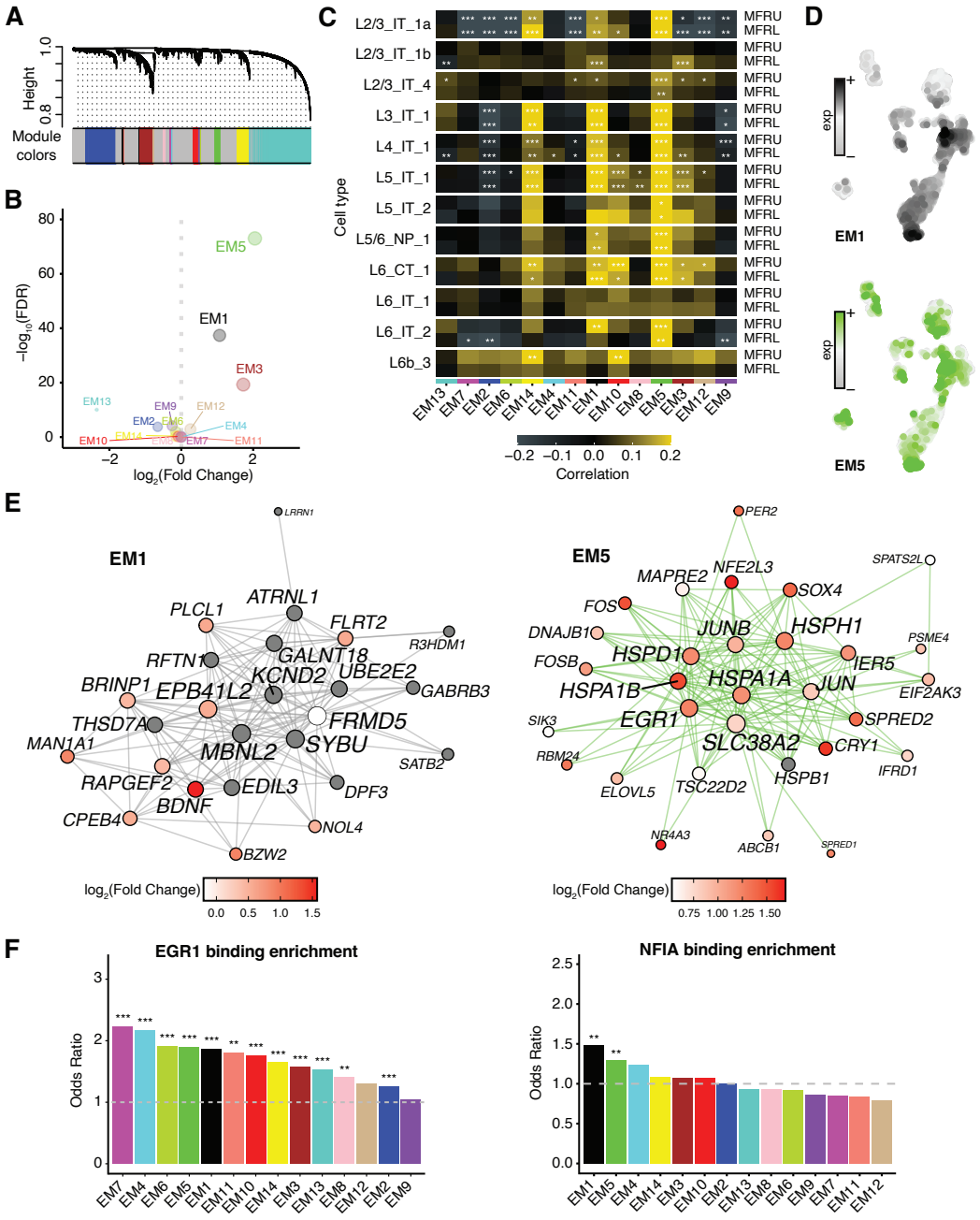


Figure S10. hdWGCNA reveals excitatory cell type and layer specific links between firing rate and gene expression networks. (A) Network dendrogram. **(B)** Significance of each module

correlated with mean firing rate fold change in pyramidal neurons. **(C)** Correlation of each module with cell type and mean firing rate of upper- and lower-layer pyramidal neurons (MFRU and MFRL, respectively). **(D)** Feature plot of gene expression in black and green modules in the original multiomic UMAP space. **(E)** Visualization of the top hub genes in the EM1 and EM5 modules. Node color indicates $\log_2$(fold change) of gene expression in stimulated versus control cells. **(F)** Degree of EGR1 and NFIA binding enrichment in each module. EM = excitatory module; MFRU = mean firing rate upper layer; MFRL = mean firing rate lower layer.

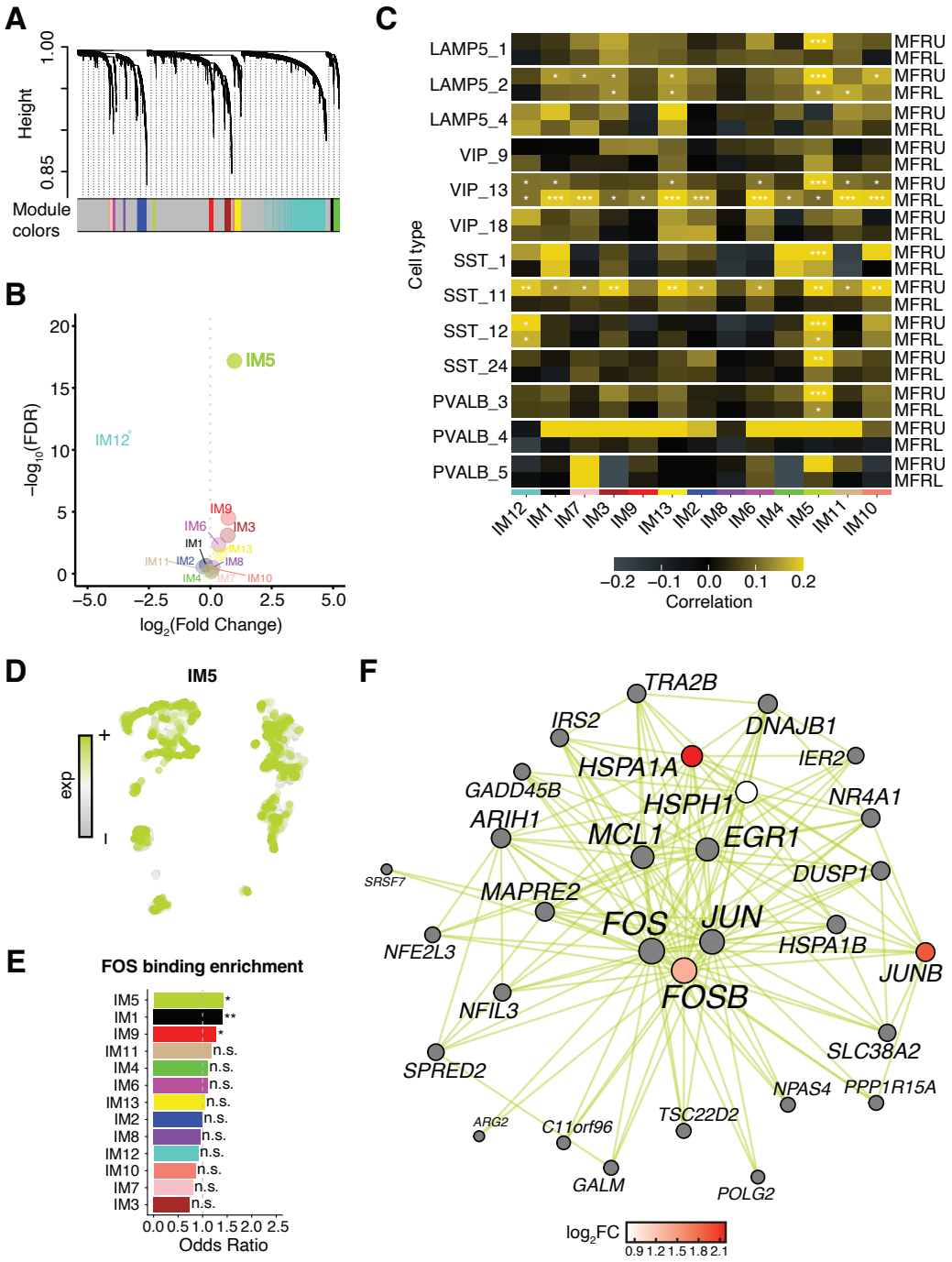


Figure S11. hdWGCNA reveals inhibitory cell type and layer specific links between firing rate and gene expression networks. (A) Network dendrogram. **(B)** Significance of each module correlated with mean firing rate fold change in interneurons. **(C)** Correlation of each module with cell type and mean firing rate of upper- and lower-layer interneurons (MFRU and MFRL, respectively). **(D)** Feature plot of gene expression in the IM5 module in the original multiomic

> UMAP space. **(E)** Degree of FOS binding enrichment in each module. **(F)** Visualization of the top hub genes in the IM5 module. Node color indicates log_2(fold change) of gene expression in stimulated versus control cells. IM = inhibitory module; MFRU = mean firing rate upper layer; MFRL = mean firing rate lower layer.

- The introduction of the paper provides an excellent background and framing to the key questions being pursued by the investigators, providing a compelling rationale for the study. One discussion angle that could factor into this introduction, however, would be the introduction of prior work on activity dependent gene expression, including the pioneering work by Michael Greenberg. What key immediate early genes and activity dependent molecular processes have been discovered so far and what remains to be discovered. I think this kind of discussion could help strengthen the rationale.

We had previously referenced work from a protégé of Dr. Greenberg (Dr. Jesse Gray; PMID 29681534; Reference #21 in the original manuscript) and Dr. Greenberg himself (PMID 20393465; Reference #22 in the original manuscript) in our description of activity related genes. We don't use the term "immediate early genes" but adopt the updated nomenclature of activity regulated genes (ARGs) separated by rapid primary response genes (rPRGs) delayed primary response genes (dPRGs) and secondary response genes (SRGs). However, we now include additional references from Dr. Greenberg in the introduction to our revised manuscript. The relevant section is copied below for the reviewers' convenience:

> Assembly activity is detectable both *in vivo* (*14*) and in *ex vivo* (*15*) preparations of rodent brain tissue (*16*), providing a common physiological scale that may bridge cellular- and network-level findings across experimental contexts. Though assemblies have been identified in the human cortex *in vivo* (*17*), there has yet to be an exploration of true assemblies in the human cortex *ex vivo.* Despite the progress made in animal models, the genetic and molecular mechanisms that shape assembly formation in the human brain, particularly in response to therapeutic stimulation, remain essentially unknown. Early transcriptional responses, in the form of immediate early genes (IEGs), more recently referred to as rapid primary response genes (rPRGs) (*18*), show enhanced expression minutes following neuronal activation. Decades of work have established that neuronal activity triggers a rapid transcriptional response, notably with the induction of genes such as *ARC*, *FOS*, and *EGR1* (*19-21*). Previous work has shown that rPRGs are not only markers of activity but effectors of synaptic plasticity, hinting at their influence on neuronal assembly formation and stabilization (*22*). Inactivation of rPRG expressing assemblies has been shown to impair memory recall, further underscoring activity dependent transcription as a functional requirement for assembly dependent mnemonic function (*23, 24*). Although individual rPRG expression patterns are well studied, there is little known to what extent these early response genes shape assembly activation in the cortex. Despite this mechanistic foundation, whether therapeutic stimulation coordinates rPRG responses in a cell type-specific manner in the human cortex is unknown. We therefore sought to address these outstanding ambiguities linked with neuromodulation therapies using a novel experimental approach in the human cortex *ex vivo* and *in vivo*.

- The authors apply stimulation to human organotypic brain slices, which is a powerful approach to functionally interrogating functional connectivity with their tissue preparations. Their current analysis focuses overwhelmingly on co-variation of neural activity as a mechanism for inferring neuronal assembles, however this seems fairly limited and leaves opportunities for more sophisticated analyses unexplored, including measurements of oscillatory behaviors, recurrence, or inference of connectivity from not just measurements of co-firing dynamics but also latency in spike timing. The analysis could also better leverage the spatial distribution of units on the planar HD-MEA in the cortical tissue, with reference to the known patterns of cortical

connectivity. Currently, there is only one Supplemental figure panel 2D that offers a very limited insight into this type of analysis. I hope the authors can elaborate on this point.

We agree with the reviewer that several additional electrophysiological analyses are feasible with these data, although we note that the reviewer does not recommend a specific approach that examines the effects of stimulation differently. We initially focused on cofiring in key 25 msec time windows due to the importance of neuronal assemblies to in vivo physiology in both humans and animal models. However, we performed additional analyses that take advantage of the spatial resolution and precise temporal precision of our recording system in which we mapped the cofiring structure across all neurons, identifying 1) stability within the pre-stimulation baseline and 2) increased cofiring across several time scales, consistent with our previous reports. Also, in response to this query, we added an analysis in which we describe cofiring in short time scales across neuronal layers (3 msec), thought to be characteristic of monosynaptic connectivity especially for pyramidal/interneuron dyads. Below, we have copied these new results within the Supplemental Figure from the updated manuscript. More broadly, the number of additional analyses one can perform with this dataset is extensive and will require dedicated explication in subsequent manuscripts. We agree that future work can incorporate local field potentials and interventions such as optogenetic activation of neuronal assemblies to understand the circuit dynamics using complementary tools, but we believe these new analyses will directly complement our other findings and address the reviewer concerns.

