nature portfolio

Peer Review File

Modelling Synaptic Dysfunction in Childhood Dementia Using Human iPSC-Derived Cortical Networks

Corresponding Author: Professor Cedric Bardy

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, Mazzachi et al., have utilised 5 MPSIIIA patient and 5 healthy control iPSC lines which they differentiated in parallel into neural stem cells for prolonged maturation into cortical cultures for functional study. MPSIIIA is one type of lysosomal storage disorder caused by dysfunction in one of the genes encoding a lysosomal enzyme involved in the degradation of heparan sulfate. Investigating functional deficits in human models is highly important as MPSIIIA patients present severe and early-onset neurodegeneration. In these derived cortical cultures analysed at different time points (30-60 and 90-120 days of differentiation), authors have identified functional changes present in the MPSIIIA cultures. Interestingly, authors do not find any differences in the electrophysiological properties of individual neurons but do identify clear changes at the network level of functionality. The authors also achieve fantastic morphological reconstructions of the cells to assess the structure of mature Synapsin:GFP neurons. Finally, authors use this cortical model in combination with approved pharmacological treatments to investigate whether they can achieve amelioration of the functional changes.

The work covers a wide range of functional analyses, and the techniques utilized are of a high standard within the field. Although of interest, the manuscript presents some weaknesses that could be addressed to improve the study and support the conclusions.

Major concerns:

1. The authors claim in Line 114 & Figure 1 that the cultures were formed of post-mitotic neurons at Day 14 – however there is no clear support of this claim in protein immunohistochemistry or other analyses. The single cell RNAseq data found in Figure 1B counters this with the presence of radial glia, astrocytes and immature neurons at Day 120 in culture. Overall neurons represented < 35% of the culture and only 15% of the total cells were mature neurons.

2. Individual cell lines present a high variability in their final fate in playground cultures assessed by scRNAseq at Day 120. In Figure S10-A it is clear in the MPSIIIA culture that many lines are high underpresented e.g. 2 lines appear to make up >60% of the culture, with 1 line making up more than >60% of the total excitatory (MPSIIIA7) or inhibitory (MPSIIIA10) neuron population. These cells will be vastly over-represented in the excitatory or inhibitory analyses performed and will provide the majority input to network level activity. Unlike the ‘Village in a dish’ model proposed by Neavin et al., (Nat. Comm., 2023, DOI: 10.1038/s41467-023-38704-1) the authors have not demonstrated high reproducibility of the fraction of cells that were used between preparations of the ‘Playground cultures’. Likewise, this brings into question the changes in excitatory and interneuron post-synaptic currents as they could be due to shifts in excitatory or interneurons proportions within the cultures.

3. With this inherent variability and the majority of cells at 120 days being astrocytic, it is suggested to provide individual panels of the cell types in Figure 1B within the panel that identify whether the clustering has been successful, and split by genotype to demonstrate that similar populations are present in healthy control and MPSIIIA patients.

4. The manuscript shows limited biological replicates. As shown in figure S2 the authors only independently differentiate N=2, or in many cases N=1, biological replicates of neural progenitor cells. There is also concern regarding the N=1 synaptic staining despite the technical replicates as initial plating density or other variabilities in cell culture could have influenced the distribution of these markers. Likewise, regarding concern 2 there is a high inherent variability in the cultures, and this may be more evident with multiple preparations from each line.

5. In Line 919 the authors note that they discard inactive wells prior to full analysis, the reviewers suggest that the total number of inactive wells should be described in the supplementary data to identify whether there is a particular phenotype based on the total number of active/inactive wells and to demonstrate the actual number of electrodes used per condition

6. Throughout, there is an odd statistical comparison, given that many of the figures depend on data generated from multiple lines, an ANOVA should be performed instead of a presumed t-test.

7. The reviewers primary concern with the interpretation of the data presented is that there is a high possibility that by selectively patching the 'healthiest' cells in the MPSIIIA condition (e.g. selected for good morphology, good neuronal properties, etc.) then the authors could be artificially selecting for newer-born neurons wherein the disorder has not had time to significantly progress. There are evident progenitors identified by the scRNAseq and a high proportion of immature neurons in the total neuron population. There is no evidence presented of accumulation of heparan sulfate to support that these neurons are under progressive load. Therefore, by not attempting to include any sick or dying cells, or at least address the proportion of these cells between culture conditions, there could be a bias for healthy cell types that does not reflect conditions when examining the whole network function, as shown by the altered MEA function. In particular, the type of tonic firing observed during the MEA recordings is a newborn neuron phenotype that gives rise to synchronised burst firing in more mature neurons, as shown in Kirwan et al. (Development, 2015, DOI: 10.1242/dev.123851). This could imply that the continuous renewal of the MPSIIIA cultures leads to a continuously reset network development, hence the more immature or hypoxia-like phenotype. For example, the capacitance is lower in early timepoints, then recovers to a higher point, is this due to the presence of newly-formed cells that are being patched. There are also more active neurons in MPSIIIA despite reported neuronal death in the accompanying manuscript.

8. Instead of barplots, boxplots or violin plots should be used when examining data with very high numbers of individual dataplots. Datapoints should also always be shown if they will be used e.g. in Figure 4 many barplots (A-D) show individual datapoints but then the authors switch to reporting only the N number. It would be better in this scenario to show all the datapoints so the reader can assess if there are many outliers.

Minor concerns:

1. The term 'isogenic' is used inappropriately to describe the cultures at Line 110, this does not fit with the established use of isogenic in iPSC culture to describe lines which have the same genotype except for a single or multiple disease-causative genes that have been introduced or corrected.

2. Figure S4 and the Cell stress mentioned in the methods but not referred to in the main text other than to state a number of cells imaged.

3. In figure S10 percentages should be provided for each of the conditions in the pie-charts to determine actual change.

4. There is reference to 'STAR methods' if this is the case for this manuscript then a workflow of the pipelines for the image analysis should be included

5. Clumping covering 104263um2 is quite a large clumping area, there is no number for the clumping events, it would be ideal if the authors could indicate discarded data amounts in the total number of imaged regions

6. Significant differences in the capacitance at 30 days (Figure 2D) are not mentioned in the text, whereas non-significant trends are mentioned in the text at Line 253 (Figure 4N/P).

7. Examples of synaptic colocalization should be shown as part of Figure 4, as the current figures it is difficult to clearly identify due to the scale. A XY-XZ 3D colocalization plot would also greatly benefit the manuscript.]

8. Inhibitory staining was only performed in one timepoint as shown in figure 7, and at this point there are high variations in patient-neuron fate ratios. As such the changes seen could be dependent on an individual patient.

9. In figure 4 it is mentioned that TTX shows disproportionate hyperactivity in the MPSIIIA cultures but then the figure shows zero significance.

10. Authors should comment on the role of glial support and glial neurotransmitter uptake.

11. The decrease in PSD95 may be just a consequence and not a disease mechanism since at day 30 neurodegeneration is already reported.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

This manuscript , focuses on studying the excitation/inhibition imbalances in patient-derived induced pluripotent stem cells (iPSC) from children Mucopolysaccharidosis Type IIIA (MPS IIIA). Authors describe MPS IIIA phenotypes based on hyperactive excitatory synapses and dysregulated gene expression linked to synaptic homeostasis. A drug screen with whole-cell patch-clamping, multi-electrode arrays and high- content confocal imaging identified a cocktail of repurposed drugs effective in restoring synaptic and neural network activity to healthy levels. Similarly to the accompanying Greenberg et al., manuscript, the use of neuronal 'playgrounds' composed of 5 patient/healthy subject lines with 2 clones per line contributes to a high rigor of this study. The diversity in experiment design on neuronal synaptic integrity and neuronal activity are of high value, with multiple detailed aspects of these phenotypes being evaluated in parallel. Little is known regarding the molecular mechanisms of the MPS IIIA, and no drugs are currently available for these patients. This study is impactful and paves a path towards further in vivo and pre-clinical screen to identify FDA-approved therapeutics. That being said, I do have some concerns to bring to the authors' attention below:

Major comments:

• Authors describe to generate cortical neuronal cultures, dominating with MAP2 multipolar/pyramidal neurons. It is therefore puzzling that they detect GABAergic synaptic Gephryn+ staining in these cells. It would be of high benefit to include a better characterization of these neuronal-astrocytic cultures, with specifications on the inhibitory/excitatory inputs. In addition to a detailed characterization of their cell culture system, it is suggested to move results from Fig6A to Figure 1.
• Why are the treatment timelines different in the high content image-based drug screening assay (treatments at DIV40-55), the neuronal activity assays (treatments start at DIV65-DIV80), and the patch clamp assays (treatments at DIV85-99)? Since the drugs do rescue synaptic changes already at DIV40-55, one would expect to observe neuronal activity changes from those early treatments. Authors should elaborate why they performed three different experimental set up for these linked and complementary experiments.
• Similarly to the accompanying Greenberg et al., manuscript, one concert that needs to be discussed is the heterogenicity of the 'playground' samples, with fact that MPSIIIA 10 and 7 being the most prominent cell lines in the MPSIIA 'playground', with MPSIIIA 8 lines to compose the least of 'playground'. This is a common issue with such village-based approaches. As different lines show variable response to neuronal and astrocytic pathologies (Suppl. Fig 8), there may be a variance in cellular responses to the tested drug, highlighting 'responders' and nonresponders'. Authors should deeply analyze their scRNAseq data, to 1. Determine if the drug effects are not driven by the dominant patient lines, and 2. Determine which lines may be more 'astrogliotic' that could potentially skew the neuronal activity analysis
• It is currently standard in the field to work with isogenic gene-corrected controls, especially relatively easily generated for a point mutations. The authors might want to validate their key findings and their drug selection process in an isogenic gene-edited pair as a 'gold standard', which would be much cleaner system accounting for other genetic variance in the specific patient line, further elevating the 'playground' approach.
• As the authors identify combination #2 to be the most efficacious therapeutic strategy impacting neuronal activity, they also discuss the potential of this drug combination on other preclinical disease phenotypes, as identified by the companion manuscript Greenberg et al.,. The companion article is not exploring the impact of combination #2. As the authors strongly suggest for this therapeutic strategy to be clinically relevant, these MPSIIIA phenotype rescue experiments should be performed here, as described in Greenberg et al.,
• Authors observe that the combination #2 shows sustained effect on the number of active neurons after treatment ceased. Yet they do not comment on the other two MEA parameters which went back to the MPSIIIA - untreated levels. These differences in readouts should be elaborated, as the current conclusion that combination #2 has long term benefits is not fully clear.
• The authors did not state the number of differentiations/ biological replicates their results consist of. Drug screening assays, when established, are acceptable to be performed on one differentiation, but all of the proof of concept/phenotype assays (Fig. 2,3,4) should be done in at least 2-3 independent differentiations per current standards. Also, the single cell RNAseq data should be at least followed up with target validation in an independent differentiation.
• It is unclear how the compound doses were determined prior to the drug screen (Fig 7), and there is no information on how the drug combination #1 and combination #2 were designed.

Minor comments:
• Typo line 168, should be Figure S7

(Remarks on code availability)

Reviewer #3

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

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I would like to congratulate the authors for the extensive and excellent work performed. The addition of new replicates for synaptic stainings, new data presentation, and the new scRNAseq data are clearly improving the quality of the work and clarity of the manuscript.

My only remaining concern is about the BIAD03 line for several reasons. First, this line shows a quite higher expression of SOX2 at the iPSC level (companion manuscript), which could have an impact in the differentiation from NSC to neurons since SOX2 needs to be downregulated during neuronal differentiation. In fact, this is the line with lower excitatory postsynaptic communication, and interestingly, in the scRNAseq data most of excitatory neurons generated from this line cluster separately from all other excitatory neurons generated by disease or healthy lines (Fig 1B, Supplementary Fig 1B and Fig 6A). What are the differences in between these two populations and what can be the functional impacts of these differences? In Supplementary Fig. 12, it actually seems that for some of the measurements, 1 or 2 individual patient lines could be driving some of the differences and one wonders if BIAD03 is that line and the driver of the functional changes in the playground networks since it is clearly dominating it at the later time points.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
The authors have adequately addressed all of my previous comments and concerns. They have conducted additional experiments and provided detailed information on the biological replicates for improved clarity. These additions enhance the reproducibility of the reported phenotypes and compound rescue, significantly strengthening the overall rigor of the study.

