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Supplemental Information

Functional Characterization of Three-Dimensional Cortical Cultures for *In Vitro* Modeling of Brain Networks

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Supplementary Information

Transparent Methods

Silk fibroin scaffold fabrication

A 6 % (w/v) silk fibroin solution was used to prepare silk fibroin porous sponge scaffolds (pore size 425 – 500 µm) as previously described (Chwalek et al., 2015; Tang-Schomer et al., 2014). Briefly, *Bombyx mori* cocoons (Tajima Shoji Co., Ltd, Japan) were cut into small pieces and boiled in 0.02 M sodium carbonate (MilliporeSigma #57795) solution for 30 min to remove sericin. Dry silk fibroin fibers were dissolved in 9.3 M lithium bromide (LiBr, MilliporeSigma #213225) solution at 60°C for 4 hrs. The solution was dialyzed against water in dialysis tubing (Fisher #21-152-9, molecular weight cutoff 3,500 Da) for 2 days with six 4 L water changes over 48 hours to remove LiBr. Undissolved material was removed by centrifuging twice at 12,700 rcf at 4°C for 30 min followed by passing through a cell strainer with 100-µm pore size (Corning #431752). The resulting silk fibroin solution was adjusted to 6 % (w/v) with water. Sodium chloride (NaCl) was sieved to 425 – 500 µm particle size. Thirty mL of 6% silk fibroin solution was poured into a 10-cm petri dish, and 60 g of NaCl was poured evenly into the solution. The silk fibroin was left at room temperature for 2 days and incubated at 60°C for 1 hr to allow the silk fibroin to form insoluble β-sheet crystalline structure. The resulting material was freed from the petri dish and rinsed in 4 L water for 2 days with 6 water exchanges to remove the NaCl particles and leave behind the resulting pores. Silk scaffolds for the biomimetic cortical cultures were cut into 3-mm diameter and 1.5-mm height cylinders using a biopsy punch (Integra #12-460-406) and a razor blade.

3D cortical cultures and AAV infections

All 3D cortical cultures were maintained, unless otherwise noted, in Neuro Medium: Neurobasal Medium (ThermoFisher #21103) or Neurobasal Medium minus phenol red (ThermoFisher #12348) supplemented with 2% B27 (ThermoFisher #17504), 1% Antibiotic-Antimycotic (ThermoFisher #15240), and 2 mM GlutaMax (ThermoFisher #35050).

Scaffolds were placed in water and sterilized by autoclaving. Prior to cell seeding, scaffolds were coated overnight with poly-D-lysine (PDL; 0.1 mg/mL in water, MilliporeSigma #P6407) and laminin (LN; 10 µg/mL, MilliporeSigma #MFCD00081739). Typically, in a 12-well we coat 20-30 sponges in 2 mL coating solution. On the day of cell seeding, the scaffolds were washed twice with PBS (10-min incubation), then incubated at least for one hour with Neuro Medium and at least for one hour in Neuro Medium containing 5% (v/v) fetal bovine serum (FBS). Excess medium was aspirated immediately before cell seeding.

Brain cortices were harvested from E16 C57BL/6 mouse embryos (Charles River Laboratories) and digested with 0.25% Trypsin (Thermo Fisher #325200) and 0.3 mg/mL DNase (MilliporeSigma #10104159001). All animal procedures were approved by Tufts University Institutional Animal Care and Use Committee (IACUC, Protocol #M2018-06). Neurons were resuspended in Neuro Medium containing 5% FBS at the density of $2x10^6$ cells per 5 µL. PDL-LN-coated scaffolds aspirated to dry and transferred to a 96-well plate (one scaffold per well). A cell suspension of 5 µL was pipetted directly onto the scaffold, and the seeded scaffolds were incubated in 37˚C for 30 min to allow for cell adhesion, followed by addition of 200 µL Neuro Medium + 5% FBS. The day of cell seeding was referred as 0 days-in-vitro (DIV). The next day (1 DIV), cultures were transferred to 500 µL of medium in 48-well plates. For neurite density image analysis, 3D cultures were infected on 1 DIV with AAV-hSyn1-TurboRFP (pENN.AAV.hSyn.TurboRFP.WPRE.RBG, Addgene #105552-AAV1, titer 2.7 x 10^{13} GC/mL, a gift from Dr. James M. Wilson, unpublished) diluted in Neuro Medium at 1:1000. 3D cultures for DNA quantification, RNA analysis, and immunostaining were transferred to fresh 500 µL Neuro Medium without AAV. For Ca^{++} imaging experiments, 3D cultures were infected with AAV-hSyn1-GCaMP6f (AAV-hSyn1-GCaMP6f-P2A-nls-dTomato, Addgene, #51085-AAV1, titer 2 x 10^{13} GC/mL, a gift from Dr. Jonathan Ting, unpublished) diluted in Neuro Medium at 1:1000. The 3D cultures where then incubated overnight at 37˚C.