Stimulation increases cofiring within pyramidal neuron pairs

We next investigated whether cofiring dynamics were impacted by stimulation in a cell type or layer-specific manner. On shorter time scales (3-5 ms), thought to be consistent with monosynaptic connectivity, we observed an overall increase in cofiring changes across neuronal subtypes and layers (Wilcoxon, p=1.23*10^-137, n=3660 unit pairs with distinct nonzero synaptic delay times) in response to stimulation compared to temporal dynamics in the pre-stimulation recording (**Fig S2A**). The increased cofiring changes were most prevalent in the pyramidal-pyramidal unit pair subpopulation (One-Way ANOVA: F=10.05, p=4.4*10^-5. Tukey post-hoc test: pyr-pyr change larger than pyr-int change p=3.1*10^-5, pyr-pyr change larger than int-int change p=2.7*10^-3, pyr-int change not significantly different from int-int change p=0.70. n=852, 1753 and 1055 pyr-pyr, pyr-int and int-int pairs with distinct nonzero synaptic delay times respectively) (**Fig S2B**). Meanwhile, cofiring changes were of similar magnitude between neuron pairs within and across different cortical layers (One-Way ANOVA: F=1.04, p=0.35. Tukey post-hoc test: upper-upper change not significantly different from upper-lower change p=0.32, upper-upper change not significantly different from lower-lower change p=0.53, upper-lower change not significantly different from lower-lower change p=0.83. n=463, 1467 and 1730 upper-upper, upper-lower and lower-lower pairs with distinct nonzero synaptic delay times respectively) (**Fig S2C**). The cofiring changes in the pyr-pyr subpopulation were driven by an overall increase in cofiring after stimulation (Wilcoxon, p=4.15*10^-17, n=852 pyr-pyr pairs with distinct nonzero synaptic delay times) (**Fig S2D**). These trends persist despite normalizing for the reported firing rate increases induced by stimulation.

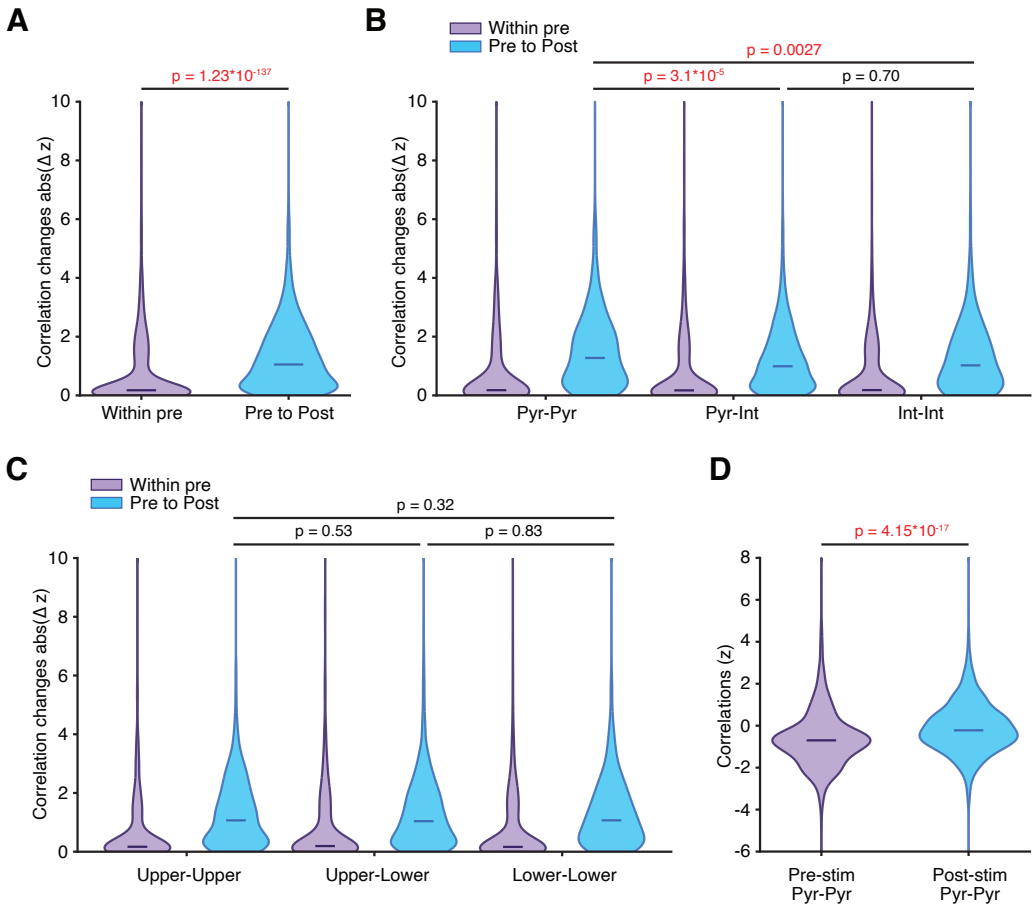


Figure S2. Effect of stimulation on co-firing by cell type and location. (A) Increase in change in cofiring on the order of 3-5 ms when comparing pre-stimulation to post-stimulation versus the first half of the pre-stimulation scan to the second half of the pre-stimulation scan (two-sided paired Wilcoxon signed-rank test. W=1753298, p=1.23*10^-137, n = 3660 unit pairs with distinct nonzero synaptic delay times). **(B)** Pyramidal-pyramidal dyads show the most prominent increase in cofiring changes (One-Way ANOVA for effect of unit pair subpopulation on induced correlation difference: F=10.05, p=4.4*10^-5. Tukey post-hoc test: pyr-pyr change larger than pyr-int change p=3.1*10^-5, pyr-pyr change larger than int-int change p=2.7*10^-3, pyr-int change not significantly different from int-int change p=0.70. n=852, 1753 and 1055 pyr-pyr, pyr-int and int-int pairs with distinct nonzero synaptic delay times respectively). **(C)** No difference in co-firing changes between different layers (One-Way ANOVA for effect of unit pair spatial location on induced correlation difference: F=1.04, p=0.35. Tukey post-hoc test: upper-upper change not significantly different from upper-lower change p=0.32, upper-upper change not significantly different from lower-lower change p=0.53, upper-lower change not significantly different from lower-lower change p=0.83. n=463, 1467 and 1730 upper-upper, upper-lower and lower-lower pairs with distinct nonzero synaptic delay times respectively). **(D)** Overall increase in cofiring in pyr-pyr pairs after stimulation (two-sided paired Wilcoxon signed-rank test n=852 pyr-pyr pairs with distinct nonzero synaptic delay times, W=151482, p=4.15*10^-17).

- Molecular analysis of gene expression changes after stimulation is a particularly novel and interest aspect of the manuscript. I find the lack of a strong signal in GABAergic interneurons to be particularly fascinating, and would encourage the authors to include this contrast to excitatory neurons as part of the main text figure. It would also be interested to investigate nominally significant DEGs in interneurons to see if they might correlate with FDR-significant DEGs from glutamatergic neuron analysis.

We agree that the gene expression in interneurons is certainly of interest. We focused on excitatory neurons because that is where we observed the most change physiologically in the MEA data. To address the reviewer's point though, we have **now carried out a RRHO analysis of the excitatory and inhibitory gene expression and found both overlapping and distinct gene expression programs.** The new results from the revised manuscript are copied below:

Commonalities between high-priority pyramidal cell and interneuron subtypes

We next used RRHO2 (*44*) to find similarities in the transcriptional effects of MEA-based stimulation between excitatory and inhibitory neuron subtypes in the temporal cortex. RRHO2 uses a stratified approach to find significant overlap between gene lists ranked according to the significance, magnitude, and direction of differential expression following stimulation. L2/3 IT-1a upregulated genes exhibit high overlap with differentially regulated genes in several types of interneurons, including cell clusters marked by *LAMP5*, *PVALB*, or *VIP* expression (**Fig S9A**). ARGs, including rPRGs, were not significantly differentially regulated in L2/3 IT-1a cells or these interneuron subtypes after MEA-based stimulation. However, L2/3 IT-1a cells were flagged as the third highest priority in the Augur analysis (**Fig 3F**), had the second highest increase in peak-to-gene linkages after stimulation (**Fig 3G**), and showed activation of IEG-regulated GRNs (**Fig 4A**). The convergence seen in the upregulated genes of L2/3 IT-1a cells and several interneuron cell types may represent an activity-dependent gene program that is commonly activated during stimulation in an otherwise diverse group of cells. L3, L4, and L5 IT neurons showed similar gene regulation patterns in our analyses, and we also see similar degrees of convergence with different interneuron subtypes. For example, both upregulated and downregulated genes in these three excitatory neuron types converge well with PVALB-5, SST-11, and SST-24 interneurons (**Fig S9B**). These three interneuron subtypes were also identified as the most highly responsive in terms of differential gene expression (**Fig S5B**) and differential gene regulatory network activation (**Fig S5D**). Although interneurons appear to show nominal changes in response to stimulation, there is clear overlap between several interneuron subtypes and the most stimulation-responsive pyramidal neurons. *SST*+ interneurons emerged as particularly interesting in this regard, which is also supported by literature showing *SST*+ interneurons pivotal role in regulating layer-specific network activity in the mouse cortex during sensory processing and development *in vivo* (*45, 46*).

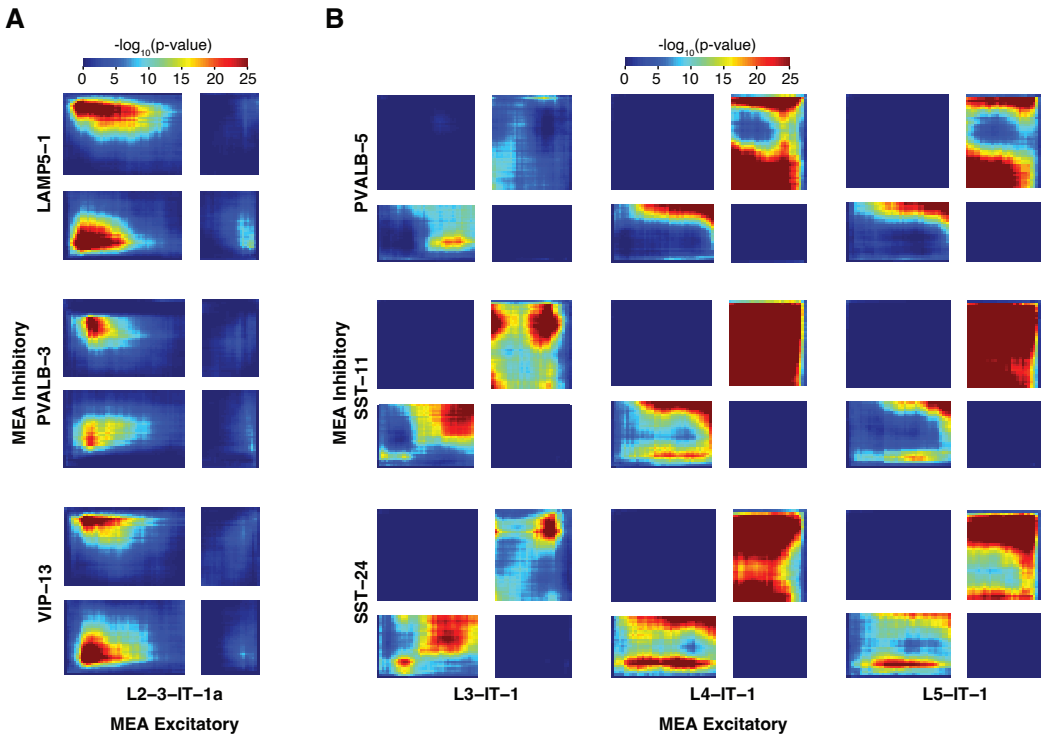


Figure S9. Overlap between excitatory and inhibitory neuron transcriptional responses to MEA stimulation. (A) Upregulated genomic repertoire in L2/3_IT_1a excitatory neurons is similar to differentially expressed genes in VIP_13, PVALB_3, and LAMP5_1 inhibitory neurons in the MEA stimulation paradigm. **(B)** L3_IT_1, L4_IT_1, and L5_IT_1 exhibit significant convergence across up and downregulated genes compared to SST_24, SST_11, and PVALB_5 interneurons in the MEA stimulation paradigm.

- Analysis of the epigenetic data leaves some additional room for improvement. Currently, it is unclear how many open chromatin regions are differentially accessible upon stimulation, including in which cell types, and whether specific TF motifs are enriched among those altered open chromatin regions. Prior work by the Greenberg lab also identified human specific activity regulated gene

We agree with the reviewer that our SCENIC+ analysis presented in Figure 4 does not directly address the reviewer's specific comments. To address this, we have performed the requested analysis and now include new text and a Supplementary Figure that explicitly visualizes: the number of stimulation-associated DARs in each cell type and the enrichment of transcription factor motifs within these regions. We also now cite the Carter et al. preprint by the Greenberg lab but believe that their human-chimpanzee iPSC-derived tetraploid system is too different from our human brain tissue to directly compare. These updated results and citation are copied below:

GRNs driven by ARGs are activated by stimulation in specific excitatory neuron types

Synchronized neuronal activity is known to induce genome-wide alterations in chromatin structure in the process of generating a coordinated activity-dependent transcriptional response (*33*). Chromatin accessibility and gene expression data can be jointly analyzed to give us a view of the overarching gene regulatory networks (GRNs) at play following stimulation. Toward this end, we first uncovered many cell type-specific differentially accessible regions (DARs) in non-coding as well as coding elements across the genome when comparing stimulated and control snATACseq data (**Fig S8A-B**). We found that DARs associated with stimulation in excitatory cell classes are significantly enriched for motifs corresponding to canonical activity-dependent transcription factors, including FOS, JUN, and EGR1, consistent with well-established activity-regulated gene programs (**Table S4-6**). Recent work in homogenous populations of excitatory neurons derived from human-chimp tetraploid cells *in vitro* identified human-specific ARGs and enrichment of FOS activity in human neurons versus chimpanzee neurons (*30*). Our data similarly show FOS motif enrichment in human excitatory neurons in the context of organotypic cortex slices.