(Remarks on code availability)

Reviewer #3

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

(Remarks on code availability)

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I thank the authors for their thorough characterization of the BIAD03 line differentiation to address my previous concern and agree that some of the differences could be due to inter-individual specific features.

Paper 2: Hyperactive synaptic circuits in a human neuronal model of childhood dementia

We sincerely thank the reviewers and editor for their detailed, and constructive feedback, which we have carefully considered throughout the revision process. In response, we undertook a comprehensive series of new experiments, including single-cell genotyping to accurately demultiplex donor identities, immunocytochemistry analyses assessing cell proliferation in astrocytes and neurons (Ki67, GFAP, DAPI, MAP2) and detailed synapse density profiling (excitatory and inhibitory markers) at multiple key developmental timepoints (15, 30, and 60 days), as well as a evaluating the effects of APAT (formerly Combination #2) on neurodegeneration and astrogliosis at 30 days. To enhance data transparency and robustness, we generated several new supplementary figures showing representative synaptic co-localisation images, breakdowns of synaptic activity by individual iPSC-derived neuronal lines and playground models, and detailed tables and histograms documenting data exclusion and quality control across all assays. Major figures were updated to include these new experimental replicates, improved statistical analyses, clearer data presentation with added data points and box plots replacing histograms where appropriate. Additionally, multiple supplementary figures now include metadata on independent neuronal differentiations and batch information for greater reproducibility and clarity. Throughout the manuscript, we have revised text sections in Results, Methods, Discussion and Limitations to comprehensively reflect these updates, clarify methodological details such as field-of-view filtering criteria and drug dosing rationales, discuss potential biases and limitations candidly, and refine terminology for more precise scientific communication.

Changes in the text are highlighted in red in the revised manuscript and responses to each specific comment are outlined below in blue.

This extensive revision demonstrates our commitment to addressing every reviewer concern thoroughly, improving the rigor and transparency of our study, and ultimately strengthening the overall impact and clarity of the manuscript. We truly appreciate the opportunity to improve our work and hope these revisions meet the high standards expected.

A summary of the figure changes is outlined below:

- Figure 1B: Updated to include separate UMAP panels for healthy and MPSIIIA samples, demonstrating comparable cell type proportions between genotypes.
- Figure 2D-E, Figure 3E, H, J and Figure 4H, L, G, K: Updated statistical analysis to a Kruskal-Wallis one-way ANOVA with Dunn's correction for multiple comparisons.
- Figure 4A-C: New data from an independent neuronal differentiation for Synapsin, Gephyrin and PSD-95.
- Figure 4F, J: Updated formatting includes dot points to represent individual donors and playground models.
- Figure 4-R: Updated formatting including dot points to represent individual neurons patched in the playground model.
- Figure 5C-G: Updated statistical analysis to a mixed-effects model (REML), with cell line and time as fixed effects and condition as a random effect, followed by Šídák's multiple comparisons test.
- Figure 6A, C: Updated cell type classification.
- Figure 6F: New panel examining the expression of glutamatergic and GABAergic postsynaptic genes in excitatory and inhibitory neurons.
- Figure 7: Updated formatting to align with the colour scheme used in companion manuscript.

- Figure 7F: Updated analysis of MEA data using the average recordings from 45 to 64 days as pretreatment baseline (previously 14 to 64 days).
- Supplementary Figure 1B-H: New data showing the distribution of various cell types among healthy and MPSIIIA donors and proliferation/cell cycling analysis of our playground cultures.
- Supplementary Figures 2-5A, 7A, 8A, 10, and 12A: New data summarising the number of independent neuronal differentiations for each cell line, and the total number of neurons, wells and differentiations per healthy and MPSIIIA conditions.
- Supplementary Figure 5C-D: Data for capacitance and cell body surface area data represented as boxplots to highlight data distribution for Figure 2D-E.
- Supplementary Figure 7D-F: Data for maximum AP amplitude, upstroke:downstroke ratio, AP duration, trough voltage and resting membrane potential represented as boxplots to highlight data distribution for Figure 3E.
- Supplementary Figure 8B-C: Data for voltage-gated potassium channel analysis represented as box plots to highlight data distribution for Figure 3H & 3J.
- Supplementary Figure 9: New figure highlighting synaptic co-localisation in high-content imaging.
- Supplementary Figure 11: New figure showing AMPA- and GABA-mediated spontaneous synaptic activity over neurodevelopment split by ESC lines, iPSC cell lines and playground model.
- Supplementary Figure 13A: New data from a 30-day snRNAseq experiment and updating formatting to include percentage contribution per donor and labelling of donors after genotyping and demultiplexing.
- Supplementary Figure 16: Updated analysis of MEA data using the average recordings from 45 to 64 days as pretreatment baseline (previously 14 to 64 days).
- Supplementary Figure 17: New figure and data validating efficacy of APAT on phenotypes from companion manuscript.
- Supplementary Figure 18-20: New figures indicating the amounts of data discarded during quality control assessment of high-content imaging data.
- Supplementary Figure 21: New figure indicating the number of inactive wells and electrodes discarded from network event frequency metric analysis of MEA data.

Reviewer #1 (previously 5)

In this manuscript, Mazzachi et al., have utilised 5 MPSIIIA patient and 5 healthy control iPSC lines which they differentiated in parallel into neural stem cells for prolonged maturation into cortical cultures for functional study. MPSIIIA is one type of lysosomal storage disorder caused by dysfunction in one of the genes encoding a lysosomal enzyme involved in the degradation of heparan sulfate. Investigating functional deficits in human models is highly important as MPSIIIA patients present severe and early-onset neurodegeneration. In these derived cortical cultures analysed at different time points (30-60 and 90-120 days of differentiation), authors have identified functional changes present in the MPSIIIA cultures. Interestingly, authors do not find any differences in the electrophysiological properties of individual neurons but do identify clear changes at the network level of functionality. The authors also achieve fantastic morphological reconstructions of the cells to assess the structure of mature Synapsin:GFP neurons. Finally, authors use this cortical model in combination with approved pharmacological treatments to investigate whether they can achieve amelioration of the functional changes.

The work covers a wide range of functional analyses, and the techniques utilized are of a high standard within the field. Although of interest, the manuscript presents some weaknesses that could be addressed to improve the study and support the conclusions.

We appreciate the reviewer's thoughtful and constructive summary of our manuscript. We are grateful for their recognition of the importance of studying functional deficits in MPSIIIA using human-derived cortical cultures, and for acknowledging the strength and quality of the techniques employed, including our morphological reconstructions and functional analyses. We sincerely thank the reviewer for their valuable feedback and comments regarding areas of weakness. We have carefully considered each of their suggestions and have addressed all of these points in the revised manuscript. We hope that the clarifications and additional data provided help to strengthen the study and support our conclusions.

Major Concerns:

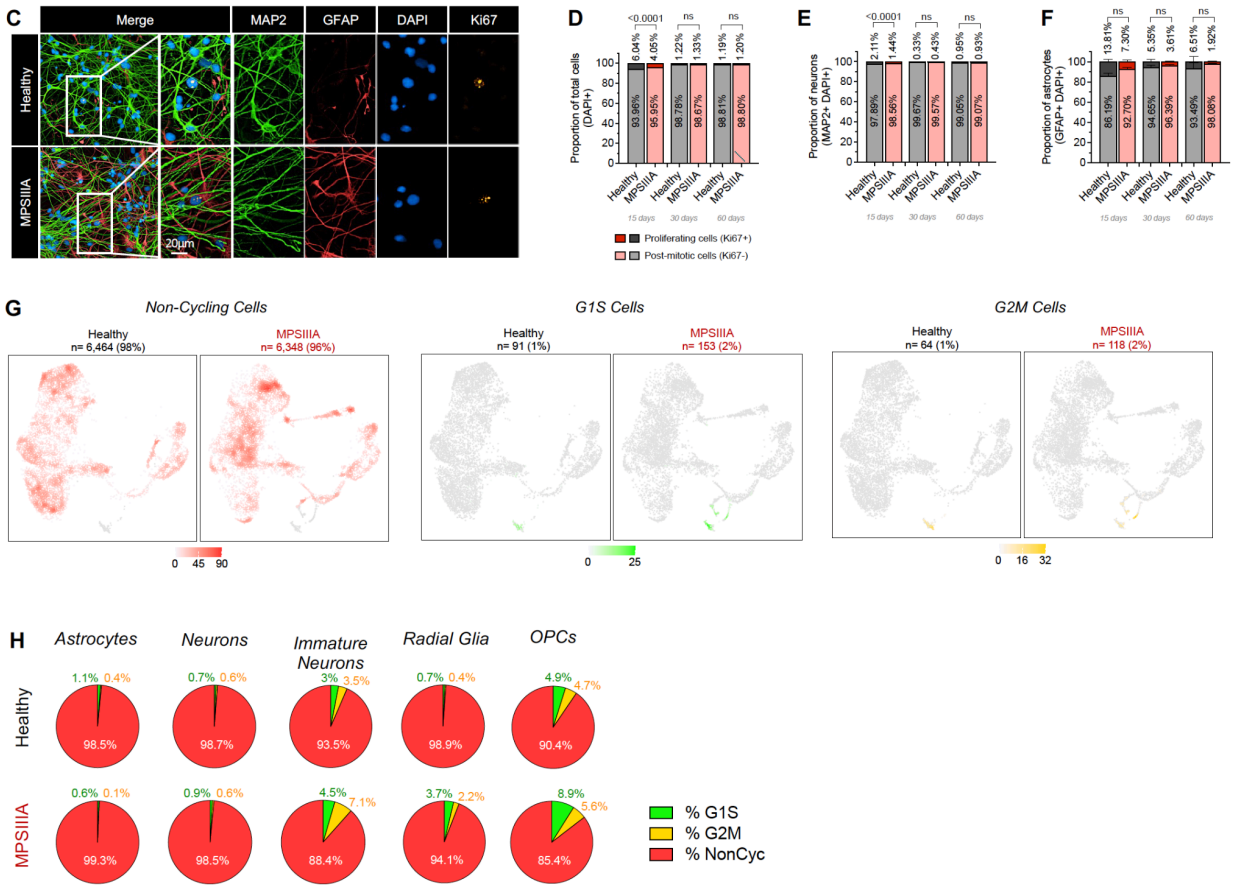
1.1 The authors claim in **Line 114 & Figure 1** that the cultures were formed of post-mitotic neurons at Day 14 – however there is no clear support of this claim in protein immunohistochemistry or other analyses. The single cell RNAseq data found in **Figure 1B** counters this with the presence of radial glia, astrocytes and immature neurons at Day 120 in culture. Overall neurons represented < 35% of the culture and only 15% of the total cells were mature neurons.

We thank the reviewer for raising this important point and giving us the opportunity to tone down our language and add new experiments to better support it. For this we added new imaging and transcriptomic data at various time points (**Lines 129-132**, **Supplementary Figure 1)**. The new data shows that most cells in our cultures are post-mitotic, with <6% proliferating cells by day 15, <2% by day 30, and very few proliferating neurons (<0.4% at day 30) (**Supplementary Fig 1A-B**).

More specifically, first, we conducted immunocytochemistry for Ki67, a proliferation marker, at Days 15, 30 and 60 (**Supplementary Figures 1C-F**). At Day 15, Ki67+ cells represented 6.04% (healthy) and 4.05% (MPSIIIA) of DAPI+ cells. Within this population, Ki67+ neurons (MAP2+) represented 2.11% (healthy) and 1.44% (MPSIIIA), while Ki67+ astrocytes (GFAP+) made up 13.81% (healthy) and 7.30% (MPSIIIA). By Day 30, the proportion of Ki67+ cells had further decreased to 1.22 (healthy) and 1.3% (MPSIIIA) of total DAPI+ cells, with Ki67+ neurons comprising 0.33% (healthy) and 0.43% (MPSIIIA) of the neuronal population. Ki67+ astrocytes were reduced to 5.35% (healthy) and 3.61% (MPSIIIA). These trends continue at 60 days in culture, where minimal Ki67+ cells were detected.