On 2 DIV, 3D cultures were transferred to 96-well plates, and 10 µL of 3 mg/mL collagen type I solution (Corning #354236) was pipetted onto each scaffold followed by incubation at 37˚C for 30 min to allow for collagen gelation. Each 3D culture was then transferred to a 24-well containing 2 mL Neuro Medium. For cultures intended for Ca^{++} imaging (AAV-hSyn1-GCaMP6f infected), glass-bottom plates (Cellvis #P24-1.5P) and phenol red-free Neuro Medium were used. Half-media changes were carried out every 3-4 days.

Assessment of 3D neurite density

3D cultures infected with AAV-hSyn1-TurboRFP were chemically fixed on at 1, 2, and 3 wk with 4% paraformaldehyde/4% sucrose in PBS for 1 hr. After three PBS washes (30-min each), the 3D cultures were imaged with a Leica SP8 confocal microscope with a 40X objective (Leica HC PL APO 40x/1.10 W CORR CS2), x,y,z-dimensions= 291 x 291 x 210 µm, 0.42 µm per z-step) with excitation laser of 552 µm. 3D movie (Movie S1) was rendered with maximum projections in LAS X software (Leica). Z-stack images were analyzed using a custom MATLAB (MathWorks) script (Liaudanskaya et al., 2020). First, Otsu's thresholding was used to remove weak background fluorescence. Then, connected objects were identified using the bwlabel MATLAB function, and objects with eccentricity lower than 0.9 were removed, as they represented signals emanating from silk and cell bodies. The remaining voxels were grouped together into objects if neighboring edges or faces were in contact using the 3D bwlabel MATLAB function. The smallest ellipsoid was drawn around each object, and the major axis length of each individual ellipsoid was measured in voxel increments. The sum of all such voxels was calculated for each z-stack image to represent the volume occupied by neurites. The neurite density was presented as the percent of neurite voxel number to total voxel number per z stack.

Immunofluorescence analysis of 3D cultures

After chemical fixation of 3D cultures, the cells were permeabilized and blocked for 1 hr in blocking solution (4% (v/v) normal goat serum (Jackson Immuno Research #005-000-121) and 0.2% (v/v) Triton-X (MilliporeSigma #T8787) in PBS). Rabbit anti-GFAP primary antibody (1:2000, MilliporeSigma #G9269) and Alexa 488 goat-anti-rabbit secondary antibody (1:1000, ThermoFisher #A11034) were diluted in blocking buffer. Primary antibody incubation was done at 4°C overnight, followed by three 30-min PBS washes. Secondary antibody incubation was done in blocking solution at room temperature for 2 hrs, followed by DAPI staining (1:1000 in 0.2% Triton-X, Thermo Fisher #D1306) and three 30-min PBS washes. Confocal z stack images were acquired using a Leica SP8 confocal microscope with a 25X objective (Leica HC FLUOTAR L 25x/0.95 W VISIR) and with excitation lasers at 405 and 488 µm.

Gene expression analysis of 3D cultures

On 1, 14, 21 DIV, 3D culture samples were collected in 600 - 700 µL ice-cold Buffer RLT (Qiagen #79216) with 1% β-mercaptoethanol (MilliporeSigma #M3148) and stored in -80°C until RNA isolation. On the day of mRNA isolation, samples were thawed and homogenized, first by manually grinding into fine pieces with a homogenizer pestle (Fisher #12-141-364), followed by homogenization for 20 sec with a motorized tissue grinder (Fisher #12-1413-61), and then passed through a QIAshredder column (Qiagen #79656). RNA was isolated using the Qiagen RNeasy Mini Kit (Qiagen #79656) according to the manufacturer's protocol, with additional in-column removal of genomic DNA with DNase (Qiagen # 79254). The resulting RNA was eluted with 25-30 µL of water and the resulting concentrations measured using a NanoDrop Spectrophotometer (ThermoFisher). cDNA was synthesized using iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad #1725035) according to the manufacturer's protocol.