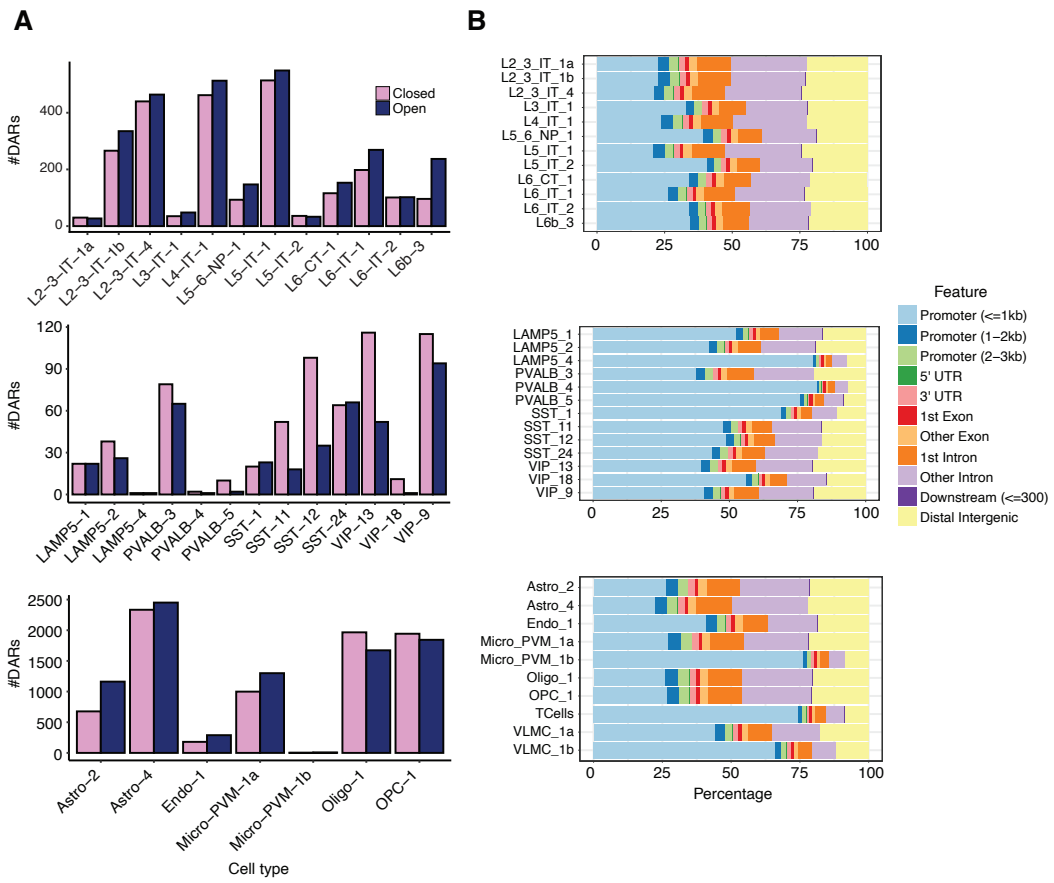


Figure S8. Cell type-specific differential chromatin accessibility after MEA stimulation. (A) Number of differentially accessible regions (DARs) in MEA stimulated versus control tissue. **(B)** Proportion of features found in MEA DARs.

- The lack of concordance between in vivo and in vitro stimulation seems particularly striking. Methodological differences in how the stimulus might have been applied through an MEA versus the Ojemann stimulator might contribute to some of the differences, but this does not seem to be extensively explored. The authors also do not comment on any potential effects in interneurons after in vivo stimulation. To extend the findings more broadly and provide a resource for the community, the authors could consider generating some stimulation and gene expression data for stem cell derived neurons or neural organoids. While these are highly distinct models to what is being studied here, a detailed comparison could be of broad interest to the community and elevate the impact of the paper.

We thank the reviewer for this comment. In original Figure S8, we showed some overlap in the gene regulation between IVS and MEA inhibitory neurons, just not to the extent that we see in excitatory neurons. We now include additional comparisons of the *in vivo* interneuron data with that from the MEA data in the Results section and additional text in the Discussion:

Excerpt from section "**Transcriptomic profiling of human brain stimulation *in vivo*":**

> We next turned our focus to inhibitory neurons in the IVS samples (**Fig S13A-B**). Differential gene expression analysis revealed that after IVS, *PVALB*+ interneurons had the highest number of DEGs, most of which were downregulated (**Fig S13C**; **Table S11**). We found multiple upregulated rPRGs in PVALB_5 neurons, but more interestingly found that all three *SST*+ subtypes (SST_6, SST_23, and SST_30) had significant upregulation of *FOS* and/or *EGR1* (**Fig S13D; Table S11**). SST_23 and SST_30 cells showed enrichment of synapse-related DEGs, while most other interneuron subtypes did not (**Fig S13E; Table S11**).

Excerpt from section "**L3 and L5 IT neurons mediate neuromodulation *in vivo* and *ex vivo*":**

> In interneurons, RRHO2 identified several *SST*+ subtypes exhibiting similar stimulation sensitive gene expression signatures between our *in vivo* and *ex vivo* paradigms (**Fig S13E**).

Excerpt from Discussion:

> Interneuron subtype-specific activity dependent genetic, molecular, and physiological responses have been examined in mouse models *in vitro* and *in vivo* (*77-79*), but there is far less evidence from the human cortex. We detected few changes in interneuron GRN activation and differential gene expression. However, both IVS and MEA experiments point toward *SST*+ interneurons as a population for further exploration. Our results indicate that *SST*+ interneurons may be of importance in our understanding of cortical assemblies strengthened by stimulation, which is supported by other studies which show that interneurons organize mnemonically relevant cortical network activity, including neuronal assemblies (*80, 81*). Future studies should include larger sample sizes in order to tease out differences among the diverse and unique subsets of interneurons in the human cortex (*82-84*).

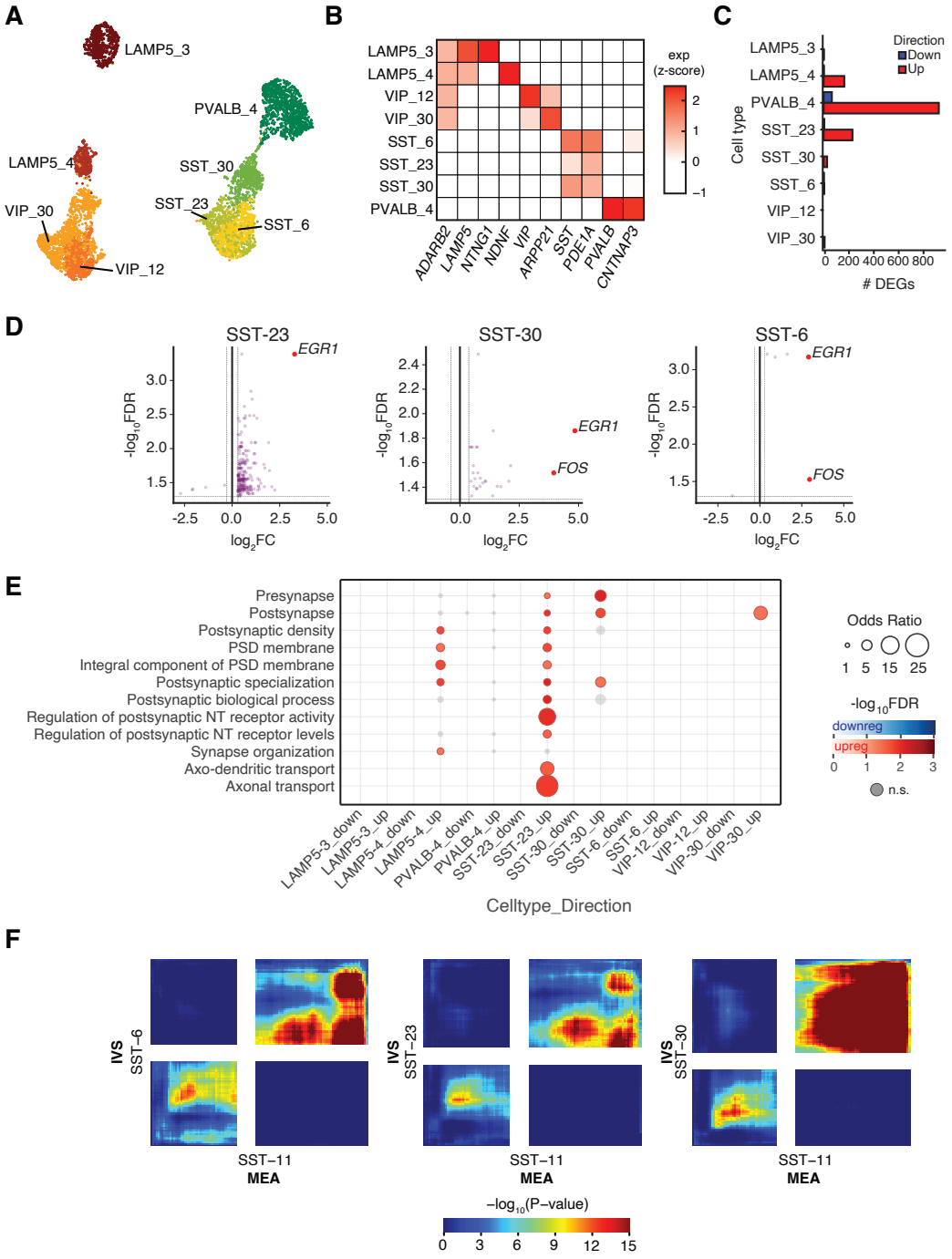


Figure S13. *In vivo* stimulation engages SST interneuron subtypes similarly to MEA-based stimulation. **(A)** UMAP of subclustered interneurons in the IVS paradigm. **(B)** Normalized expression of key marker genes for each interneuron subcluster. **(C)** Number of differentially expressed genes per interneuron subcluster. **(D)** Upregulation of rPRGs *EGR1* and *FOS* in SST+ interneurons. **(E)** Enrichment of synaptic gene ontology terms downregulated DEGs only (blues), and upregulated DEGs only (reds) by cell type. Fisher's exact test with FDR correction was used in accordance with the SynGO documentation (syngoportal.org). **(F)** RRHO2 comparison of SST subtypes from MEA and IVS paradigms shows significant convergence in differential gene regulation.

We would also like to note that our gene expression changes were in line with a preprint from Ted Abel's lab that carried out in vivo stimulation; we have expanded mention of this similarity from that in our original submission (Reference #55 in the revised manuscript). Therefore, it seems that these expression changes in the human brain are reproducible across two independent studies in different patient populations and brain regions that

were carried out using distinct in vivo stimulation parameters. We show robust overlap of a proportion of the gene expression programs between our in vivo and intro experiments, highlighting the similarities of the techniques and potential genes ripe for further characterization that we believe will be of keen interest to readers as potential programs effectively modeled using ex vivo preparations. We also note that the *ex vivo* stimulation programs may in fact be more directly analogous to deep brain stimulation, in which current is delivered at the depth of tissue layers rather than through bipolar tools applied to the pia.

Finally, while we agree that gene expression changes in iPSC-derived neurons could be informative to determine the extent of reproducibility of our results across models, we emphasize that our system of intact human brain tissue, which preserves native cellular diversity as well as spatial and circuit-level organization, offers substantial advantages over current iPSC-derived systems. While we appreciate the suggestion that any overlaps in cell systems would be valuable, our primary objective is to establish the physiology and genomic changes most relevant to *in vivo* neuromodulation rather than shifting toward more simplified model systems that are further from this goal. We do agree that directly comparisons may be a fruitful avenue of experimentation in future work.

- Although the authors do not comment on this, they also appear to detect changes in non-neuronal cells. Because the analysis is rather isolated, it is difficult to understand which transcriptomic changes are specific to non-neuronal cells versus neurons. The analysis, currently hidden in Figure S5, could be elevated and discussed in greater depth. In fact, what may be most striking is that transcriptional chance in glia are perhaps more numerous than in neurons.

We agree there are striking changes in non-neurons and thank the reviewer for pointing this out. We initially did not focus on these results given the goal of characterizing gene expression and neurophysiological responses. However, we agree that it will be of interest in the field and have now provided additional text, analyses, and supplemental figures about these data in the Results section and an updated Discussion on the subject. This includes a greatly expanded new figure that characterizes non-neuronal cells after *in vivo* stimulation, including differentially upregulated IEGs in astrocytes, and how these gene expression changes overlap with non-neurons in the MEA multiome experiments.

> **Non-neuronal facilitation of neuromodulation across paradigms**
>
> We have focused primarily on stimulation-induced circuit modulation in neurons and the associated cell type-specific gene expression changes. However, our *ex vivo* and *in vivo* preparations also allow us to interrogate the effects of stimulation in non-neuronal populations in the context of the intact cerebral cortex. Models of brain stimulation posit that non-neuronal populations, particularly astrocytes, may play important roles in shaping circuit remodeling over longer timescales (*54*). Consistent with this, our MEA data provide evidence of stimulation responses in non-neuronal populations (**Fig S6**). Differential gene expression was induced by stimulation across all non-neuronal cell types, with enrichment of synaptic genes in several (**Fig S6C-D**). One microglial cluster was found to be particularly responsive to stimulation by Augur and scMultiMap analysis (**Fig S6E-F**), which is consistent with findings from another group that implicates microglia in the mediation of stimulation's effects on the cortex (*55*). Stimulation engaged regulatory programs most prominently in astrocytes and oligodendrocyte-lineage cells, including enrichment of immediate early gene networks (MAFF, KLF4, EGR1) and activation of regulons linked to synaptic support, metabolic coupling, and myelination (**Fig S6G**).
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> We identified the same populations of non-neuronal cells in the IVS experiments (**Fig S15A-B**). After IVS, oligodendrocytes show the largest number of DEGs which are nearly all downregulated (**Fig S15C**). Though astrocytes have a smaller number of DEGs comparatively, they upregulate rPRGs to a high degree (**Fig S15D-E**) suggesting that they are indeed being engaged by stimulation either directly via cell membrane depolarization (*56*), or indirectly via neurotransmitter-mediated astrocytic synaptic remodeling (*57-60*). Comparing the non-neuronal response *ex vivo* versus *in vivo*, we see hotspots of overlap in the upregulated genes of some subsets of astrocytes,

microglia, OPCs, and endothelial cells (**Fig S15F**). In summary, we found evidence that non-neuronal cells are impacted in a way that may strengthen short-term neuronal assembly dynamics via astrocyte, oligodendrocyte-lineage, or microglial actions at the synapse. Further characterization, particularly under semi-chronic stimulation paradigms, will be required to define the full extent of these glial contributions.