Secondly, we examined the cell cycle phase distribution of our single-nuclei RNA sequencing data at Day 120 **(Supplementary Figure 1G)**. This revealed that 98% of healthy control cells and 96% of the MPSIIIA cells were non-cycling, with only 1-2% of cells in G1S and G2M phases for both genotypes. The small proportion of proliferating cells consists primarily of immature neurons, radial glia and oligodendrocyte precursor cells, not mature neurons (**Supplementary Figure 1H**), reinforcing the post-mitotic nature of the neuronal population. These findings align with our 30 and 60-day transcriptomic analysis for cell cycle states in our companion paper (Greenberg, et al., **Supplementary Figure 4**) showing minimal neural proliferation.

We appreciate the reviewer bringing this to our attention and believe this additional data strengthens the characterisation of these *in vitro* cultures.



1.2 Individual cell lines present a high variability in their final fate in playground cultures assessed by scRNAseq at Day 120. In Figure **S10-A** it is clear in the MPSIIIA culture that many lines are high underpresented e.g. 2 lines appear to make up >60% of the culture, with 1 line making up more than >60% of the total excitatory (MPSIIIA7) or inhibitory (MPSIIIA10) neuron population. These cells will be vastly over-represented in the excitatory or inhibitory analyses performed and will provide the majority input to network level activity. Unlike the 'Village in a dish' model proposed by Neavin et al., (Nat. Comm., 2023, DOI: 10.1038/s41467-023-38704-1) the authors have not demonstrated high reproducibility of the fraction of cells that were used between preparations of the 'Playground cultures'. Likewise, this brings into question the changes in excitatory and interneuron post-synaptic currents as they could be due to shifts in excitatory or interneurons proportions within the cultures.

We appreciate the reviewer's raising this important point as over or underrepresentation of single patients is a legitimate concern when using a village model.

First, in addition to snRNAseq at 60 and 120 day, we have now also performed single-nuclei RNA sequencing at 30 days in culture, which shows an almost equal proportion of all cell lines in playground culture for both the healthy and MPSIIIA cohorts (companion manuscript Greenberg, et al., **Supplementary Figure 3**). This new data demonstrates the quality of the playground preparation. Any disproportionate representation of specific patients in the disease cohort at 120 days reflects disease characteristics rather than a technical experimenter bias, or low quality preparation of the playground culture.

Second, to further address this concern, we performed SNP genotyping analysis to link the identity of each patient and healthy control line in the playground of our snRNAseq data. We then used this new data to correlate the playground snRNAseq results with electrophysiological assays that were performed on single clones rather than playgrounds.

We have identified BIAD03 (formerly MPSIIIA 7), which displays a lower level of excitatory postsynaptic communication compared to other patients (**Supplementary Figure 11**). This patient also shows slower progressing neurodegeneration and astrogliosis phenotypes (companion manuscript Greenberg, et al., **Supplementary Figures 14D-I, 15C-E**), which may cause it to dominate the neuronal population as it is dying slower compared to others. This is supported by our 30 day transcriptomic data which shows roughly equal proportions of patients in our playground model (**Supplementary Figure 13A-B**). However, looking at the synaptic activity from BIAD03 in **Supplementary Figure 11** it has some of the lowest levels of AMPA and GABA-mediated activity and therefore isn't dominating our observed phenotypes. MPSIIIA 10 was identified as BIAD10, and while it does have high inhibitory postsynaptic communication, these levels are comparable to other patient lines such as BIAD01 and BIAD11 **(Supplementary Figure 11**). These results highlight that these two patients, while highly represented in our 120-day single-nuclei data, are not driving the synaptic phenotype. Similarly, at the network level all our patients have similar levels of active neurons, active bursting cells and network event frequency, which is not driven by a specific patient as the majority are consistently higher than their healthy counterparts across development (**Supplementary Figure 12**).

It is also important to note that disease progression among individual lines will influence the proportion of each patient in single-nuclei sequencing. Patients with a more severe neurodegenerative phenotype are more likely to have a lower proportion of neurons to sequence at late time points, such as 120 days in culture. For example, BIAD02 has a severe neurodegenerative phenotype (companion manuscript Greenberg, et al., Supplementary Figures 14D-I), and as such makes up a smaller proportion of our 120-day neuronal population. It is likely that this is caused by neurodegeneration as our 30 day transcriptomic data shows roughly equal proportions of our MPSIIIA patients (**Supplementary Figure 13A**). We acknowledge this is a caveat of playground experiments at late time points and thus have updated our limitations section (**lines 560-570**) to include the following:

- "Some MPS IIIA donors were over-represented in the 120-day playground, unlike the more balanced 30-day cohort (**Supplementary Fig. 13A**; see also companion paper Greenberg et al. **Supplementary Figure 3A**). This likely reflects variable disease severity and progression: over a long period in vitro, patient lines with extreme neurodegeneration and astrogliogenesis yielded fewer neurons and more reactive astrocytes. We verified these correlations with donor-resolved snRNA-seq after genotyping (**Supplementary Fig. 13A**; see also companion paper Greenberg et al. **Supplementary Figures 14 & 15**). We mitigated this patient-bias by characterising each line individually to confirm that the phenotypes and drug rescue were reproducible in all patients and not solely driven by a single patient in our playground neural model (e.g., **Supplementary Fig. 17**). Although it is expected that some patients will respond differently to a treatment, our data indicate that APAT may have broad benefit on most MPS IIIA patients."

We also characterised the single-cell and network activity of each individual patient line prior to the introduction of our playground model. Throughout our playground model experiments we closely compared this to our individual line data to ensure the model followed the same trends. This gives us confidence that the phenotypes we identify are true as they appear in different models.

We acknowledge that this is always going to be a compromise when performing late-stage experiments

in a disease-based village model. However, we appreciate the reviewers raising this important point and hope these explanations clarify their concerns.

1.3 With this inherent variability and the majority of cells at 120 days being astrocytic, it is suggested to provide individual panels of the cell types in **Figure 1B** within the panel that identify whether the clustering has been successful, and split by genotype to demonstrate that similar populations are present in healthy control and MPSIIIA patients.

We thank the reviewer for this insightful suggestion regarding the cell type clustering. We have updated **Figure 1B** to include separate UMAP panels for healthy and MPSIIIA samples, demonstrating comparable cell type proportions between genotypes. Additionally, to clarify individual patient contributions, we have included a patient-specific breakdown in **Supplementary Figure 1B.** When we initially performed the cell type identification scoring, we only used the Nowakowski, et al., 2017 list of genes for various cell types. To further validate our cell type assignment, we have since incorporated additional datasets into our scoring system (Cell 2022, DOI: 10.1016/j.cell.2022.09.039; Nature 2017, DOI: https://doi.org/10.1038/nature21029; and a dataset provided by scMayo in R/RStudio) and assigned cell types based on the most matches across the datasets. This enhanced approach minimally affected the original cell type assignments and proportions, increasing our confidence in our results. We appreciate your feedback and believe these updates strengthen our results.

1.4 The manuscript shows limited biological replicates. As shown in **figure S2** the authors only independently differentiate N=2, or in many cases N=1, biological replicates of neural progenitor cells. There is also concern regarding the N=1 synaptic staining despite the technical replicates as initial plating density or other variabilities in cell culture could have influenced the distribution of these markers. Likewise, regarding concern 2 there is a high inherent variability in the cultures, and this may be more evident with multiple preparations from each line.

We appreciate the reviewer's concern regarding the low number of biological replicates in some experiments and the potential variability in cell culture. We understand that balancing the number of biological replicates with the depth of analysis is always a necessary compromise. We have performed further synaptic staining experiments to address this, and also clarified how we report biological replicates for each assay throughout our manuscript.

We agree the synaptic staining experiments needed to be repeated in another biological replicate. We have completed additional synaptic imaging experiments at Days 15, 30, 60 and 90 in an independent neuronal differentiation of the playground model (increasing our number of biological replicates to n=3). This new data confirms the original phenotypic trends and has been incorporated into **Figure 4A-C** and **Supplementary Figure 4**, strengthening the reproducibility of our findings.

We have improved transparency by detailing the biological and technical replicates for all experiments in the manuscript in **Supplementary Figures 2-5,7,8,10 and 12**. For most assays, we used two clones per genotype to account for variability, except in labour-intensive patch-clamping experiments, where one clone was used. This is outlined in **Lines 110-116** of the manuscript.

For patch-clamping experiments, we analysed 1,169 neurons from 12 cell lines (5 healthy control iPSCs, 5 MPSIIA iPSCs, 1 ESC Wt, 1 ESC SGSH knockout, plus a playground model with 2 clones per donor [excluding BIAD05]) at 30-60 and 90-120 day time points (**Supplementary Figure 2**). This surpasses typical studies in the field, which often rely on fewer replicates (two biological replicates from a single iPSC or embryonic WT/KO line, with a smaller neuron sample size) (Neuron 2024, DOI:

10.1016/j.neuron.2024.09.020; Science 2019, DOI:10.1126/science.aav5386; Science 2016; DOI: 10.1126/science.aaf2669). To further enhance clarity, we have updated **Supplementary Figures 2-4** with additional information including neuronal differentiation dates, the number of coverslips patched per cell line, and the average neurons patched per coverslip. We hope this additional information provides greater transparency regarding the experimental timeline and reproducibility of our data.

We appreciate the reviewer's concerns regarding biological replicates in our multielectrode array (MEA) experiments. Our MEA experiments included 19 iPSC-derived cell lines (biological replicates; 9 healthy control and 10 MPSIIIA-derived lines) plus 4 independent playground models (2 healthy control and 2 MPSIIIA) and 8-72 wells per condition (technical replicates), with data collection spanning over a three-year period. While MEA technical and biological replicate numbers are often not explicitly reported in published studies, making comparisons challenging, we believe this extensive dataset exceeds the standard in the field. For example, a recent study in *Nature Communications* reported 5 cell lines, with 2 wells per condition per independent experiment across 3 independent experiments (Vahsen et al. 2024, DOI: 0.1038/s41467-023-41603-0), and a *Neuron* study reported 2 cell lines, with 4-8 wells per condition per independent experiment, across 3 independent experiments (López-Erauskin et al. 2018, DOI: 10.1016/j.neuron.2018.09.044).

We also include multiple complementary assays, which further confirms the reliability of our findings. For example, our MEA data aligns closely with our patch-clamping results, validating the reproducibility of our functional phenotypes. And noting that the experimenters were blinded for all our data collection and analyses, which is particularly important for patch-clamping.

We believe our comprehensive study design, with an extensive number of cell lines, clones independent differentiations, robust data collection and new synaptic staining experiments, provides strong evidence for our conclusions while exceeding the standards in the field.

1.5 In **Line 919** the authors note that they discard inactive wells prior to full analysis, the reviewers suggest that the total number of inactive wells should be described in the supplementary data to identify whether there is a particular phenotype based on the total number of active/inactive wells and to demonstrate the actual number of electrodes used per condition.

Thank you for this suggestion, we have clarified the description of well exclusion criteria for the Network Event Frequency metric analyses in our manuscript and expanded on our reporting of excluded wells and electrodes to ensure greater transparency.

Previously, **Line 919** stated that "wells with a network event frequency of 0Hz were excluded from network analyses". To clarify, this exclusion applies only to the Network event frequency (Hz) metric (**Figures 5F & 7F**) as the absence of detectable network activity in these wells artificially lowered the average, masking true differences in network frequency between groups. All wells were included in the other network, firing and bursting analyses, including Firing rate per active neuron (Hz), Number of active neurons per well, Number of active bursting cells, and Burst frequency (Hz) and Spike-sorted synchrony index (**Figures 5B-E, G & 7F**).

We apologise for the confusion and have updated **Lines 1002-1006** (previously Line 919) in the manuscript to read:

> "All wells were included in firing (Firing rate per active neuron (Hz) and Number of active neurons per well), bursting (Burst frequency (Hz) and Number of active bursting cells) and network

(Spike-sorted synchrony index) metric analyses. Wells with a network event frequency of 0Hz were excluded from the Network event frequency (Hz) metric only (**Supplementary Fig. 21**)."

In response to the reviewer's request, we have added **Supplementary Figure 21** which details the percentage of discarded wells and electrodes for each individual cell line (**Figure 5F**) and playground (**Figure 7F**) for the Network Frequency (Hz) metric (**Supplementary Figure 21A**). Given variability across recordings, we provided averaged data for the 30-50, 55-70 and 80-94 day timepoints.

To determine whether well exclusion may have introduced bias, we also generated a histogram comparing the number of excluded wells between Healthy and MPSIIIA groups at each timepoint **(Supplementary Figure 21B)**. This revealed no significant differences between conditions, supporting that the well exclusion does not alter the observed Network Event Frequency phenotype.

We also note that in **Figure 5**, each data point represents the average of all wells per condition, such that each condition contributes equally regardless of the number of excluded or active wells. This ensures that the exclusion of 0Hz wells does not skew the representation of individual lines or artificially bias our Network Event Frequency phenotype. In **Figure 7**, however, all wells are shown individually to maintain consistency with the drug screening data presentation, which also displays data at the individual well level.

We thank the reviewer for this suggestion and hope these changes and clarification address previous concerns.

1.6 Throughout, there is an odd statistical comparison, given that many of the figures depend on data generated from multiple lines, an ANOVA should be performed instead of a presumed t-test.

We thank the reviewer for their thoughtful comment regarding the statistical approach used in our analyses. We acknowledge the oversight in our original use of Mann-Whitney U tests in instances where a group-level comparison involving multiple cell lines would have warranted a more appropriate statistical model.

In response, we have carefully reviewed our statistical analyses throughout the manuscript. Where relevant, we have updated the tests to a Kruskal-Wallis one-way ANOVA with Dunn's correction for multiple comparisons. For experiments involving repeated measures across timepoints, we have applied a mixed-effects model (REML), with cell line and time as fixed effects and condition as a random effect, followed by Šídák's multiple comparisons test. These changes ensure that our analyses more accurately reflect the structure of the data and reduce the risk of misleading conclusions.

We apologise for the initial oversight and are grateful to the reviewer for drawing attention to this issue. Our results and conclusions remained consistent with the change in statistical testing, but we believe these revisions improve the rigour and clarity of our findings.

1.7 The reviewers primary concern with the interpretation of the data presented is that there is a high possibility that by selectively patching the 'healthiest' cells in the MPSIIIA condition (e.g. selected for good morphology, good neuronal properties, etc.) then the authors could be artificially selecting for newer-born neurons wherein the disorder has not had time to significantly progress. There are evident progenitors identified by the scRNAseq and a high proportion of immature neurons in the total neuron population. There is no evidence presented of accumulation of heparan sulfate to support that these

neurons are under progressive load. Therefore, by not attempting to include any sick or dying cells, or at least address the proportion of these cells between culture conditions, there could be a bias for healthy cell types that does not reflect conditions when examining the whole network function, as shown by the altered MEA function. In particular, the type of tonic firing observed during the MEA recordings is a newborn neuron phenotype that gives rise to synchronised burst firing in more mature neurons, as shown in Kirwan et al. (Development, 2015, DOI: 10.1242/dev.123851). This could imply that the continuous renewal of the MPSIIIA cultures leads to a continuously reset network development, hence the more immature or hypoxia-like phenotype. For example, the capacitance is lower in early timepoints, then recovers to a higher point, is this due to the presence of newly-formed cells that are being patched. There are also more active neurons in MPSIIIA despite reported neuronal death in the accompanying manuscript.

We appreciate the reviewer's concerns regarding potential bias in our selection of the best-looking cells for patch-clamping and its implications on MPSIIIA phenotypes. To reduce experimenter bias in the patch-clamping, those involved were blinded during the data acquisition and analysis process until completion. Therefore, to maintain consistency between conditions across years of data collection, we selected cells with complex neuronal morphology, as highlighted in **Figure 2** and **Supplementary Figures 5 & 6**, that were surrounded by other neurons. We saw no significant differences in the morphology of our patched neurons, highlighting that our synaptic phenotype isn't driven by alterations in neurite growth and development. However, we acknowledge that this approach introduces bias toward "healthier" cells and we have updated the limitations section (**Lines 572-576**) of the manuscript to reflect this:

> "Finally, we acknowledge potential selection bias: whole-cell patch-clamp recordings may favour healthier-appearing, morphologically complex neurons, enriching for multipolar/pyramidal and under-representing bipolar/fusiform types (Fig. 2A). To mitigate this, experimenters were blinded to genotype and treatment throughout acquisition and analysis to minimise the chance that selection criteria confounded disease phenotypes."

Regarding progressive load in MPSIIIA cultures, our companion manuscript (Greenberg et al.) demonstrates increased heparan sulfate release in the media by Day 30. To further address the reviewer concern, we have also quantified intracellular heparan sulfate accumulation at days 30, 60 and 90 **(Greenberg et al., Figure 3A-C).** This confirms the progressive accumulation of heparan sulfate in our MPSIIIA cultures and cells, indicating that they are under progressive load during whole-cell patch-clamping. However, there is also no way for us to identify specific cells under progressive load during neuron selection for patch-clamping.

We appreciate the reviewer's concerns about newborn neurons potentially skewing results. Although, we do not believe this is the case. A bias towards patching less mature neurons in MPSIIIA will result in less complex morphology (e.g., Bardy et al 2016). We found no difference in morphological complexity of patched clamped neurons in the MPSIIA vs Healthy cohort (**Figure 2**). Similarly, if we had been biased to patched less mature neurons in either cohort, we would have seen differences in their action potential firing properties with patch clamping, which clearly was not different (**Figure 3**). Furthermore, in an attempt to address part of this concern, we confirmed minimal proliferation through Ki67 staining at 15, 30, and 60 days in culture, and cell cycle analysis of single-nuclei RNA sequencing data at Days 30, 60 and 120 (30 and 60 day data can be found in **Supplementary Figure 4C-F, Greenberg et al.;** 120 day data can be found in **Supplementary Figure 1**). These assays show minimal proliferation in our cultures, and that the proliferative cells are primarily astrocytes, not neurons, suggesting limited neuronal renewal in MPSIIIA cultures.

Further, the decreased capacitance in the early timepoint that appears to recover over the later timepoint may be reflective of MPSIIIA neurons compensating for neurodegeneration occurring, where they increase membrane area through other mechanisms such as neurite outgrowth that support synaptic function. As our companion paper reports neurodegeneration in MPSIIIA as early as 30 days in culture (**Greenberg et al., Figure 4A-C & Supplementary Figure 14**), the recovery in capacitance is unlikely to be caused by newborn neurons being patch clamped. Therefore, our MEA and patch-clamping phenotypes are likely to reflect disease-specific changes that are influenced by both neurodegeneration and astrogliosis, leading to excitotoxicity and hyperactivity that further exacerbates disease progression.

We thank you for raising this discussion surrounding our results. We hope that our explanation and additional data showing that our cultures undergo progressive heparan sulfate accumulation have helped to address the reviewer's concerns.

1.8 Instead of barplots, boxplots or violin plots should be used when examining data with very high numbers of individual dataplots. Datapoints should also always be shown if they will be used e.g. in **Figure 4** many barplots (**A-D**) show individual datapoints but then the authors switch to reporting only the N number. It would be better in this scenario to show all the datapoints so the reader can assess if there are many outliers.
We thank the reviewer for their attention to detail. Ensuring transparency with our data is crucial to us and something we strive to do. To address these concerns, for datasets with a large n number, such as the majority of our patch-clamping histograms, we have included box plot formats of our main figure data into **Supplementary Figures 5C-D, 7D,F and 8B-C**. This enables readers to view the distribution of our data and ensures we are being transparent with data variability. However, for data with much smaller n numbers, such as our imaging, multielectrode array data and a few patch-clamping graphs (**Figure 4F, J and N-R**), we have presented these as histograms with data points as the variability is easier to visualise with a smaller sample size. We hope that these changes address the reviewer's concerns and further increase our transparency with our data.

Minor Concerns:

- The term 'isogenic' is used inappropriately to describe the cultures at **Line 110**, this does not fit with the established use of isogenic in iPSC culture to describe lines which have the same genotype except for a single or multiple disease-causative genes that have been introduced or corrected.
  We appreciate the reviewer's attention to the terminology used in our manuscript. Our use of the term 'isogenic' was intended to reflect that the neural cultures derived from each individual (patient or healthy control) originate from genetically identical neural progenitor clones.

  In our study, the two neuronal cell lines per individual are derived from two independent neural progenitor clones that originate from the same iPSC line. This ensures that the two cell lines are genetically identical but also biologically independent. This approach allows us to capture potential variability in differentiation while still maintaining a controlled genetic background.

  To avoid confusion, we have clarified our terminology in **Lines 110-111** of the revised text to state that these are genetically identical neural cultures derived from distinct neural progenitor clones rather than referring to them as 'isogenic'. We hope this addresses the reviewer's concern.

- **Figure S4** and the Cell stress mentioned in the methods but not referred to in the main text other than to state a number of cells imaged.

Thank you for bringing this to our attention, we apologise for the confusion. To ensure clarity and consistency, we have updated the wording in the **Results (Lines 244-259)**, **Discussion (Lines 465-483)** and **Methods (Lines 894-899)** to refer to 'cellular stress' rather than just 'stress' or 'cell stress'. This aligns more with the terminology used in the Discussion and ensures the reader can follow the context more easily.

- In **figure S10** percentages should be provided for each of the conditions in the pie-charts to determine actual change.
  Thank you for this suggestion. We have now updated this supplementary figure (now **Supplementary Figure 13**) to include the percentages of each donor in our excitatory and inhibitory neuron populations.

- There is reference to '**STAR methods**' if this is the case for this manuscript then a workflow of the pipelines for the image analysis should be included.
  We thank the reviewer for bringing this to our attention and apologise for the oversight. The reference to 'STAR methods' was included in error, as this framework is not required for our manuscript. To address this, we have renamed the section to 'Methods'.

- Clumping covering 104263um2 is quite a large clumping area, there is no number for the clumping events, it would be ideal if the authors could indicate discarded data amounts in the total number of imaged regions.
  We acknowledge that the description of the clumping area could have been clearer, and appreciate the opportunity to clarify our quality control approach.

  For each condition, we have individual well replicates. Each well is divided into fields of view (FOVs) which represent 1/25th of a well. Each FOV within the well is assessed for quality control before being included in further analyses.

  The clumping area of 104,000μm² corresponds to 25% of a field of view (FOV) within a well, which is a small region when considered in the context of the total well area (~104,263,000μm²). This area was used as an exclusion criterion for FOVs with clumps, as neurites, puncta and other morphological features cannot be accurately detected in this region.

  New supplementary figures and tables (**Supplementary Figures 18-19, Supplementary Information Tables 2-3**) have been created to indicate the total number of fields imaged, the number of fields excluded and the percentage of fields that were excluded per well, condition and time point. To ensure quality control outcomes were not influencing our phenotype, we also compared the percentages of discarded image regions across conditions in another new supplementary figure (**Supplementary Figure 20**). This demonstrated that the conditions and/or treatments did not affect the amounts of discarded data.

  We have also amended the Methods (**Lines 959-966**) to provide more information about how FOVs were excluded and improve clarity regarding the distinction between the FOV area and the entire well area.

  Lines 959-966: Cellular detachment and clumping can alter the accuracy of neuronal morphology analysis pipelines. Each imaged well is composed of 25 fields of view (FOV). Each FOV underwent a quality control assessment before being included in further dendritic and synaptic analyses. FOVs were filtered to exclude fields with a total clumping area of

≥104000μm^2 (25% of the total FOV), ≥4 clumping events (clumping with an area of more than 5000μm^2) or a neuronal density (MAP2+DAPI+ cells) of less than 1 (Supplementary Fig. 18-20, Supplementary Information Tables 2-3).

- Significant differences in the capacitance at 30 days (**Figure 2D**) are not mentioned in the text, whereas non-significant trends are mentioned in the text at **Line 253 (Figure 4N/P)**.
  Thank you for bringing this to our attention, we appreciate the reviewer's attention to detail and have revised the manuscript to ensure clarity and consistency.