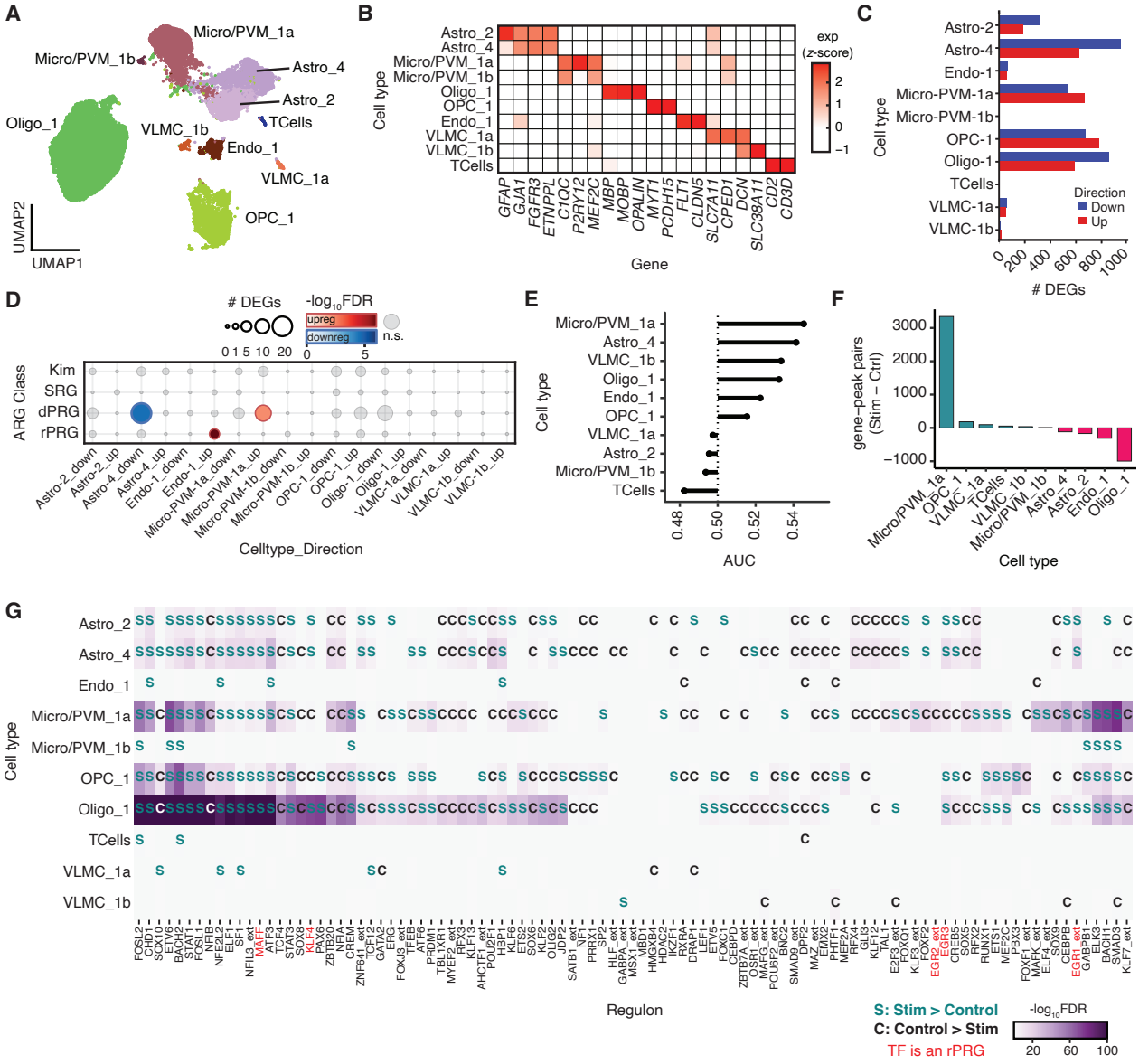


Figure S6. Stimulation engages non-neuronal cell types through gene regulatory network modulation. A. UMAP of subclustered non-neurons. **B.** Normalized expression of key marker genes for each non-neuron subcluster. **C.** Number of differentially expressed genes per non-neuron subcluster. **D.** Enrichment of differentially expressed activity regulated genes in several non-neuron subclusters. **E.** Augur cell type prioritization. **F.** Number of chromatin-to-gene linkages increases after stimulation in Micro/PVM_1a cells determined by scMultiMap. **G.** Stimulation differentially activates many regulons in non-neurons.

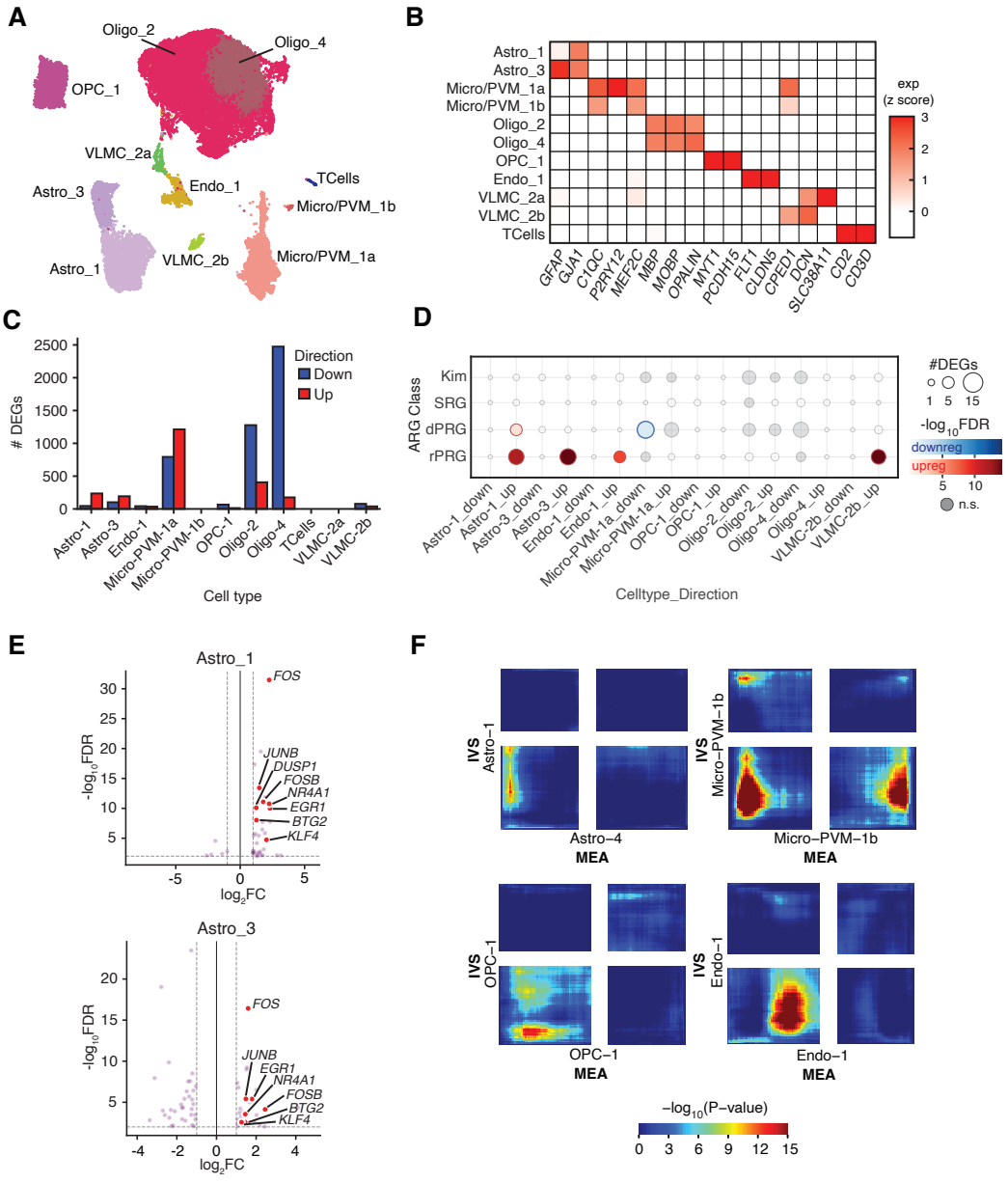


Figure S15. Impact of *in vivo* stimulation on non-neuronal cell types. (A) UMAP of subclustered non-neurons in the IVS paradigm. **(B)** Normalized expression of key marker genes for each non-neuron subcluster. **(C)** Number of differentially expressed genes per non-neuron subcluster. **(D)** Enrichment of differentially expressed activity regulated genes in several non-neuron subclusters. **(E)** Upregulation of rPRGs in astrocytes. **(F)** Convergence and divergence of transcriptional responses in different non-neuronal cell types in MEA and IVS paradigms.

Excerpt from the discussion on non-neurons:

Non-neurons of many classes were affected by stimulation in ways that may support stimulation-induced circuit modulation. Oligodendrocyte-lineage cells exhibited broad differential gene expression and substantial GRN activation after stimulation, which is congruent with studies on activity-dependent oligodendrocyte maturation and myelination as a means of neural plasticity in central nervous system injury models (*85, 86*). Our data also show that astrocytes upregulate rPRGs and enhance expression of synaptic gene networks after stimulation. This is in line with the astrocyte hypothesis of stimulation, which formed out of mounting evidence that astrocytes respond to and modulate neuronal activity at local synapses and across wide interconnected

networks (*54, 87*). Future experimentation could utilize AAVs to induce the expression of GFAP-controlled genetically encoded calcium indicators (*88, 89*) in human organotypic slice culture to directly visualize the astrocytic response to stimulation and understand how this relates to neural assembly dynamics and cell type-specific gene expression.

Referee #2 (Remarks to the Author):

The study by Moore et al. reports analysis of an exceedingly rare data set of the electrophysiologic and transcriptomic response to electrical stimulation of ex vivo human brain samples acquired following temporal lobectomy and then organotypic slice culture preparation. The ex vivo results were then confirmed with samples taking following in vivo stimulation of the prefrontal cortex, comparing stimulated vs control sites excised in this region during surgical treatment of epilepsy.
They find that with stimulation over 15 min, they tend to see greater spiking activity in the following 10-15 min block of time, with this effect driven by principally excitatory neurons based on spiking waveform features. They show that the recorded electrical activity could be reliably clustered into co-activating 'assemblies' and that across their recordings, of 19 defined assemblies, 11 showed stronger co-activation following stimulation, 5 showed weaker co-activation following stimulation, and 3 showed no change. They then delve into the rich data set of gene expression changes focusing principally on the changes in neurons seen following stimulation in their preparations. Both ex vivo and in vivo they see a wide panoply of changes of known activity-dependent genes and genes related to synaptic structure and maintenance, with different changes seen across cortical layers with the most concordant changes between the preparations seen in layers 3 and 5.

Overall, the principal advance in this study is the generation of these gene expression datasets from human brain tissues, which the authors are nicely making available to the community. The results reported in this analysis are incredibly interesting but fundamentally descriptive in a way that unfortunately falls short of yielding the 'clear mechanistic explanation of how exogenous stimulation reshapes circuit dynamics to produce…behavioral effects' to which the authors allude in the introduction. There appears to be some missed opportunities to provide further potential mechanistic insight in these datasets and similar experimental preparations, if it were logistically feasible to complete.

We thank the reviewer for appreciating the rarity, value, and challenges associated with compiling the data in this manuscript. We agree that we can provide further context in the text of the manuscript to link our data more thoroughly to circuits and behavior. We would like to emphasize though that we are limited ethically by our ability to directly manipulate circuits or behavior in living humans and are therefore only able to measure certain features in tissue removed from the patients.

Specific comments:
• The term 'plasticity' in describing the assembly level changes (Fig. 2) is used a bit loosely and are reported in response to a single stimulation protocol. Long-term plasticity is usually reported over the course of ~1 hour, not the 10-15 min over which these recordings were completed. Further, it is not clear how long these changes may have persisted or not over the 10-15 min recording period in these data.

We agree that improved precision in the use of these terms is warranted. We have updated our Discussion to include the points raised by the reviewer in terms of the timing of plasticity. We now describe the cofiring changes we observed as "short term plasticity" to differentiate from sustained changes elicited by LTP. However, we also note that the cofiring on the 25 msec time scale is thought to be critical for fostering LTP among the assembly member neurons, and that future experimentation can examine these recordings over longer time scales with alternative stimulation paradigms or optogenetics.

Copied below are the results and figure with the updated "short-term plasticity" language:

Neuronal assemblies are active in the human temporal cortex *ex vivo*

Neuronal assemblies represent a basic units of coordinated circuit activity relevant for cognition, and their detection provides a window into how stimulation reorganizes local network dynamics (*9, 10*). We employed a well-validated assembly identification method combining principal component analyses (PCA) and independent component analysis (ICA) to extract cofiring relationships at the 25 ms scale, iterating the ICA step to ensure the robustness of detected assemblies (**Fig 2A**; **Fig S3A**) (*25*). Using this approach, we identified 19 assemblies, each comprising independent, functionally connected member neurons (**Fig 2B**; **Fig S3B**). This number exceeded that expected by chance in every iteration of our shuffle control procedure we implemented (permutation test, $p < 0.0001$; see *Methods*; **Fig S3C**). These results demonstrate the presence of neuronal assemblies in our organotypic slice preparations.

We next examined the identity of assembly member neurons based on putative cell type cluster and cortical layer location. Assembly member neurons were distributed across all seven cell type clusters without bias (**Fig S3D**). No overall preference was observed when comparing neurons in upper versus lower cortical layers (estimated as described in *Methods*). However, member neurons were significantly overrepresented in the 2000-2500 μm range of the MEA, corresponding approximately to cortical layer 4 (Fisher's exact test, odds ratio = 1.93, $p = 0.025$; **Fig S3E**). These findings indicate that assembly member neurons are diverse in both cell type and cortical location, with a notable enrichment near the layer 4 region.