  In **Line 149** (now **Line 157-161**), we originally stated, "While MPS IIIA neurons showed a lower capacitance at 30–60-days". To better reflect the significant differences observed in **Figure 2D**, we have updated this to: "While MPSIIIA neurons had a significantly lower capacitance at 30–60-days", emphasising the significance of our capacitance findings in early neurodevelopment.

  Regarding the mention of non-significant trends in **Figures 4N & P**, we consistently report both significant and non-significant results throughout the manuscript to provide a comprehensive overview of our data. We believe this approach ensures transparency in our findings and hope these revisions address the reviewer's concerns.

- Examples of synaptic colocalization should be shown as part of **Figure 4**, as the current figures it is difficult to clearly identify due to the scale. A XY-XZ 3D colocalization plot would also greatly benefit the manuscript.
  Thank you for this suggestion. We acknowledge that the scale of our current representative images makes it difficult to clearly visualise co-localised synaptic puncta in **Figures 4** and **7**. Our high-throughput analysis software quantifies synaptic puncta in 2D space, determining synaptic pairs based on a 0μm distance threshold, prioritising the less abundant synaptic puncta type to ensure accuracy and avoid overcounting. While we recognise the potential for miscounting in 2D space, our approach uses a high number of data points across multiple fields of view and wells to average variability, minimizing any impact on our results. Due to logistical limitations of our current software, generating an XY-XZ 3D colocalization plot is not feasible at this stage, and plotting XY coordinates alone would not accurately reflect the number of colocalized synaptic puncta.

  To address this concern, we have added **Supplementary Figure 9,** featuring magnified regions of the representative images from **Figures 4** and **7**, with and without the MAP2 neuronal marker to enhance the puncta resolution. Additionally, we have added white arrows in **Supplementary Figures 9A & C** to highlight examples of co-localised synapses and improve visualization.

  We hope that this addition allows for clearer visualization of colocalized puncta, helps clarify our findings and addresses the reviewer's concern.

- Inhibitory staining was only performed in one timepoint as shown in **figure 7**, and at this point there are high variations in patient-neuron fate ratios. As such the changes seen could be dependent on an individual patient.
  We thank the reviewer for raising this important point. We have conducted additional inhibitory synapse immunostaining at Days 15, 30, 60 and 90 in the playground model, now included in **Figure 4B** and **Supplementary Figure 4.** These new experiments further confirmed that both healthy and disease cultures showed a gradual increase in inhibitory synapse density over 90

days of maturation, and that the inhibitory synaptic density increased faster in healthy cultures, resulting in significantly fewer inhibitory synapses at 90 days in MPSIIIA. These findings align with and extend our original conclusion of a progressive excitation/inhibition imbalance in favour of overexcitability in MPS IIIA.

We acknowledge the reviewer's concern that variability in patient-neuron fate ratios, or reliance on an individual patient, could influence these results. While we have not fully characterised patient-specific neuron fate ratios in individual lines, our patch-clamping data shows consistent GABA-related trends across individual patient lines (**Supplementary Figure 11**), suggesting that inhibitory synapse variability is unlikely to be driven by a single patient.

- In **figure 4** it is mentioned that TTX shows disproportionate hyperactivity in the MPSIIIA cultures but then the figure shows zero significance.
  Thank you. We apologise for the confusion surrounding our TTX results. We stated that TTX shows disproportionate hyperactivity because our cumulative distribution plots show a significant shift in each curve (**Figures 4N, 4P and 4R**), indicating altered excitation/inhibition balance. However, we acknowledge that this may be misleading for readers as the grouped histograms alongside our cumulative distribution plots shows the same trend but without significance.

  To make this clearer, we have updated **lines 278-290** of the manuscript to the following:
  - "When we repeated these experiments in the presence of tetrodotoxin (TTX), a voltage-gated sodium channel antagonist that blocks action potentials, we observed similar shifts in synaptic excitation and inhibition (**Fig 4N-R**). Specifically, the frequency of miniature AMPA-mediated events showed an increase in MPS IIIA (**Fig 4N**), although it only became significant when analysed as a cumulative distribution. In contrast, the frequency of miniature GABA-mediated events showed a trend towards a decrease in MPS IIIA (**Fig 4P**). We found no significant changes to the amplitude of miniature AMPA and GABA events, indicating that the machinery responsible for stochastic postsynaptic release of single neurotransmitter vesicles remains functional in MPS IIIA (**Fig 4O, Q**). The opposing polarity of changes in AMPA- vs. GABA-mediated miniature synaptic event frequencies in MPS IIIA, without alterations in their amplitudes, caused a shift in E/I balance that was statistically significant when assessed via cumulative distribution (**Fig. 4R**). Overall, whether or not TTX was present, these results indicate altered synaptic homeostasis and hyperactive excitatory synaptic transmission in MPS IIIA neurons **(Fig. 4)**."

  We hope this updated wording addresses the reviewer's concern.

- Authors should comment on the role of glial support and glial neurotransmitter uptake.
  We acknowledge that this is a very important point and thank the reviewers for raising it given the critical role that astrocytes play in synapse development and maintenance.
  We have now mentioned the potential role of astrocytes in our discussion from **Lines 477-483**. The changes made can be seen below:
  - "Astrocytes, which regulate glutamate uptake and synaptic milieu[102], were over-reactive in our cultures, and APAT treatment reduced GFAP reactivity while also improving neuronal integrity (MAP2). These observations suggest that synaptic hyperexcitability and astrocytic dysregulation may act in concert to promote neurodegeneration (see also companion paper Greenberg et al.). Causality remains to be established, but the

alignment between synaptic hyperactivity, astrogliogenesis, and neuronal loss argues for a model in which early synaptic dysfunction amplifies vulnerability[103].”

- The decrease in PSD95 may be just a consequence and not a disease mechanism since at day 30 neurodegeneration is already reported.
  We want to thank the reviewer for raising this point about the role of PSD-95 in our MPSIIIA model and its potential as a consequence rather than a disease mechanism.

  We would like to clarify that our data demonstrates an increase in PSD-95 puncta in MPSIIIA playground cultures compared to healthy controls as early as Day 30 (**Lines 231-235, Figure 4A-B**). We propose that this increase may contribute to an excitatory/inhibitory (E/I) synapse imbalance and neuronal hyperexcitability, which could precede or potentially exacerbate network dysfunction and neuronal death in MPSIIIA. Given that heparan sulfate accumulation, a hallmark of MPSIIIA, is observed in our cultures from Day 30 onwards **(Greenberg et al., Figure 3A-C),** this may drive the early synaptic changes, such as the PSD-95 increase. However, we acknowledge that we cannot definitively determine whether this increase is a cause or consequence of the disease pathology, and we do not claim PSD-95 changes as the primary disease mechanism but rather one of several factors in a complex disease process.

  We also acknowledge that the reviewer noted a decrease in PSD-95 and would like to apologise for the confusion. A decrease in PSD-95 was mentioned in our discussion (now **Line 574**) when referencing prior studies of post-mortem MPSIIIA and MPSIIIC brain tissue. As these models represent late or end-stage disease, it is possible that the reduction in PSD-95 is a late consequence of severe neurodegeneration. We hope these explanations address the concern.

Reviewer #2 (previously 6)

This manuscript, focuses on studying the excitation/inhibition imbalances in patient-derived induced pluripotent stem cells (iPSC) from children Mucopolysaccharidosis Type IIIA (MPS IIIA). Authors describe MPS IIIA phenotypes based on hyperactive excitatory synapses and dysregulated gene expression linked to synaptic homeostasis. A drug screen with whole-cell patch-clamping, multi-electrode arrays and high- content confocal imaging identified a cocktail of repurposed drugs effective in restoring synaptic and neural network activity to healthy levels. Similarly to the accompanying Greenberg et al., manuscript, the use of neuronal 'playgrounds' composed of 5 patient/healthy subject lines with 2 clones per line contributes to a high rigor of this study. The diversity in experiment design on neuronal synaptic integrity and neuronal activity are of high value, with multiple detailed aspects of these phenotypes being evaluated in parallel. Little is known regarding the molecular mechanisms of the MPS IIIA, and no drugs are currently available for these patients. This study is impactful and paves a path towards further in vivo and pre-clinical screen to identify FDA-approved therapeutics. That being said, I do have some concerns to bring to the authors' attention below:

We would like to thank the reviewer for their thoughtful and encouraging assessment of our manuscript. We are grateful for the recognition of our study's rigor, the value of the experimental diversity, and the potential impact of our findings. We appreciate the reviewer's careful consideration of our work and their constructive concerns. We have addressed all of these points in detail below and have made the necessary revisions to strengthen the manuscript. We hope our responses and clarifications meet the reviewer's expectations.

Major Concerns:

2.1 Authors describe to generate cortical neuronal cultures, dominating with MAP2 multipolar/pyramidal neurons. It is therefore puzzling that they detect GABAergic synaptic Gephryn+ staining in these cells. It would be of high benefit to include a better characterization of these neuronal-astrocytic cultures, with specifications on the inhibitory/excitatory inputs. In addition to a detailed characterization of their cell culture system, it is suggested to move results from **Fig6A** to **Figure 1**.

We thank the reviewer for bringing this point to our attention. We apologize if there was a misunderstanding in our communication of these results. We have attempted to clarify this point in the revised version as follows:

We did not mean to claim that our cortical culture is dominated by pyramidal/multipolar neurons, but simply that our patchclamping cohort is. Our approach of selecting the "best-looking" cells for patch-clamping is used to maintain consistency while being blinded to genotypes. It is standard in the field that Patch-clampers tend to select more of a specific morphological type. However, we appreciate that it may introduce a level of experimenter bias, which in our case may cause a higher proportion of multipolar/pyramidal types in the patch-clamp neuron cohort (**Figure 2A**), which may not be reflective of the entire culture. Due to the laborious nature of patch-clamping, we can only select a few cells on a coverslip out of thousands. This may leave many simpler or other morphologies, such as those that might be associated with interneuron subtypes, unselected. This can then skew our morphological types to contain more multipolar/pyramidal neuronal types, which makes it appear as though our cultures do not contain interneurons. We are confident that our cultures contain excitatory neurons and inhibitory interneurons based on functional and molecular characterisations.

To clarify possible misunderstanding, the functional GABAergic synaptic inputs mentioned are detected postsynaptically by surrounding inhibitory neurons releasing GABA that binds to its

receptors on our selected neuron during patch-clamping. It is important to note that both excitatory and inhibitory neurons express GABA receptors and receive inhibitory synaptic inputs. In this study, we performed immunostaining for Gephyrin, a general marker of inhibitory synapses, on our entire neural culture which contains multiple neuronal subtypes (glutamatergic, GABAergic, etc.). The overlap of Gephyrin puncta with most MAP2+ cells further suggest that inhibitory synapses are not restricted to a particular neuronal identity.

We also wish to clarify that we only classify cells as excitatory or inhibitory when performing our single-nuclei RNA sequencing analysis (**Figure 6**), as we are unable to confirm neuronal identity during whole-cell patch clamping. To address the reviewer's concerns, we have now split our neuronal population in **Figure 1B** into excitatory and inhibitory neurons to improve the characterisation of our cultures. The intent of **Figure 1** is to provide a general overview of the cell types in our culture, with a more extensive characterisation of these cultures throughout the remainder of the manuscript. Our companion paper (**Greenberg, et al.**) also provides extensive characterisation of the astrocytic, lysosomal and neurodegenerative aspects of these cultures. In line with this, we wish to keep Figure 6A in its current position as the remainder of the data further characterises these specialised neuronal populations.

However, to clarify this point in the revised manuscript, we have updated the limitations section of our discussion (**lines 605-609**) to include the following:

> "Finally, we acknowledge potential selection bias: whole-cell patch-clamp recordings may favour healthier-appearing, morphologically complex neurons, enriching for multipolar/pyramidal and under-representing bipolar/fusiform types (Fig. 2A). To mitigate this, experimenters were blinded to genotype and treatment throughout acquisition and analysis to minimise the chance that selection criteria confounded disease phenotypes."

Again, we thank the reviewers for raising an important point for discussion, and we hope that this resolves their concerns.

2.2 Why are the treatment timelines different in the high content image-based drug screening assay (treatments at DIV40-55), the neuronal activity assays (treatments start at DIV65-DIV80), and the patch clamp assays (treatments at DIV85-99)? Since the drugs do rescue synaptic changes already at DIV40-55, one would expect to observe neuronal activity changes from those early treatments. Authors should elaborate why they performed three different experimental set up for these linked and complementary experiments.

We thank the reviewers for raising this point and apologise for the lack of clarification surrounding our treatment timelines and apparent discrepancies in the earliest version.

The explanation between these discrepancies is mostly practical. First the imaging assays tend to be easier and more consistent (cleaner) at earlier timepoints. In contrast functional assays tend to be more reliable at later time points, when neuronal cultures have functionally matured. In addition, we tried to design some of our experiments sequentially from less labour intensive to most labour intensive to be able to narrow down the number of treatments tested in subsequent assays. By doing this, we only progressed the top performing candidates from the imaging assays to our MEA and then patch-clamping assays. This strategy helped us focus our lower throughput experiments on treatments that were most likely to be successful.

Finally, an important aspect of this study was to investigate in a preclinical model whether late-stage treatment, i.e. 65-99 days in culture, may provide therapeutic benefit. Although we acknowledge the

challenge of extrapolating timeline *in vitro* to a clinical setting, it may still provide some relevant pre-clinical insights, for a disease where delayed diagnosis and access to treatments are common, highlighting a critical unmet need to find therapeutics that are effective in late-stage disease (functionally mature neurons). While we also appreciate the importance of early intervention, understanding the therapeutic potential of later-stage neurodevelopment is equally as crucial for predicting the clinical translation of these drug candidates.

Again, we want to thank the reviewers for bringing this to our attention and apologise that this may have been poorly articulated in the original manuscript. We have since updated our methods (**Lines 944-949**) to read as follows:
"Drug treatment timelines were staggered to capture MPS IIIA-relevant phenotypes and meet assay-specific maturation requirements. High-content imaging was performed at days 40-55, MEA at days 65-80, and whole-cell patch from 85 days onwards, ensuring sufficient maturation for reliable assessment of neuronal synaptic function. These timepoints coincide with the emergence of structural and functional phenotypes in our cultures, enabling assessment of both early- and later-stage treatment effects."

2.3 Similarly to the accompanying Greenberg et al., manuscript, one concert that needs to be discussed is the heterogenicity of the 'playground' samples, with fact that MPSIIIA 10 and 7 being the most prominent cell lines in the MPSIIA 'playground', with MPSIIIA 8 lines to compose the least of 'playground'. This is a common issue with such village-based approaches. As different lines show variable response to neuronal and astrocytic pathologies (**Suppl. Fig 8**), there may be a variance in cellular responses to the tested drug, highlighting 'responders' and nonresponders'. Authors should deeply analyze their scRNAseq data, to

- 1. Determine if the drug effects are not driven by the dominant patient lines, and
- 2. Determine which lines may be more 'astrogliotic' that could potentially skew the neuronal activity analysis

Heterogeneity within village-based models is a valid concern and we thank the reviewer for raising this point and how it may affect subsequent analyses. To further address this concern, we have performed further experiments and analysis.

1. We have now genotyped our RNA-sequencing data to determine how our patient proportions align with phenotypes identified in individual cell lines in other assays. Across experiments, we observe that the phenotypes reported in the playground model recapitulate the trends seen in individual patient lines. However, the severity of these phenotypes varies between patients, reflecting differences in disease progression.

   For example, BIAD10 (originally MPSIIIA 10), which is over-represented in our single-nuclei dataset, exhibits a severe astrogliosis phenotype as identified in our companion paper. Contrastingly, BIAD03 (originally MPSIIIA 7) exhibits a slower-progressing phenotype, evident in the low number of astrocytes and higher number of neurons in our single-cell data (**Supplementary Figure 1B**), again consistent with the imaging data in our companion manuscript. Notably, BIAD02 (originally MPSIIIIA 8) shows less severe astrogliosis but greater lysosomal dysfunction and a similar rate of neurodegeneration to BIAD10. These differences in phenotype severity contribute to variation in the rate of neuronal loss and, consequently, to unequal patient proportions at 120 days in culture. This is further supported by data in our companion manuscript showing that patient proportions are similar at 30 days in culture (Greenberg et al., Supplementary Figure 33), excluding the possibility of a technical bias in the preparation of the playground. Instead the changes over time appear correlated with differential

disease progression. As a result, our patient lines have varying levels of activity at both the single-cell and network level, aligning with varying levels of disease progression which reflect the clinical heterogeneity of MPSIIIA.

2. To address the reviewer's concerns about responders and non-responders, we treated cultures with four drugs (Theophylline; Acetyl-D-Leucine and Allopurinol + Probenecid) from 15-30 days in culture and performed single-nuclei RNA sequencing transcriptomic analysis at 30 days (**Supplementary Figure 17D**). This data showed that while patient responses vary, each patient responds to treatment as evidenced by the rescue in proportion of neurons and astrocytes. Additional immunofluorescence staining for each individual line with an astrocyte marker (GFAP) also shows a significant rescue after treatment with APAT (Allopurinol + Probenecid + Acetyl-D-Leucine + Theophylline) from 15-30 days in cultures **(Supplementary Figure 17C)**.

Taken together, our new single-cell genotyping, phenotypic analysis, and patient-specific drug response data suggests that the observed phenotypes and drug responses are not solely driven by one or two dominant patient lines.

2.4 It is currently standard in the field to work with isogenic gene-corrected controls, especially relatively easily generated for a point mutations. The authors might want to validate their key findings and their drug selection process in an isogenic gene-edited pair as a 'gold standard', which would be much cleaner system accounting for other genetic variance in the specific patient line, further elevating the 'playground' approach.

We appreciate the reviewer's suggestion and acknowledge that isogenic gene-corrected controls can be very valuable. We have included a CRISPR edited *SGSH* knockout embryonic stem cell (ESC)-derived neuronal line in our analysis which shows the same trends we see in our patient lines and playground model. We have included a breakdown of this data in **Supplementary Figure 11**, highlighting the same trends across ESC WT/KO and patient lines.

Additionally, we utilised multiple cell lines for MPSIIIA and healthy age-matched individuals as we believe it better represents the variance among the disease community compared to neurotypical children. While we acknowledge that gene-corrected controls for each patient may be beneficial to further validate our key phenotypes, we decided to focus limited resources in including more patient lines and clones instead. It is outside the current scope of this paper and is perhaps something that we could investigate in the future as we expand this project.

2.5 As the authors identify combination #2 to be the most efficacious therapeutic strategy impacting neuronal activity, they also discuss the potential of this drug combination on other preclinical disease phenotypes, as identified by the companion manuscript Greenberg et al.,. The companion article is not exploring the impact of combination #2. As the authors strongly suggest for this therapeutic strategy to be clinically relevant, these MPSIIIA phenotype rescue experiments should be performed here, as described in Greenberg et al.,

Thank you for bringing this up. To address this, we have completed new experiments treating neural cultures with APAT (previously referred to as Combination #2) to see if it rescues the phenotypes identified in our companion manuscript (neurodegeneration and astrogliosis). This data has now been included in **Supplementary Figure 17**. These results demonstrate that APAT significantly rescues the neurodegeneration and astrogliosis phenotypes, aligning with our discussion about combination therapies rescuing other preclinical phenotypes. This strengthens the clinical relevance of APAT by presenting the broader phenotypic rescue data in the companion manuscript, which may indirectly rescue synaptic dysfunction.

APAT was not tested against phenotypes in Greenberg, et al. as it was designed based on each drug's individual success rescuing the phenotypes identified in our companion manuscript and the hypothesis that combining multiple drugs would have a synergistic effect. This decision was also influenced by each drug's capacity to reduce the excitatory synapse density (**Figure 4A-D**), which prompted us to pursue the APAT combination.

We appreciate the reviewer raising this important point as it brings into question the clinical relevance of our combination therapy. We hope that the addition of this data in our companion manuscript addresses their concerns about the therapeutic benefit of APAT. We would also like to mention that further work to optimise the combination therapy for clinical trials is important and under way, but beyond the scope of this first study.

2.6 Authors observe that the combination #2 shows sustained effect on the number of active neurons after treatment ceased. Yet they do not comment on the other two MEA parameters which went back to the MPSIIIA - untreated levels. These differences in readouts should be elaborated, as the current conclusion that combination #2 has long term benefits is not fully clear.
Thank you so much for bringing this up. We apologise for the oversight. Upon reviewing our Python script for analyzing MEA data, we identified an error in the averaging of recordings to establish the pretreatment baseline. Initially, we intended to average recordings before treatment starting at 65 days. Due to an oversight, the initial analysis averaged recordings from 14 to 64 days, which included a significant portion of the neuronal maturation phase and is not suitable for inclusion in a baseline average. We have corrected this oversight by averaging recordings from 45 to 64 days for the pretreatment baseline. This correction, reflected in updated **Figure 7F** and **Supplementary Figure 14 A-G**, does not alter our conclusions and enhances their robustness. The methods section (**Lines 1057-1058**) has also been amended accordingly.

To further address the reviewer's observation about the MEA parameters, we have also elaborated in the results section (**Lines 430-433**) on the sustained effects of APAT (previously referred to as Combination #2). The treatment maintains a significant decrease in the number of active neurons post-treatment indicating a sustained effect. However, the number of active bursting cells and network frequency show a partial return toward MPSIIIA untreated levels. This suggests that APAT achieves a sustained rescue of neuronal activity, however the extent of rescue varies across metrics, with the active neuron counts showing the most persistent effects. These differences likely reflect the complex mechanisms of disease-specific network dynamics.

2.7 The authors did not state the number of differentiations/ biological replicates their results consist of. Drug screening assays, when established, are acceptable to be performed on one differentiation, but all of the proof of concept/phenotype assays (**Fig. 2,3,4**) should be done in at least 2-3 independent differentiations per current standards. Also, the single cell RNAseq data should be at least followed up with target validation in an independent differentiation.

We appreciate the reviewer's recommendations regarding the number of biological replicates and the need for validation in our proof-of-concept and phenotype assays. We have performed additional synaptic imaging and single cell RNA sequencing experiments and updated our reporting of biological replicates used for each assay to enhance the transparency and robustness of our findings.

To address the concern about phenotype assays (**Figures 2-4**), we performed additional synaptic staining (Synapsin-I, PSD-95 and Gephyrin) in an independent neuronal differentiation at 15, 30, 60 and 90 days. This new data confirms and extends our original phenotypic trends, demonstrating a progressive shift in the excitation/inhibition balance in MPSIIIA cultures, towards greater excitation. Despite a brief transient increase in inhibitory synapse density at Day 15 compared to healthy, a consistent gradual decline followed at Days 30 and 60, reaching significance by Day 90. In contrast, excitatory synapse density showed a significant increase from Day 30 onwards. These findings, supported by complementary MEA and patch-clamping data, support and strengthen our original conclusion that excitatory and inhibitory synapse density fluctuates in opposite directions in MPSIIIA.

We agree that the single-nuclei RNA sequencing should be repeated with target validation to confirm the effectiveness of the drugs. While we have not repeated RNA sequencing at the 120-day timepoint in another independent differentiation, our updated Figure 6F demonstrates that the phenotypic changes in glutamatergic and GABAergic postsynaptic gene expression is consistent across multiple patient-derived MPSIIIA lines. This consistency across patients supports the robustness of our identified targets. However, to address another reviewer comment we have now repeated single-nuclei RNA sequencing on newly differentiated Healthy and MPSIIIA playground cultures as well as MPSIIIA cultures treated with APAT (Allopurinol, Probenecid, Acetyl-D-Leucine and Theophylline) and each drug individually (with the exception of Allopurinol and Probenecid, which were combined due to similar mechanisms of action). This was completed at 30-days to align with the treatment window used in our companion paper and the data corresponding to this can be found in Greenberg, et al. **Figures 8-10**. This new data reveals consistent transcriptional dysregulation across patient lines and identified key neuronal and astrocytic pathways targeted by our top drug candidates, which show widespread rescue effects. Together, these findings provide cross-patient validation of our model and mechanistic insight into the mechanistic pathways of our potential therapeutics.