Stimulation promotes local circuit remodeling through assembly activation and flexibility

To understand how stimulation affected the extracted assemblies, we calculated *E*, representing the strength cofiring activity among members relative to overall population spiking activity (see *Methods*; **Fig 2C**) (*17*). Following stimulation, the magnitude of *E* during activation events was significantly higher than in pre-stimulation activation events (paired t-test, $t(18) = 2.375$, $p = 0.0289$; **Fig 2D**). Moreover, *strong* activation events (see *Methods*) occurred more frequently after stimulation (paired t-test, $t(18) = 2.372$, $p = 0.0291$; **Fig 2E**). Together, these results indicate that stimulation promotes organized assembly activity rather than non-specific changes in the firing rate of individual neurons.

We further investigated the relationship between stimulation and assembly activation by examining correlations between activation event magnitude and time. A significant correlation reflects an assembly activation profile, which we term positive or negative short-term "plasticity," indicating that the connections driving cofiring within an assembly were significantly strengthened or weakened in the short period of time following stimulation (**Fig 2F**). Out of 19 assemblies, 11 exhibited a positive short-term plasticity profile, showing a significant increase in activation magnitude over time (**Fig 2G**). We implemented a shuffle control and confirmed that these correlations were unlikely to occur by chance (**Fig S3F**).

As an additional analysis of local short-term circuit plasticity, we applied established methods to assess assembly member flexibility, defined as the number of neurons drifting into or out of assemblies specifically during periods of assembly activation (*17*). We observed a greater number of neurons exhibiting flexibility after stimulation compared to baseline, as measured by drift fraction (Fisher's exact test, odds ratio = 2.03, $p < 0.0001$; **Fig 2H**). Using a shuffle control, we confirmed that the increase in drift fraction following stimulation was highly unlikely to occur by chance (permutation test, $p < 0.0001$; **Fig S3G**). Importantly, we note that since assembly activation events are calculated relative to overall spiking activity for member neurons, these results are not explicable due to non-specific firing rate changes. Overall, the evidence of increased assembly flexibility after stimulation both through new member neuron recruitment and enhanced assembly coactivation supports the hypothesis that stimulation promotes short-term plasticity in local neural circuits.

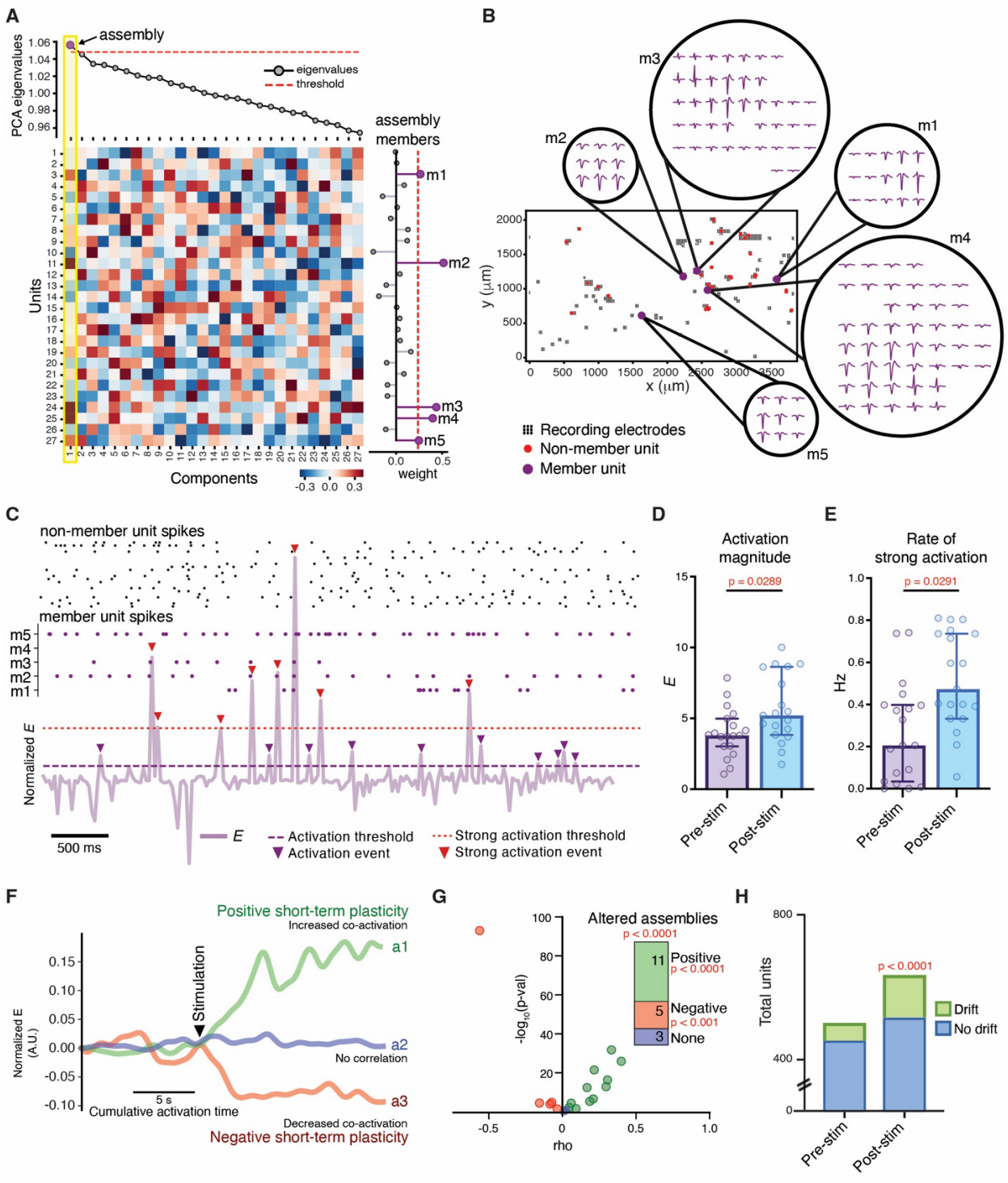


Fig. 2. Cell assemblies are activated and strengthened after stimulation. (A) Example of assembly identification with the combined PCA/ICA method. Heatmap shows the PCA components with associated eigenvalues in line plot at the top. Values for the identified assembly are highlighted in yellow. Right stem plot shows assembly members (m1-m5) passing the unit vector membership threshold derived from ICA results. (**B**) Member unit locations and spike waveform footprints of assembly identified in (A). **(C)** Magnitude of assembly activation strength *E* corresponds with co- activation of member neurons. **(D)** Activation event ($E > 95^{th}$%) magnitude increases after stimulation (paired t-test, t(n=19) = 2.375, p = 0.0289). **(E)** The rate of strong activation events ($E > 99^{th}$%) per second is higher after stimulation (paired t-test, t(n=19) = 2.372, p = 0.0291). **(F)** Line graphs of activation strength over cumulative time are examples of three

assemblies with different correlation (short-term plasticity) profiles. Values shown are normalized activation strength *E* during activation events, with baseline set to zero and smoothed with a gaussian kernel. Tick indicates the transition from pre-stimulation to post-stimulation. **(G)** Correlation between assembly event activation magnitude and the time of activation events is mostly positive. All comparisons are Spearman rank correlations. Inset shows proportion of assemblies with positive, negative, or insignificant Spearman's rho. Permutation tests on total number of assemblies, total positive and total negative assemblies. **(H)** Neuron drift into and out of assemblies increases after stimulation (Fisher's exact test, $p < 0.0001$, odds ratio = 2.03).

PCA = principal component analysis; *E* = assembly expression/activation strength; A.U. = arbitrary units.

Excerpt from the discussion on the timing of plasticity:

> We identified neuronal assemblies on time scales defined by a cycle of low gamma oscillations based on previous work in both rodents and humans (*10, 14, 17*). This time scale facilitates long-term potentiation (LTP) and organization of individual firing through hierarchical interactions of theta and gamma oscillations (*62, 63*). We consider the changes in assembly activation and flexibility we observe as reflecting *short-term plasticity* to distinguish them from the sustained synaptic strengthening that characterizes classical LTP, which is typically assayed over timescales of an hour or more. The 10 to 15-minute recording window available in our preparations following stimulation does not permit conclusions about whether the cofiring changes we detect are maintained; rather, we interpret coordinated cofiring at this timescale as a potential precondition for downstream consolidation into durable circuit remodeling. These are however timescales over which dendritic spines have been shown to exhibit activity-dependent remodeling (*64*), and analogous to *in vivo* two photon assays using basic cofiring methods (*65*). More generally for this assembly assay, we acknowledge that cofiring on other time scales is possible, and that further characterization of *ex vivo* physiology and response to stimulation will require explication of optimal frequencies that modulate cofiring. We note however that the identification of assemblies, especially with the computationally rigorous methods we employ (*25*), provides remarkable correspondence with *in vivo* experimentation to facilitate predictions about the impact of stimulation on cortical tissue (*17*). Alternative mechanisms to assay stimulation-related circuit plasticity represent a goal of subsequent experimentation. This may include methods such as optogenetics to specifically activate neurons of interest (e.g., L4 pyramidal cells) in order to gauge their direct role in cell assembly modulation. The feasibility of utilizing optogenetics in human organotypic slices in conjunction with MEA recordings was recently demonstrated (*66*). Similar approaches have been used to define and enhance the cell type-specific mechanisms of DBS for motor disorders and therefore may be translated to our paradigm as well (*67, 68*).

Finally, it is already known that electrical stimulation can yield changes in functional brain activities in humans in the off period following brain stimulation – for instance, Parkinson's symptoms recur on a somewhat stereotyped time course following turning off of DBS, with different delays in different symptoms with tremor recurring within 10s of seconds (as Fig. 2F suggests), then bradykinesia/rigidity coming back later, and so on. As is the electrical recording results themselves are limited in terms of novel mechanistic insights.

The reviewer points out that the neurophysiological responses we observed have some echo in known results of in vivo deep brain stimulation. We think this is absolutely a strength of our experimental paradigm, in that we observe convergence with existing clinical literature, which supports the choice of stimulation parameters we utilized. However, we respectfully disagree that such correspondence indicates that our results are not novel insights, especially as they are directly linked with specific gene expression programs in humans as well as layer specific neurophysiology, which have not otherwise been reported. We also note that examining these physiological effects in our OSC system complements work done in rodent models of motor-related circuits. We do think this comment indicates an opportunity to clarify one of the principal motivations we had in executing our

electrophysiological analysis, which is to validate that gene expression programs including ARGs are reflected in expected circuit changes, and to begin describing these changes neurophysiologically.

• To gain further mechanistic insight into the electrical recordings, the response to differing stimulus paradigms, and how those correlate to gene expression data, would be of great interest given the known differential response of human neural circuits to DBS or TMS of different stimulation frequencies, which is thought to be mediated by different neuronal and perhaps non-neuronal cell types.

The reviewer offers an excellent suggestion for future experimentation. For this initial, groundbreaking work we selected previously validated MEA stimulation parameters. The availability of these tissue specimens is rare; as such, we made the decision to focus on a single stimulation protocol for the current manuscript. But we have updated the Discussion to include explicit mention of these alternative stimulation parameters. We would like to emphasize that it would take years to collect enough tissue to study different stimulation parameters, though we do acknowledge this future program in the updated Discussion. We do think that our results will be critical to focus subsequent hypothesis generation and mold future experimentation.

Excerpt from Discussion:

> Our organotypic slice preparations, which preserve cortical lamination, offer a powerful platform to study how stimulation engages local circuits at cellular resolution. To maximize the interpretability of these experiments, we selected stimulation parameters with three deliberate goals: (1) avoid specifically inducing local LTP or long-term depression, which could bias assembly dynamics toward a particular activation state (*69-71*); (2) use parameters with a demonstrated safety profile and no evidence of tissue injury (*72*); and (3) maintain charge delivery comparable to that used in clinical DBS paradigms (*73*). This approach allowed us to stimulate across the entire tissue section, engaging distributed networks rather than single synapses, and to observe physiologic circuit-level responses without driving synaptic plasticity through supraphysiologic protocols. Importantly, this design enabled us to detect stimulation-related changes in assembly activation strength and membership flexibility that likely reflect endogenous network-level plasticity rather than artificially imposed potentiation. The robust, cell type-specific transcriptional and electrophysiological changes we observed suggest that our parameters were sufficient to capture biologically meaningful effects. Nonetheless, the tunable nature of neuromodulation therapies demonstrates that different stimulation parameters can induce or disrupt neural circuit function to varying degrees. Disease-relevant neuronal populations can be targeted with bespoke DBS parameters to ameliorate motor symptoms in rodent models of Parkinson's disease (*68*). It has been theorized that similar targeted approaches could be applied in humans to improve neuromodulation outcomes (*74*), but it is unknown whether such an approach could be harnessed for stimulation-enhanced cognitive function. Systematic exploration of additional stimulation waveforms, intensities, and patterns will be critical in future work to define how specific parameters shape circuit and molecular outcomes and to refine the translational relevance of these findings.

• What correlated analysis could be completed between the electrical and gene expression datasets? Could we understand which gene expression profiles correlate to increasing neuronal assembly strength following stimulation vs the decreased neuronal assembly strength?

Please see our response to reviewer #1, which is copied below for the reviewer's convenience, where we detail our new and extensive hdWGCNA analysis that directly integrates single cell gene expression with the firing rate parameters from the MEA**:**

"We thank the reviewer for this positive summary of our manuscript that highlights the strength of our approach, as well as the reasonable request for expanded integration across gene expression and neurophysiological data. First, we have updated our framing to explain that the neurophysiological data provide a detailed understanding of the specific circuit level changes elicited by the differential gene expression programs characterized in the

second part of the manuscript. We believe these data will add considerably to reader interest in our findings as they precisely link these gene programs to neurophysiology highly relevant for cognition (assembly formation) rather than non-specific firing changes alone. We further wish to emphasize that linking these gene expression changes to circuit dynamics described using assembly analysis highlight the relevance of different expression programs."