We have now detailed all biological and technical replicates for each assay in the manuscript in **Supplementary Figures 2-4, 5, 7, 8, 10 and 12**, which includes the number of independent neuronal differentiations and neuronal differentiation batch dates for each cell line and clone. Most assays used two clones per genotype to account for variability. For whole-cell patch-clamping, which is labour-intensive, we used one clone per cell line. This is outlined in **Lines 110-116** of the manuscript.

Patch-clamping experiments were conducted over four years and involved analysing 1,169 neurons from 14 different cell lines (5x healthy iPSCs, 5x MPSIIIA iPSCs, 1x ESC WT, 1x ESC SGSHKO and a playground model with 2 clones per donor [excluding BIAD05]) at 30-60 and 90-120 day in culture (**Supplementary Figure 2**). **Supplementary Figure 2** has been updated to include the number of coverslips patched per line, and average neurons patched per coverslip, ensuring transparency in our experimental timeline.

Our study was designed to ensure biological and statistical confidence across all assays, including whole-cell patch-clamping and multielectrode arrays (MEA). For patch clamping, our design exceeds typical studies which often rely on fewer replicates (two biological replicates from a single iPSC or embryonic WT/KO line, with a smaller neuron sample size)(Neuron 2024, DOI: 10.1016/j.neuron.2024.09.020; Science 2019, DOI: 10.1126/science.aav5386; Science 2016, DOI: 10.1126/science.aaf2669). For MEA experiments, we recorded 8-72 wells per condition over three years, using 9 control and 10 MPSIIIA iPSC-derived lines, and 4 playground models (2 healthy controls and 2 MPSIIIA). Although high-impact MEA studies do not consistently report technical and

biological replicates, we have detailed these in our supplementary figures. Our dataset included a larger number of cell lines and replicates than recent studies, which typically have fewer cell lines and wells (2-5 cell lines, with 2-8 wells per condition across 3 independent experiments) (Nature Communications 2024, DOI: 0.1038/s41467-023-41603-0; Neuron 2018, DOI:10.1016/j.neuron.2018.09.044). Therefore, our dataset reflects a rigorous and well-powered design that supports the reproducibility of our findings.

2.8 It is unclear how the compound doses were determined prior to the drug screen (**Fig 7**), and there is no information on how the drug combination #1 and combination #2 were designed.

We apologise for the oversight. We have addressed this by amending the Methods and Results sections of our manuscript to include the rationale dosing and combination therapy design as follows:

Lines 370-373:
"AP was chosen as both compounds (allopurinol and probenecid) target overlapping pathways relating to neuroinflammation and oxidative stress, offering a complementary mechanism of action that may be enhanced by their combination."

Lines 394-398:
"Each APAT component (allopurinol, probenecid, acetyl-D-leucine and theophylline) (i) rescued preclinical MPS IIIA phenotypes in our companion study (Greenberg et al.), (ii) reduced excitatory synapse number, (iii) sustained the rescue of electrophysiological activity of MPS IIIA neural cultures on MEA and (iv) engages distinct targets, providing a rationale for potential synergy."

Lines 917-949:
"Drug screen on iPSC-derived neural cultures
10 repurposed drugs were selected for drug screening. Initial drug concentrations were determined based on an extensive literature search focusing on previous reports of these compounds' efficacy in cell models of neurodegenerative diseases or related phenotypes [*companion manuscript Greenberg et al.]*. Compound doses were further optimised based on previous findings [*companion manuscript Greenberg et al.*] and to address solubility limitations (Supplementary Fig. 14). Perampanel was supplied by Compounds Australia at a 5mM stock in DMSO. Allopurinol (Sigma, PHR1377), N-acetyl-D-leucine (Sigma, A0876), cannabidiol (CBD) (generously donated by the Lambert Initiative for Cannabinoid Therapeutics, University of Sydney), carbamazepine (Sigma, PHR1067), lithium chloride (Sigma, L9650), lumiracoxib (Sigma, SML2928), memantine hydrochloride (Sigma, PHR1886), probenecid (Sigma, PHR2608) and theophylline (Sigma, PHR1023) were supplied separately. Appropriate vehicle controls were used for all drugs and drug combinations.

Drug combinations were designed in collaboration with paediatric neurologists based on their likelihood to produce synergistic effects, informed by their mechanism of action and drug class classification. AP consisted of allopurinol and probenecid, which are uric acid lowering agents that target pathways related to neuroinflammation and oxidative stress, and their complementary mechanisms indicate potential to enhance their combined effect. APAT consisted of allopurinol, probenecid, acetyl-D-leucine and theophylline. Theophylline and acetyl-D-leucine have both previously demonstrated a high efficacy in rescuing heparan sulfate accumulation, neurodegeneration and astrogliosis phenotypes [companion manuscript Greenberg et al.]. Theophylline also has broad-spectrum neurophysiological effects indicating a potential to modulate

synaptic activity[123], while acetyl-D-leucine has demonstrated efficacy in slowing disease progression, improving cognition and motor deficits in another lysosomal storage disorder Niemann Pick Type-C[124].

Drug treatment timelines were staggered to capture MPS IIIA-relevant phenotypes and meet assay-specific maturation requirements. High-content imaging was performed at days 40-55, MEA at days 65-80, and whole-cell patch from 85 days onwards, ensuring sufficient maturation for reliable assessment of neuronal synaptic function. These timepoints coincide with the emergence of structural and functional phenotypes in our cultures, enabling assessment of both early- and later-stage treatment effects."

Lines 986-991:
"6 repurposed treatments (Allopurinol, N-Acetyl-D-Leucine, Carbamazepine, Lithium Chloride and Probenecid) were chosen for drug screening using multielectrode array based on an extensive literature search, previous findings [*companion paper Greenberg et al.*] and the results from synaptic high-content imaging (**Figure 7A-E, Supplementary Fig. 15A-B**). Appropriate vehicle controls were used for all drugs and drug combinations."

Lines 1044-1049:
"5 repurposed drugs (Allopurinol, N-Acetyl-D-Leucine, Theophylline, Carbamazepine and Probenecid) were chosen for the patch-clamping drug screen. All drug concentrations were determined based on an extensive literature search, previous findings [*companion paper Greenberg et al.*] and results from synaptic high-content imaging (**Figure 7A-E, Supplementary Fig. 15A-B**) and MEA drug screens (**Figure 7F-G, Supplementary Fig. 15**). Appropriate vehicle controls were used for all drugs and drug combinations."

Briefly, the initial drug screening, as described in our companion manuscript (Greenberg et al.), was informed by an extensive literature review. This review focused on the repurposed compounds previously reported to exhibit efficacy in cell models of neurodegenerative diseases or related phenotypes.

For our study, certain compound doses were further optimised based on the findings from the initial drug screen *(*Greenberg et al.) and to address solubility limitations. For example, the dose of Theophylline was modified, after showing consistent partial rescue,in initial drug screening, we increased the concentration to see if we could improve efficacy (Greenberg et al., Figure 5). Additionally, the concentration of allopurinol and probenecid was reduced in combination treatments due to limitation in solubility and minimizing toxicity to cultures.

The drug combinations were carefully designed in collaboration with paediatric neurologists based on the following considerations:

- Compounds were combined based on their likelihood to produce synergistic effects, informed by their mechanism of action and drug class classification.
- For **AP**, both compounds target overlapping pathways related to neuroinflammation and oxidative stress, offering a complementary mechanism of action to enhance their combined effect.
- For **APAT**, both Allopurinol and Probenecid act synergistically to target inflammatory pathways and oxidative stress. Theophylline was effective in the majority of assays in initial drug screening, rescuing neuronal, astrocytes and heparan sulfate phenotypes (Greenberg et al.). It has broad-spectrum neurophysiological effects and has

potential to modulate synaptic activity. Acetyl-D-Leucine also demonstrated a high degree of efficacy in prior screens (Greenberg et al.) with robust targeting of neuroinflammatory pathways and modulation of cellular metabolism.

Minor Concerns:

- Typo line 168, should be Figure **S7**
  Thank you for bringing this to our attention. We have corrected this in the manuscript to "Supplementary Figure 7"

Paper 2: Hyperactive synaptic circuits in a human neuronal model of childhood dementia

Thank you again for submitting your manuscript "Hyperactive synaptic circuits in a human neuronal model of childhood dementia" to Nature Communications. We have now received reports from 3 reviewers and, on the basis of their comments, we have decided to invite a revision of your work for further consideration in our journal. In particular your revision should:

1. Address the remaining concerns of the reviewers,
2. Ensure that all data in this paper is independent; there can be no overlapping data with the other paper which will not be published at the same time.
3. Since the Greenberg/drug screening paper will not be published simultaneously the we suggest the drug recovery data be removed from Mazzachi/hyperactive circuits paper.
4. Remove all references to the Greenberg/drug screening paper.

When resubmitting, you must provide a point-by-point response to the reviewers' comments. Please show all changes in the manuscript text file with track changes or colour highlighting. If you are unable to address specific reviewer requests or find any points invalid, please explain why in the point-by-point response.

We sincerely thank the reviewers for their careful re-evaluation of our manuscript and thank the editors for allowing us the opportunity to submit a revised version for further consideration. We are grateful for the constructive feedback and are delighted that, with these revisions, the work may be suitable for publication.

We have made the following amendments to address the reviewers' and editors' latest comments and requests:

1. Addressed the remaining concern of Reviewer #1 with electrophysiological and transcriptomic analysis, which can be found in the two new figures attached below.
2. Ensured all data in this manuscript is independent. We confirm that the revised version has no overlapping data with the other paper. We transferred all iPSC/NPC quality control data and karyotyping from the Greenberg/drug screening paper into this manuscript to support the successful generation of neural cultures from high-quality stem cells.
3. Removed the drug screen data (originally Figure 7) from the paper and all references throughout the manuscript as requested.
4. Removed all references to the Greenberg/drug screening paper throughout the manuscript as requested.

Reviewer #1

I would like to congratulate the authors for the extensive and excellent work performed. The addition of new replicates for synaptic stainings, new data presentation, and the new scRNAseq data are clearly improving the quality of the work and clarity of the manuscript.

My only remaining concern is about the BIAD03 line for several reasons. First, this line shows a quite higher expression of SOX2 at the iPSC level (companion manuscript), which could have an impact in the differentiation from NSC to neurons since SOX2 needs to be downregulated during neuronal differentiation. In fact, this is the line with lower excitatory postsynaptic communication, and interestingly, in the scRNAseq data most of excitatory neurons generated from this line cluster separately from all other excitatory neurons generated by disease or healthy lines (Fig 1B,

Supplementary Fig 1B and Fig 6A). What are the differences in between these two populations and what can be the functional impacts of these differences? In Supplementary Fig. 12, it actually seems that for some of the measurements, 1 or 2 individual patient lines could be driving some of the differences and one wonders if BIAD03 is that line and the driver of the functional changes in the playground networks since it is clearly dominating it at the later time points.

We thank the reviewer for their positive assessment of our revisions and for highlighting the improvements in data quality and clarity, which was the result of insightful reviewers' suggestions in the first round of review. We also appreciate their detailed attention to the BIAD03 line. Below, we address BIAD03 concerns and questions using electrophysiological and transcriptomic datasets, which show that BIAD03 undergoes typical neural differentiation and only begins to diverge at a later time in culture, likely reflecting donor-specific disease variability rather than impaired lineage commitment.

We acknowledge the reviewer's concern that elevated SOX2 mRNA levels in the BIAD03 iPSC line could potentially influence downstream neuronal differentiation and function. We agree that appropriate downregulation of SOX2 is an important feature of neural differentiation. However, published work (Cell Stem Cell 2007, DOI: 10.1016/j.stem.2007.09.002) indicates that moderate elevation of SOX2 at the iPSC stage does not impair differentiation, provided SOX2 is correctly regulated at the NPC stage. Consistent with this, BIAD03 NPCs display comparable proportions of NES+/SOX2+ and NES+/SOX2+FOXG1+ cells to the other MPS IIIA lines (Figure R1A-B), supporting appropriate lineage commitment. While BIAD03 shows slightly higher SOX2 mRNA levels at the iPSC stage (Figure R1C), the absolute expression level ($2^{-\Delta Ct}$ = 0.08) is similar to that observed in BIAD10 and ESC lines and lies within the reported range for human iPSCs. BIAD03 also meets standard pluripotency and quality control criteria, including OCT4/SOX2/KLF4 co-expression (Figure R1D), typical morphology, and comparable proliferation rates. In addition, grouped analysis of SOX2 protein expression reveals no significant difference between healthy and MPS IIIA iPSC lines (Figure R1E).