"Integrating these two data types is a limitation of existing gene expression profiling techniques due to the absence of a method that measures gene expression longitudinally in the same cells over time. We note that we cannot carry out a correlation analysis similar to those we previously conducted for oscillatory measures in human brain (Berto et al., 2018 and 2021), given the sample size limitations for this type of work as such analyses would be underpowered and may not result in statistically robust results. However, to address the reviewer concerns and further link together our findings, w**e now include new single cell/high dimensional weighted gene co-expression network analysis (hdWGCNA) results that directly link transcriptional programs to neurophysiological metrics.** The new results section and supplemental figures are copied below for the reviewers' convenience. In line with the reviewer's recommendation, we have focused on the central strength of our study: a detailed molecular characterization of human brain responses to physiologically relevant stimulation."

Linking neuronal activity with transcriptional programs

Separately, the MEA and multiomics results presented here outline cell type-specific alterations following stimulation. The multiomics data represent a snapshot of the period directly following MEA stimulation and recording. Using high dimensional weighted gene co-expression network analysis (hdWGCNA), we can link this transcriptional snapshot with electrophysiological changes at the level of individual cell types (see *Methods*). With this approach, we identified hdWGCNA co-expression modules (**Fig S10A**) that were differentially weighted between stimulation and control conditions (**Fig S10B**). Next, we correlated module eigengenes with MEA-derived firing rate metrics (see *Methods*) to identify networks of genes that are significantly correlated with stimulation-induced neuronal activity (**Fig S10C**). In excitatory neurons, two modules ("EM1" and "EM5") are positively correlated with pyramidal neuron firing rate and are most highly expressed in L2/3, L4, and L5 excitatory neurons (**Fig S10D**). These modules are enriched for canonical ARGs including *FOS, JUN,* and *BDNF,* which are also top network hub genes (**Fig S10E**). Within these modules, many genes are regulated by activity-dependent transcriptional regulators including EGR1 and NFIA (**Fig S10F**).

We repeated this analysis in inhibitory neurons by integrating gene networks with interneuron firing rate (**Fig S11A**). As we did in excitatory neurons, we identified several gene modules that were differentially associated with stimulation (**Fig S11B**). Modules associated with upper layer interneuron firing rate were especially highly expressed in SST-11 inhibitory neurons (**Fig S11C**). The IM5 module exhibited significant covariation with firing rate in several cell types (**Fig S11C-D**). The IM5 module was enriched for FOS binding activity (**Fig S11E**) and contained many activity-regulated hub genes (**Fig S11F**) similar to those seen in the EM5 module in excitatory neurons.

Together, this integrative analysis links physiological and molecular components of stimulation *ex vivo* by showing that stimulation-induced changes in network firing are supported by coordinated activation of activity-dependent gene regulatory programs. These results are consistent with a model in which electrical stimulation preferentially engages specific neuron types and activates transcriptional networks that may contribute to circuit-level short-term plasticity.

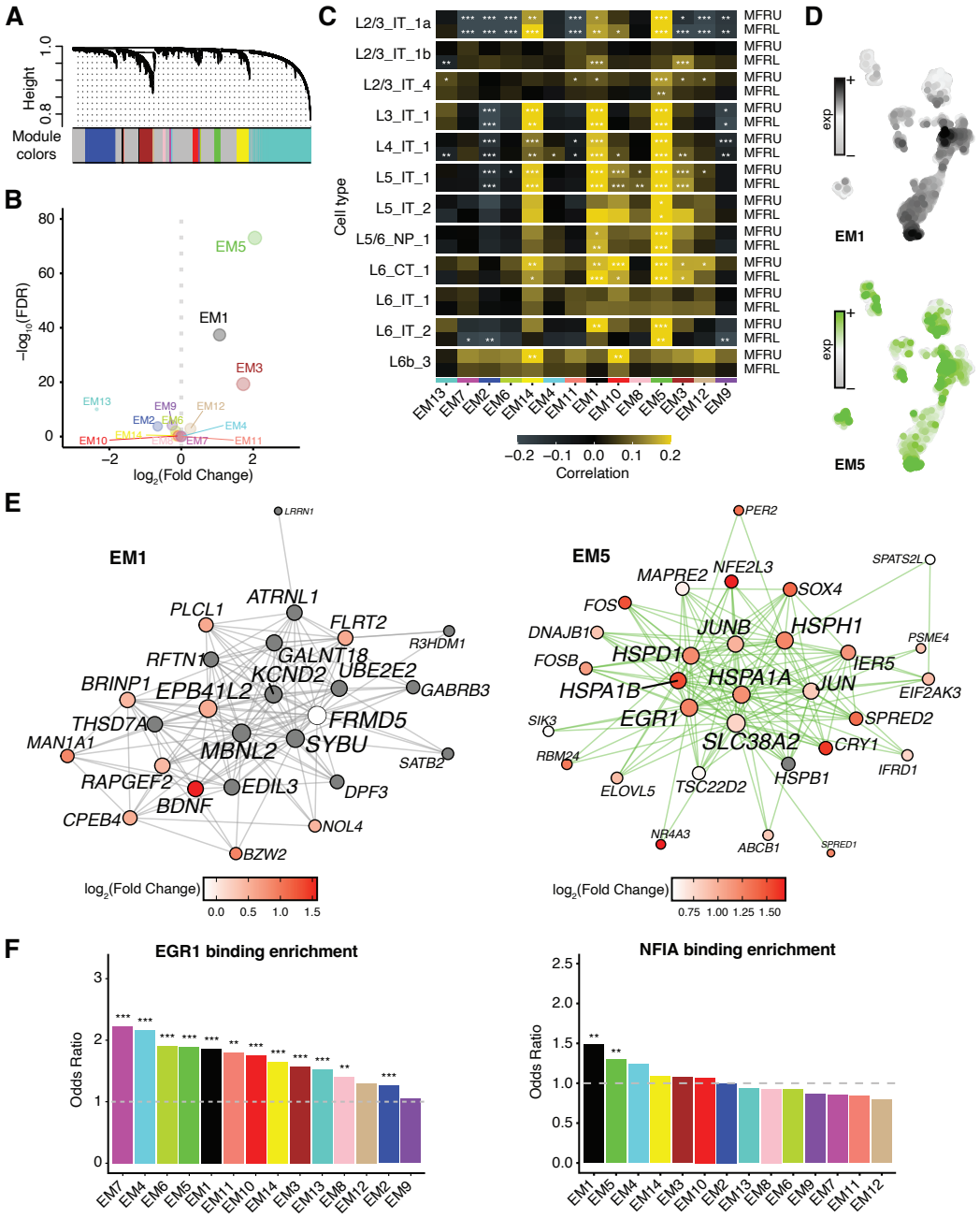


Figure S10. hdWGCNA reveals excitatory cell type and layer specific links between firing rate and gene expression networks. (A) Network dendrogram. **(B)** Significance of each module correlated with mean firing rate fold change in pyramidal neurons. **(C)** Correlation of each module with cell type and mean firing rate of upper- and lower-layer pyramidal neurons (MFRU and MFRL, respectively). **(D)** Feature plot of gene expression in black and green modules in the original multiomic UMAP space. **(E)** Visualization of the top hub genes in the EM1 and EM5 modules. Node color indicates $\log_2$(fold change) of gene expression in stimulated versus control cells. **(F)** Degree of EGR1 and NFIA binding enrichment in each module. EM = excitatory module; MFRU = mean firing rate upper layer; MFRL = mean firing rate lower layer.

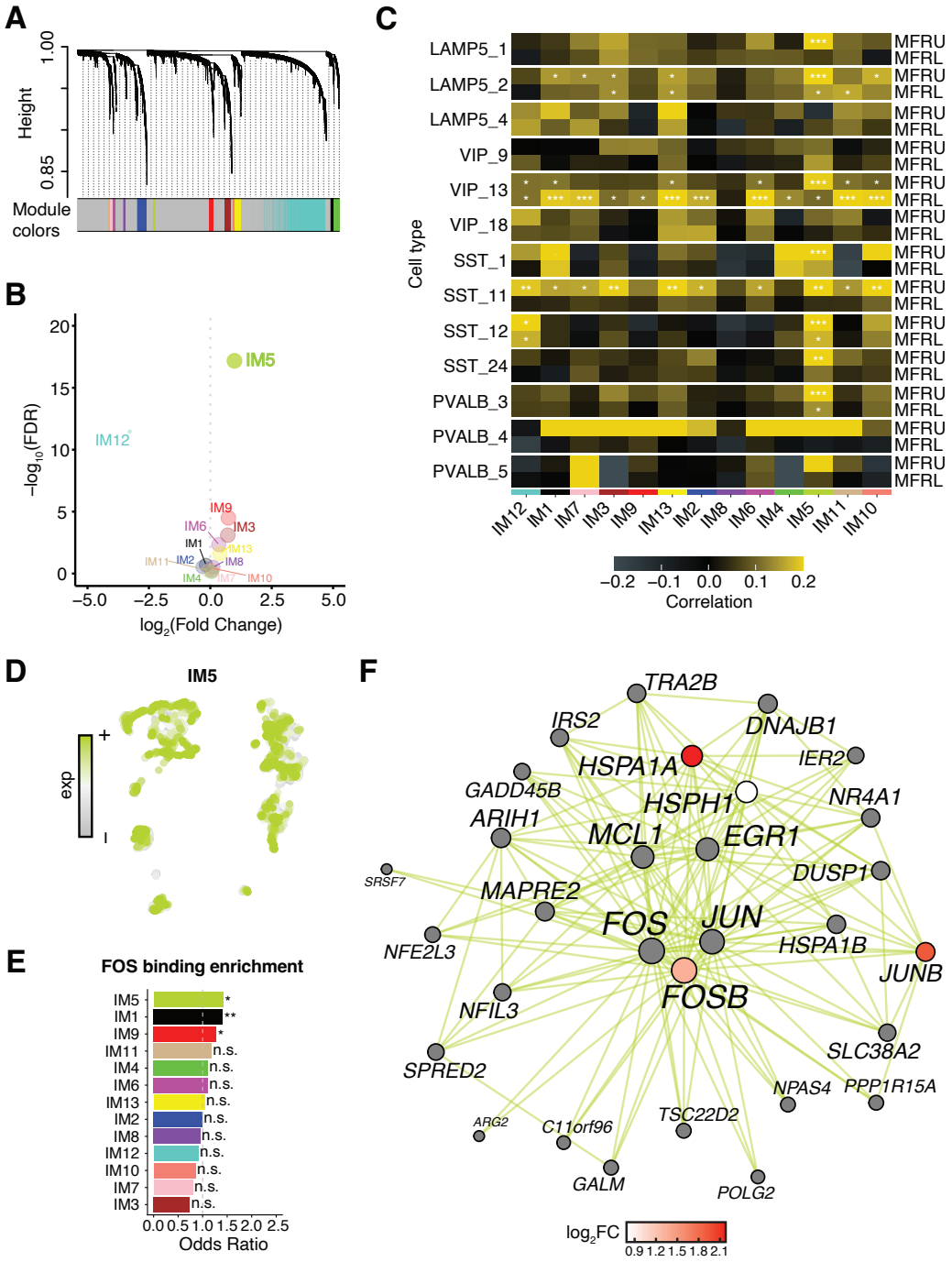


Figure S11. hdWGCNA reveals inhibitory cell type and layer specific links between firing rate and gene expression networks. (A) Network dendrogram. **(B)** Significance of each module correlated with mean firing rate fold change in interneurons. **(C)** Correlation of each module with cell type and mean firing rate of upper- and lower-layer interneurons (MFRU and MFRL, respectively). **(D)** Feature plot of gene expression in the IM5 module in the original multiomic UMAP space. **(E)** Degree of FOS binding enrichment in each module. **(F)** Visualization of the top hub genes in the IM5 module. Node color indicates $\log_2$(fold change) of gene expression in stimulated versus control cells. IM = inhibitory module; MFRU = mean firing rate upper layer; MFRL = mean firing rate lower layer.

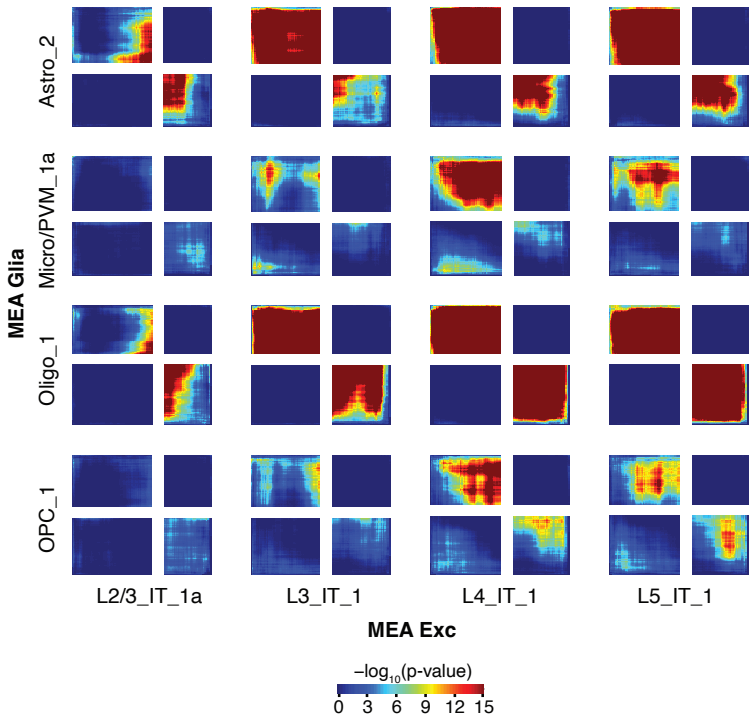
• Looking at Fig. S6, it appears the greatest DEG level changes are seen, among neurons, in layer 2/3 and layer 4 – even though these neurons show anti-correlated or minimally correlated changes between the MEA and IVS results (Fig. 6). In contrast, it appears relatively greater overall changes are seen in the non-neuronal

cells, across astrocytes, microglia/PVMs, and oligodendrocytes (both precursor and mature). From this vantage, it appears that the greater signal is in the non-neuronal cells although the authors focused principally on the neuronal changes in this analysis. How do the neuronal and non-neuronal changes correlate to each other to mediate the observed changes in electrical spiking behavior? Did the non-neuronal gene changes correlate as well with the electrical recording findings as the neuronal gene changes? How concordant are the non-neuronal changes between the MEA and IVS preparations?