Most importantly, BIAD03 neurons exhibited electrophysiological properties similar to other MPS IIIA lines, confirming successful neural differentiation (Figure R1F-H). They showed fast, repetitive action potential firing (Figure R1F), supported by functional voltage-gated channels (Figure R1G). Consistent with early synapse formation, some cells across all lines lacked AMPA inputs at the earliest time point (30-60 days). AMPA activity in BIAD03 at 30-60 days was indeed relatively low, but differing significantly only from BIAD01 (Figure R1H). BIAD03 GABAergic activity matched BIAD01 and BIAD11, while BIAD02 and BIAD10 were lower (Figure R1H). Furthermore, 60-day snRNA-seq data clustered BIAD03 excitatory neurons with other MPS IIIA patients (Figure R1I-J), supporting early similarities, and further excluding the possibility of BIAD03 not differentiating correctly.

We agree with the reviewer that the distinct transcriptomic clustering of BIAD03 excitatory neurons at 120 days is intriguing (Manuscript Figures 1B and 6A; Supplementary Figure 3B). To explore this further, we performed differential expression and gene ontology analyses comparing BIAD03 excitatory neurons with those from the other MPS IIIA lines. Genes upregulated in BIAD03 were enriched for pathways related to axonal and dendritic projection development (Figure R2A), which aligns with our morphological analysis showing increased cellular complexity in BIAD03 neurons at both time points, including greater cell body surface area, branching (node number), and total neurite length (Figure R2B).

Conversely, genes downregulated in BIAD03 were enriched for synaptic-related pathways, including glutamatergic synaptic transmission (Figure R2C). This finding is consistent with our patch-clamp data at both early and later stages, where BIAD03 exhibits lower AMPA drive relative to other patient lines (Figure R1H; Supplementary Figure 18). Importantly, these transcriptional differences do not translate

into deficits in other key functional measures, such as action potential firing capacity (Figure R2D) or voltage-gated sodium and potassium channel function (Figure R2E), which are comparable across all patient lines. Similarly, in MEA assays, BIAD03 closely follows the cohort average across maturation stages and readouts (Figure R2D), consistent with the lower AMPA activity detected by patch-clamping. Together, these data indicate that BIAD03 alone does not drive the synaptic or network phenotypes observed across the cohort.

Rather than reflecting impaired neural differentiation, we interpret these findings as an example of possible inter-patient heterogeneity in MPS IIIA, a condition known to exhibit variability in disease onset, progression, and severity despite shared genetic causes. Although this study was not designed to detect with confidence patient-specific differences, capturing population heterogeneity is a strength in this multi-donor model, as it more closely reflects the clinical spectrum of the disease than isogenic or single-donor approaches. BIAD03 exhibits characteristics suggesting that it falls on the milder side of the MPS IIIA disease spectrum, as evidenced by reduced lysosomal burden, less pronounced reactive astrogliosis, and a slower rate of neurodegeneration (data from the other paper). As a result, BIAD03 neurons tend to persist longer in the neural culture and may contribute a relatively higher proportion of excitatory neurons at later time points in the playground (e.g., 120 days), but did not bias the conclusions.

In summary, although BIAD03 shows modestly elevated SOX2 mRNA levels at the iPSC stage, this is appropriately regulated during neural differentiation, resulting in normal neuronal identity and function (at the early neural maturation timepoint; 30-60 days). The late-stage (120 day) transcriptomic divergence of BIAD03 may reflect donor-specific biological variability, including relatively increased neuromorphological complexity and reduced synaptic drive, rather than a neural differentiation issue. Importantly, electrophysiological and MEA data demonstrates that BIAD03 does not disproportionately influence the functional phenotypes observed across the cohort. We believe the inclusion of BIAD03 may strengthen the model by representing the milder end of the MPS IIIA spectrum, therefore enhancing the overall generalisability of our findings.

We thank the reviewer again for their thoughtful and constructive comments, which have helped to refine both our analyses and interpretation, and we hope that the additional data and clarification address their question.

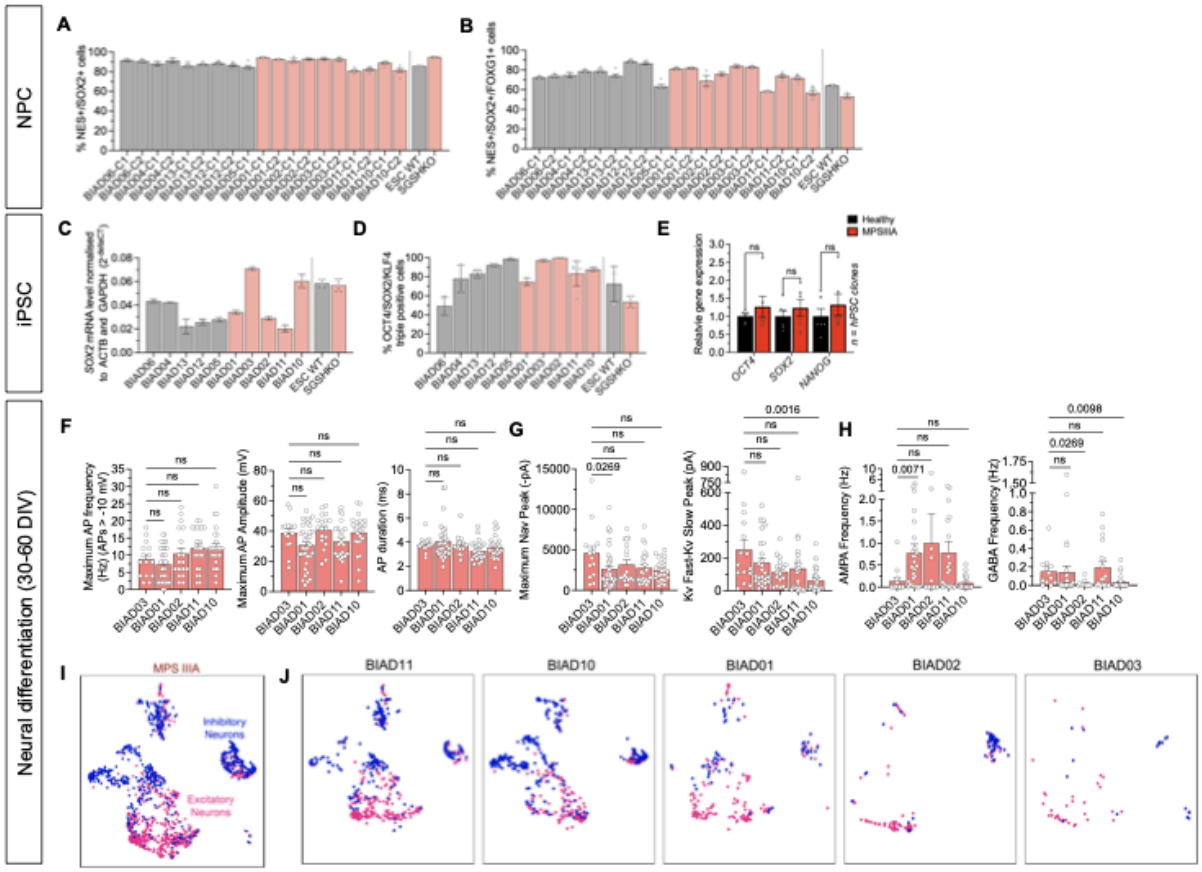


Figure R1: Heightened SOX2 expression in iPSC stage does not affect neural differentiation of BIAD03.

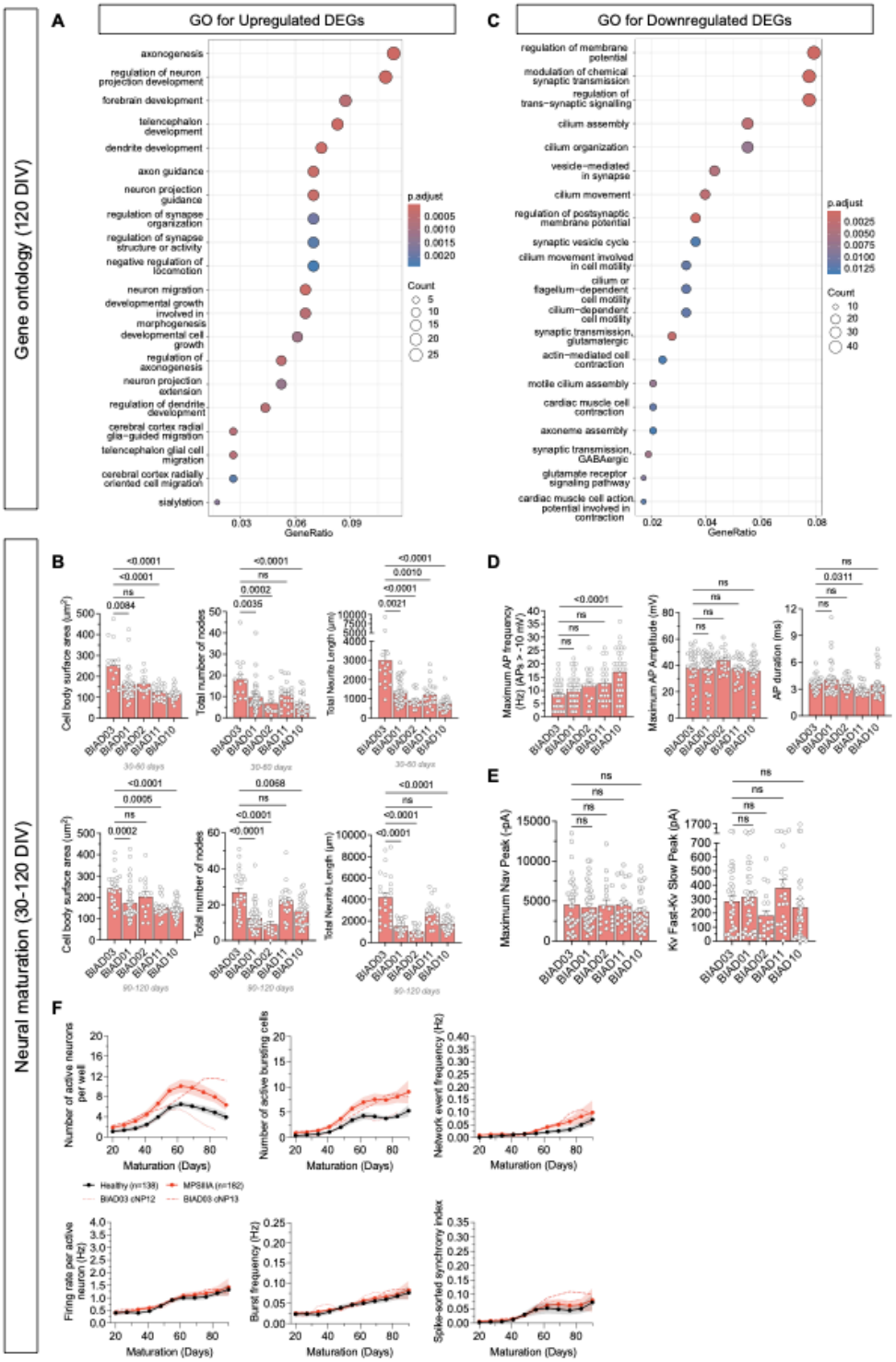


Figure R2: Synaptic and network phenotypes are not driven by BIAD03.

Reviewer #2

The authors have adequately addressed all of my previous comments and concerns. They have conducted additional experiments and provided detailed information on the biological replicates for improved clarity. These additions enhance the reproducibility of the reported phenotypes and compound rescue, significantly strengthening the overall rigor of the study.

We sincerely thank the reviewer for their very positive feedback and for recognising that all previous concerns have been addressed. We are particularly grateful for their kind words about the new experiments and increased clarity on biological replicates, and we are delighted that they feel these additions have substantially strengthened the rigor, reproducibility, and overall impact of the study.