The reviewer raises an interesting question that we have addressed in several ways. We note that a direct correlation between gene expression and neurophysiological changes in non-neurons is not feasible with the recordings available in the MEA system (which cannot record membrane potential shifts in astrocytes, for example), and we therefore hypothesize that the transcriptional responses in non-neuronal cells we observe reflect secondary or modulatory processes. Thus, strong transcriptional responses in non-neuronal cells do not necessarily translate into acute correlations with spiking metrics. This is an extremely understudied aspect of modern neuroscience and beyond the scope of our immediate study, but we have added text in the Discussion about glial/neurophysiological correlations for possible subsequent experimentation:

> Future experimentation could, for example, utilize viral manipulation to induce the expression of GFAP-controlled genetically encoded calcium indicators (*88, 89*) in human organotypic slice culture to directly visualize the astrocytic response to stimulation and understand how this relates to neural assembly dynamics and cell type-specific gene expression.

In order to understand whether there were similarities in the neuronal versus non-neuronal transcriptional responses to stimulation, we conducted RRHO analysis comparing the differential regulation of commonly expressed genes in each cell subtype. Most of these comparisons showed anti-correlation or no correlation at all. We do not show these analyses in the manuscript but have included them below in Response Figure 1:



Response Figure 1. RRHO2 comparing differential gene regulation after MEA-based stimulation between non-neuronal cell types and excitatory neuron cell types.

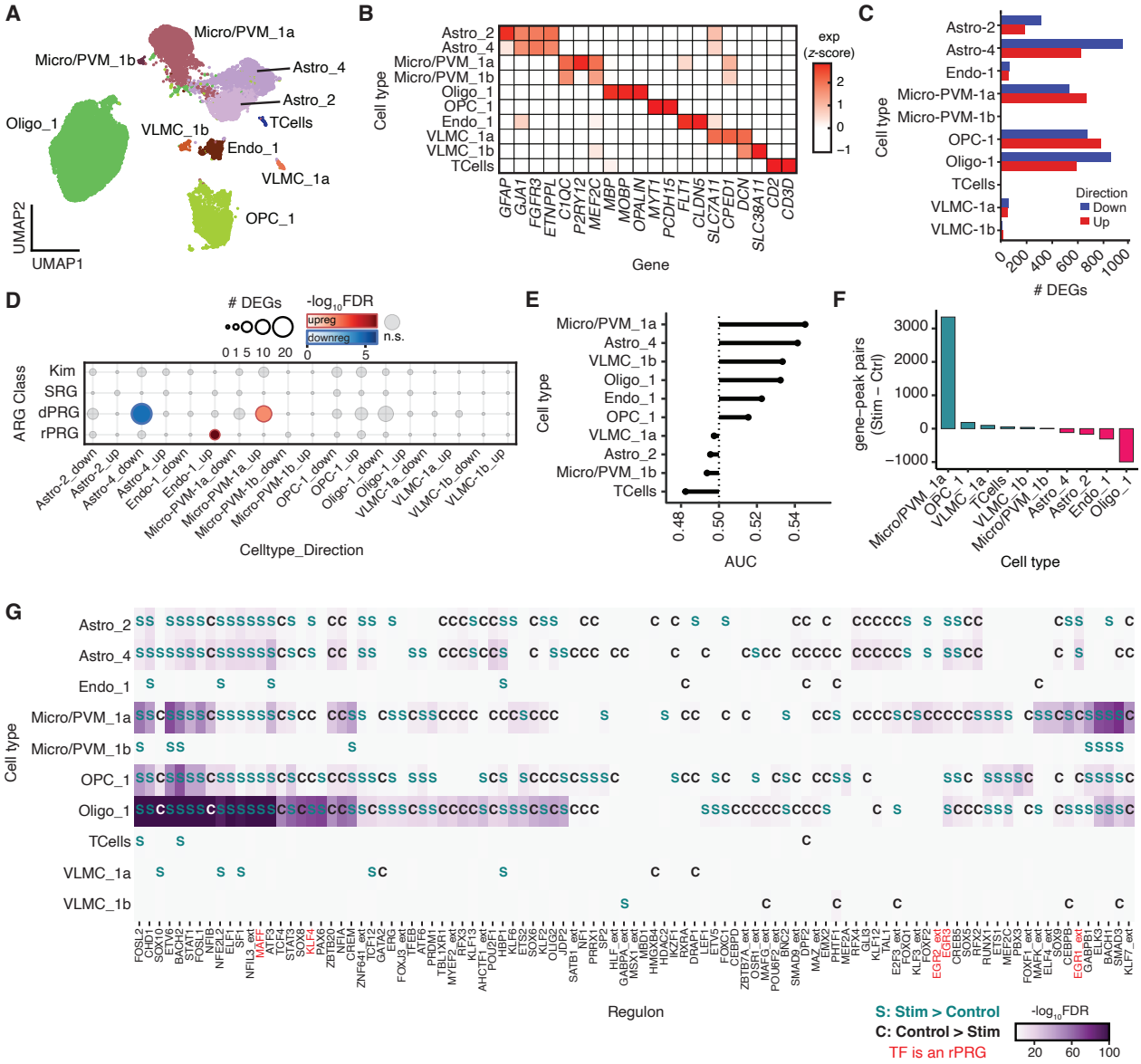
However, the findings we did observe in glia suggest an underlying gene expression substrate of chronic changes that may occur subsequently in circuits over time in response to stimulation. Stimulation-dependent DEGs in astrocytes and oligodendrocytes are enriched for functions related to energy metabolism, kinase

activity, chromatin organization, and axonal/synapse-associated processes. In this revised manuscript, we discuss that the expression changes in non-neurons are potentially secondary to activity changes elicited in neurons, as glial cells like astrocytes are known to modulate or support the effects of neuronal activity at the synapse, for example. We also carried out an RRHO approach for non-neurons in MEA vs IVS, to help explicate potential overlap in the findings across these model systems. Our updated figure includes a detailed presentation of these findings in response to these reviewer requests:

Non-neuronal facilitation of neuromodulation across paradigms

We have focused primarily on stimulation-induced circuit modulation in neurons and the associated cell type-specific gene expression changes. However, our *ex vivo* and *in vivo* preparations also allow us to interrogate the effects of stimulation in non-neuronal populations in the context of the intact cerebral cortex. Models of brain stimulation posit that non-neuronal populations, particularly astrocytes, may play important roles in shaping circuit remodeling over longer timescales (*54*). Consistent with this, our MEA data provide evidence of stimulation responses in non-neuronal populations (**Fig S6**). Differential gene expression was induced by stimulation across all non-neuronal cell types, with enrichment of synaptic genes in several (**Fig S6C-D**). One microglial cluster was found to be particularly responsive to stimulation by Augur and scMultiMap analysis (**Fig S6E-F**), which is consistent with findings from another group that implicates microglia in the mediation of stimulation's effects on the cortex (*55*). Stimulation engaged regulatory programs most prominently in astrocytes and oligodendrocyte-lineage cells, including enrichment of immediate early gene networks (MAFF, KLF4, EGR1) and activation of regulons linked to synaptic support, metabolic coupling, and myelination (**Fig S6G**).

We identified the same populations of non-neuronal cells in the IVS experiments (**Fig S15A-B**). After IVS, oligodendrocytes show the largest number of DEGs which are nearly all downregulated (**Fig S15C**). Though astrocytes have a smaller number of DEGs comparatively, they upregulate rPRGs to a high degree (**Fig S15D-E**) suggesting that they are indeed being engaged by stimulation either directly via cell membrane depolarization (*56*), or indirectly via neurotransmitter-mediated astrocytic synaptic remodeling (*57-60*). Comparing the non-neuronal response *ex vivo* versus *in vivo*, we see hotspots of overlap in the upregulated genes of some subsets of astrocytes, microglia, OPCs, and endothelial cells (**Fig S15F**). In summary, we found evidence that non-neuronal cells are impacted in a way that may strengthen short-term neuronal assembly dynamics via astrocyte, oligodendrocyte-lineage, or microglial actions at the synapse. Further characterization, particularly under semi-chronic stimulation paradigms, will be required to define the full extent of these glial contributions.



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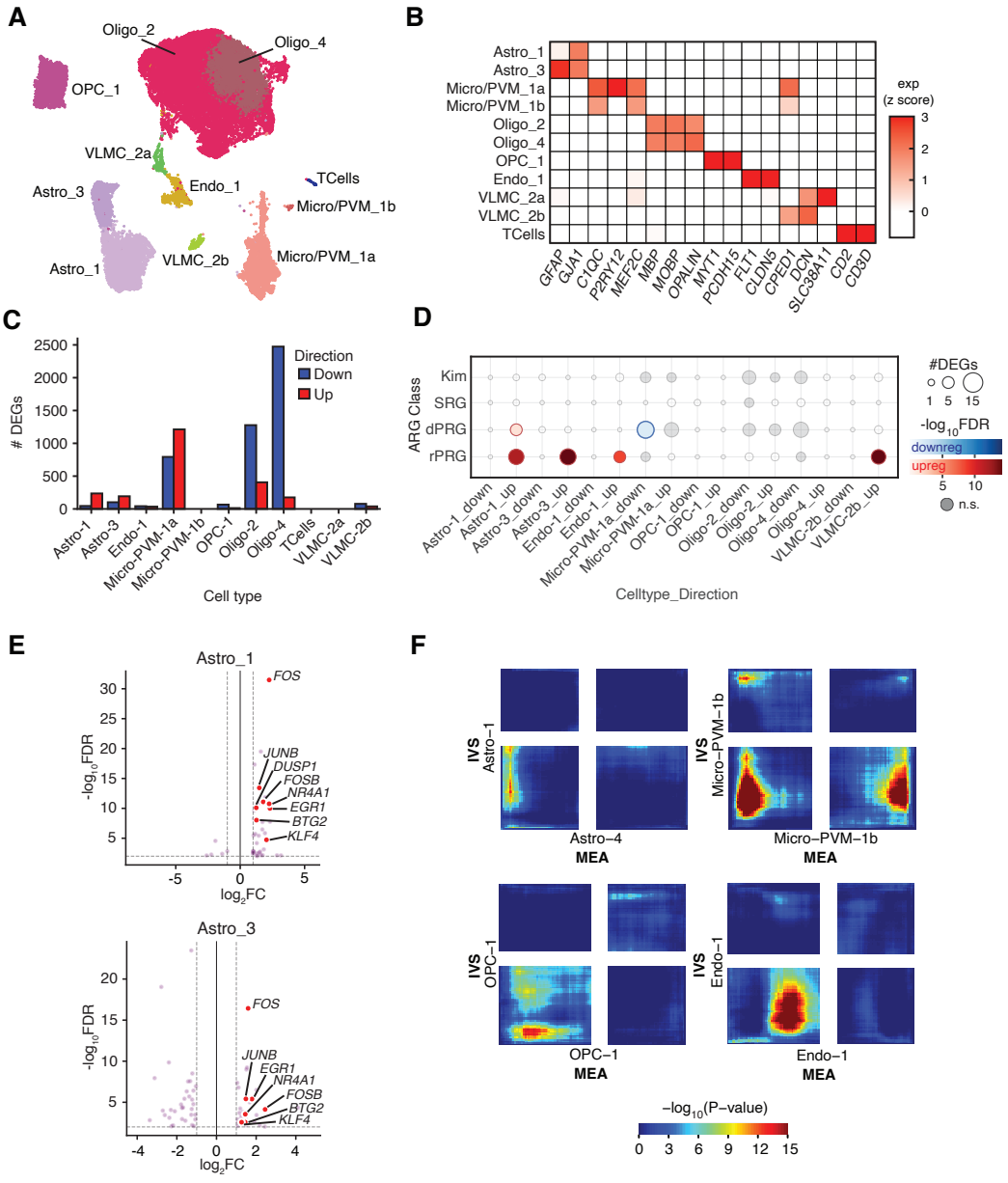

Figure S15. Impact of *in vivo* stimulation on non-neuronal cell types. (A) UMAP of subclustered non-neurons in the IVS paradigm. **(B)** Normalized expression of key marker genes for each non-neuron subcluster. **(C)** Number of differentially expressed genes per non-neuron subcluster. **(D)** Enrichment of differentially expressed activity regulated genes in several non-neuron subclusters. **(E)** Upregulation of rPRGs in astrocytes. **(F)** Convergence and divergence of transcriptional responses in different non-neuronal cell types in MEA and IVS paradigms.

Excerpt from the discussion on non-neurons:

Non-neurons of many classes were affected by stimulation in ways that may support stimulation-induced circuit modulation. Oligodendrocyte-lineage cells exhibited broad differential gene expression and substantial GRN activation after stimulation, which is congruent with studies on activity-dependent oligodendrocyte maturation and myelination as a means of neural plasticity in central nervous system injury models (*85, 86*). Our data also show that astrocytes upregulate rPRGs and enhance expression of synaptic gene networks after stimulation. This is in line with the astrocyte hypothesis of stimulation, which formed out of mounting evidence that astrocytes respond to and modulate neuronal activity at local synapses and across wide interconnected

networks (*54, 87*). Future experimentation could, for example, utilize viral manipulation to induce the expression of GFAP-controlled genetically encoded calcium indicators (*88, 89*) in human organotypic slice culture to directly visualize the astrocytic response to stimulation and understand how this relates to neural assembly dynamics and cell type-specific gene expression.

Minor comments

• How do the authors interpret that the MEA vs IVS changes are anti-correlated in layer 2/3 cells (Fig. 6)? Does this reflect that the recordings were completed in different brain regions? Or is the cell culture process altering the layer 2/3 cells in some way?

We thank the reviewer for their inquiry and agree that there are several possibilities for this finding. We note that the tissue used for multiomics in the MEA experiment was not cultured. That tissue was stimulated, recorded from, and flash frozen on the day of surgery (DIV0). We have clarified this detail in the main text and methods section:

Multiome sample preparation and nuclei isolation

Slices undergoing the MEA stimulation protocol on DIV0 were flash frozen immediately after the final recording was complete (~15 minutes after stimulation). Control slices rested without perturbation in normal aCSF for at least one hour before being flash frozen. We chose to include only DIV0 slices (i.e., slices that were prepared, stimulated, and recorded from on the day of the surgery) in the multiome experiments to prevent confounding gene expression changes that may occur during the early culture period (*107*). To have at least 20 mg of tissue for the multiome assay, we combined 4 slices originating from the same patient and condition per nuclei preparation.

Therefore, we think it is more likely that the anti-correlation is due to the differences between the MEA and IVS stimulation methods, as discussed in the original manuscript. The anti-correlation may also be due to the regional differences, as noted by the reviewer, which we now include in the discussion:

We ultimately compared stimulation-sensitive gene signatures identified *ex vivo* with those observed in human cortical tissue after intraoperative stimulation *in vivo*. Although the paradigms differ in geometry and current spread (i.e., *ex vivo* stimulation engages all six cortical layers directly, whereas *in vivo* stimulation is delivered from the cortical surface and preferentially reaches L2/3 dendrites and somata) we observed striking overlap in the cell types engaged by both. In particular, L3 and L5 excitatory neurons were consistently implicated across paradigms, suggesting that these cell types act as common hubs through which stimulation reorganizes cortical activity. L3 excitatory neurons are known to coordinate activity within cortical columns (*75, 76*), whereas L5 neurons integrate inputs from upper layers and relay signals to subcortical and distant cortical targets. Together, these findings support a model in which L3 excitatory neurons coordinate (gain-control) local columnar responses, while L5 excitatory neurons broadcast the resulting activity to downstream cortical and subcortical targets. At the same time, we observed paradigm-specific effects, such as preferential involvement of L4 excitatory neurons *ex vivo*, which may reflect the “deeper” penetration of current when the entire cortical cross-section is stimulated and may be more directly analogous to deep brain stimulation, in which the geometry of stimulation/tissue interaction may match our *ex vivo* preparations. The paradigm discordance seen in L2/3 excitatory neurons may also be due to the differences in stimulation delivery modes or perhaps due to differences between temporal and frontal cortex. L2/3 excitatory neurons have different distribution densities and have some unique genetic features in the PFC versus temporal cortex (*28*). Whether this translates to physiological differences is currently undefined, but further

large-scale comparative experimentation may yield information that could be used to develop bespoke neuromodulation therapies in different cortical regions. Together, our complementary results suggest that combining *ex vivo* and *in vivo* approaches can reveal both core, layer-generalizable effects of stimulation and layer-specific mechanisms that depend on the mode and geometry of current delivery.

• More for curiosity, were the temporal and prefrontal cortical recordings typically from a particular gyrus (STG vs parahippocampal, SFG vs IFG, etc) or was it from any frontal or temporal region that may be readily included in the surgical field?

The temporal samples were principally from the polar extent of the STG. In the frontal cortex, stimulation included MFG and SFG near BA9.

• In the Fig 1 legend, I believe the comment re log(FR+1) presentation is about panels D and F, not D and G

Yes, thank you for catching that typo. We have corrected this mistake in an updated legend:

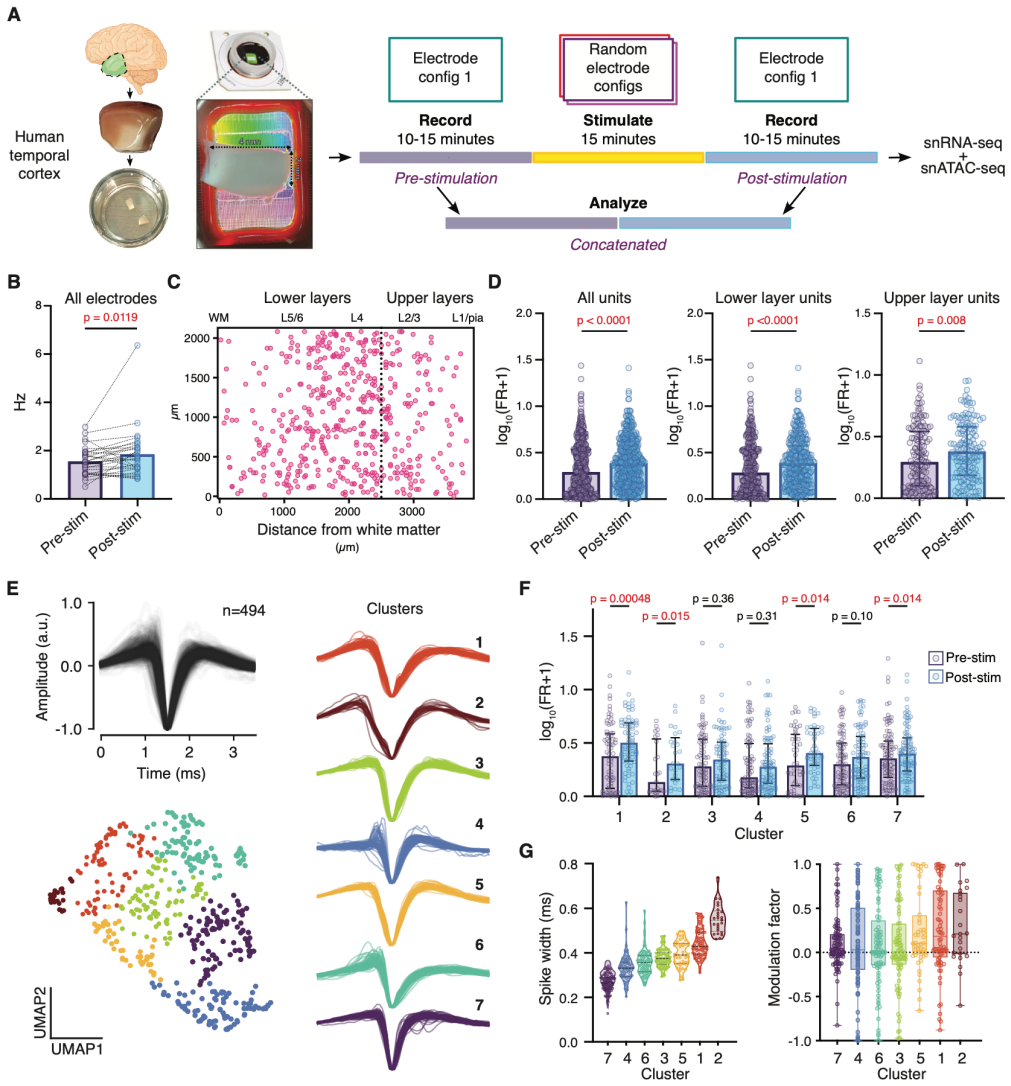

Fig. 1. MEA stimulation increases firing rate activity in a cell type-dependent manner. (A) MEA stimulation experiment overview. Left panel: a preparation of cortical slices from human

temporal lobectomy samples. Right panel: stimulation experiment timeline. (**B)** The mean 90th percentile firing rate recorded at all electrodes increases after stimulation (Wilcoxon signed-rank test p=0.012*, n* = 29 experiments) (**C**) The location of the highest amplitude spike of every unit identified with spike sorting. The boundary between the upper and lower layers is set at 1500 uM from the right short edge of the MEA, which corresponds to the pial edge of the slice. (**D)** Stimulation increases the average firing rate of all units together (paired two-tailed Wilcoxon signed- rank test $p<0.0001$*, n* = 494 units), and in upper- (paired two-tailed Wilcoxon signed-rank test $p = 0.008$, *n* = 142 units) and lower-layer groups (paired two-tailed Wilcoxon signed-rank test $p<0.0001$*, n* = 352 units). (**E)** Putative cell types identified with WaveMAP clustering. Upper left panel shows all unit waveforms that were averaged, aligned, trimmed, and normalized prior to clustering into groups in the right panel. (**F)** Effect of stimulation on firing rate in each cell-type cluster. All comparisons were made with paired two-tailed Wilcoxon signed-rank tests. (**G**) Unique characteristics of putative cell type clusters. Violin plot shows the distribution of spike trough widths at half maximum for each WaveMAP cluster. Bar and strip plot shows the firing rate modulation factor according to the calculation MF = (FRpost-FRpre)/(FRpost+FRpre). Clusters are ordered according to increasing spike width to show the positive relationship between this arrangement and the change in firing rate. In (D) and (F), $\log_{10}$(FR+1) transform of firing rate values is for visualization only. Statistics performed on non-transformed data.

WM = white matter; FR = firing rate; a.u. = arbitrary units; UMAP = uniform manifold approximation and projection.

• Were these trials registered on ClinicalTrials.gov? If so, the registration number should be included.

No, we had no intervention seeking to observe a behavioral or health outcome, so this was not a clinical trial.

Referee #2 (Remarks on code availability):

I believe the code is of use to the community, and definitely the code with the GEO database repository is a great addition. At a cursory glance the code looks appropriately constructed.

Point-by point response

Referees' comments:

Referee #1 (Remarks to the Author):

The reviewers have addressed my concerns and the manuscript represents an interesting an novel contribution to the literature.

We are pleased that we were able to address all concerns raised by Referee #1.

Referee #2 (Remarks to the Author):

In this revised manuscript the authors have admirably added clarifying discussion, methodological detail, and new analysis compared to the initial submission. In this study the investigators took human post-surgical brain samples (from the anterior temporal lobe) and cultured them in a slice preparation. Then with multielectrode arrays (MEAs) they stimulated patterns of activity in the ex vivo brain tissue, measured the spiking response of neurons in response to the stimulation, and analyzed the changes in gene expression associated with this stimulation. They then compared similar metrics to prefrontal cortical (PFC) tissue also indicated for neurosurgical resection. They stimulated the target PFC region and measured gene expression changes resultant from the stimulus following excision, and compared the results of in vivo stimulation (IVS) to MEA stimulation post culture. The authors observed that stimulation is associated with a short-term plasticity in the form of increasing neuronal co-firing in assemblies in the 10-15 min following stimulation. They further observed that neuronal assemblies – defined as co-activating/firing sets of single units – generally show an increase of co-activiation, with >half of assemblies showing strengthened co-activation following stimulation. In the genetic analysis, the authors find that stimulation is associated with a general upregulation of activity dependent and synapse associated genes. Curiously, the authors also find that simulation yields substantial changes in non-neuronal cells like microglia and oligodendrocytes. However, the genetic response to stimulation was largely anti-correlated between the in vivo prefrontal cortical stimulation case and the anterior temporal cultures MEA preparation.

Altogether, the study remains an interesting one, as it confirms that assembly architecture in response to stimulation is seen in human brain tissue like it has been observed in animal models, and is conserved between in vivo and ex vivo preparations. The added hdWGNA analysis and new discussion all help to clarify pending questions from the original submission. However, the fundamental conclusions of this study continue to fall short of revealing mechanistic insight into how neuronal assemblies of co-activating neurons form or contribute to functional effects. That the gene expression data are so different from the cultured MEA/anterior temporal and the IVS/prefrontal cortical stimulation studies indicates that the generalizability of these findings may be limited. For instance, from the MEA results, layer 4 cells are principally implicated as the mediators of the response to simulation, but after comparing to the IVS results it seems the focus should more be on layer 3 and 5 cells. To fully understand which results here would truly generalize would require a greater number of samples across different stimulation protocols and preparations that are likely impractical to implement as the authors note in their rebuttal. Finally, the more surprising and curious results in this analysis – namely that the non-neuronal cells show a similar level of gene expression changes in response to stimulation as neurons – continues to be under-explored in this study.

Overall, the revised study remains an interesting contribution to the literature, but one whose conclusions may be more appropriate for a more specialized journal and audience.

We thank the reviewer for their overall positive assessment of our study and recognition of the limitations that exist based on sample sizes. We were actually pleased with the relative overlap in response between the ex vivo and in vivo datasets considering the differences in stimulation parameters and brain regions. It remains to be determined how these results will generalize under different stimulation conditions and in alternative brain regions (if even feasible).

Few additional questions/comments:
• Could the non-neuronal cellular response to stimulation have implications for the gliosis observed in response to DBS? How may we interpret the microglial and oligodendroglia gene networks being recruited in terms of the plasticity that is observed or expected in the firing rate results? Do the genes induced in microglia and astrocytes in response to stimulation cohere more with a disease-associated phenotype or the homeostatic phenotype of each cell type?

These are excellent questions that we can only speculate upon at this point. The non-neuronal responses occur within minutes compared to the gliosis that happens in DBS over months to years. Future studies that stimulate over longer time courses ex vivo can model the extent to which the non-neuronal responses evolve over time and corresponding changes in specific glial subtypes. With respect to non-neuronal GRN changes, we do not know if these alterations are primary responses or are secondary to the neuronal responses. Therefore, it is challenging to interpret how the non-neuronal changes impact firing rates. Future studies will require manipulation/impairment of specific cell types in the presence of stimulation in order to determine direct cellular impacts. The question of the relationship between the gene networks we report and disease-specific phenotypes is interesting, as one of the motivating results

for our work was experimentation in animal models suggesting normalization of abnormal expression patterns via stimulation. Integration with disease-specific datasets may therefore suggest conditions for which stimulation can offer some therapeutic advantage.

• The current analysis comparing the MEA and IVS results focuses on the DRGs upregulated in concert between the paradigms - what more can be understood by looking also at the commonly downregulated genes between the MEA and IVS preparations?

We primarily focused on commonly upregulated genes to highlight activity regulated genes and synaptic genes in the neuronal subtypes of interest. In excitatory and inhibitory neurons, there are very few commonly downregulated DEGs (only two overlap in L6 excitatory neurons, and none overlap in inhibitory neurons). Glial cells demonstrated a larger overlap in the downregulated DEGs, particularly in microglia and oligodendrocytes. None of these are IEGs. In oligodendrocytes, transcriptional coregulators (e.g., *CRTC1*) were overrepresented in the downregulated genes by gene ontology analysis. This is an indicator of the complex gene regulatory network activity ongoing in these cells, which we were able to map with SCENIC+ analysis in the MEA multiome experiments. Though this GRN analysis was not possible in the IVS RNA-seq experiments, the commonly affected transcriptional coregulators hint that similar GRNs are affected in oligodendrocytes across conditions.