Relative gene expressions were quantified using the Taqman quantitative real-time PCR (qRT-PCR) assay and analyzed with the QuantStudio 5 RT-PCR System (Thermo Fisher). Each qRT-PCR reaction contained

10 ng cDNA, 10 µL Taqman Gene Expression Master Mix (ThermoFisher #4369016), and 1 µL Taqman assay. See Table 2 for the list of Taqman assays. All expressions were referenced to housekeeping gene Rn18s and normalized to 1 DIV. ΔΔCt method was used to calculated relative expression levels.

DNA Quantification

Relative DNA amount was quantified using Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher # P7589). Cortical cell suspensions (1 and 2 x 10^6 cells) were centrifuged at 500 rpm for 5 min. Supernatant was removed, and cell pellets were lyzed with 700 µL Buffer RLT with 1% β-mercaptoethanol and vortex. 3D cultures were lyzed as described in the Gene expression analysis of 3D cultures section above. 5 µL samples or DNA standards were diluted with 95 µL Buffer RLT with 1% β-mercaptoethanol. Assays were conducted according to the manufacturer's protocol. Raw fluorescence values were used to estimate number of cells in the 3D cultures.

Ca^{++} imaging

A half-medium change was done 1 or 2 days prior to the day of imaging. Ca^{++} imaging was performed on AAV-hSyn1-GCaMP6f infected 3D cultures with a Nikon Eclipse Ti-2 inverted fluorescence microscope with a 4X objective (Nikon CFI Plan Fluor DL 4x na 0.13 wd 16.5mm Objective). During imaging, plates were kept at 37˚C and 5% CO_2 in a stage-top incubator (Bioscience Tools #TC-MWP). Medium was removed to keep the 3D cultures from floating during image acquisition, leaving approximately 500 µL of medium to support the culture during imaging. The 3D culture was centered in the field of view and all time-lapse images were acquired with a FITC filter and the following settings: 3x3 binning, 50-ms exposure, 200-ms intervals (i.e. 5 Hz), and 1-min duration. At 2 weeks (14 DIV) only spontaneous baseline Ca^{++} activities were recorded. At 3 weeks (20-22 DIV), a 1-minute baseline image analysis was first performed. Picrotoxin (50 µM, Abcam #ab120315) and bicuculline (10 µM, Abcam #ab120107) were then applied for 7 min to the sample, after which recording was repeated for another minute. NBQX (5 µM Tocris 1044; and AP5 (50 µM, Sigma A8054) were applied for 22 min before the second recording. Picrotoxin and bicuculline were first dissolved in DMSO to make 2000X stock solutions. A 5 µL of a 100X concentrated solution, diluted in Neuro Medium, of each of the drugs was added to the cultures. Equivalent concentration of DMSO (1:2000) was added to a separate set of 3D to control for the addition of DMSO in picrotoxin and bicuculline. The time-lapse images were converted to TIFF stacks in NIS-Elements software (Nikon).

Neuronal activity and global synchronization analysis of 3D neural networks

Region of interest (ROI) assignment

A mask in the shape of a honeycomb pattern of 37 hexagonal ROIs (side-to-side distance = 500 µm, center-to-center distance = 500 µm) was first designed on Adobe Illustrator to cover the circular projection of the 3D scaffold and converted to binary in Adobe Photoshop or NIS-Elements (Nikon, Micro Video Instruments, Inc). Each ROI was defined as a node for all of the following analyses.

Ca^{++} events detection, % active ROI, and Ca^{++} event frequency calculation

Ca^{++} event detection and event frequency calculation were conducted using an open source MATLAB-based software FluoroSNNAP (Patel et al., 2015). The time-lapse TIFF stack and ROI mask were loaded in FluoroSNNAP, and the Segmentation GUI was used to create a segmentation MATLAB. The *Single-Cell Ca^{++} Transient Kinetics* module was used for Ca^{++} event detection and to calculate total events for each individual ROI in a 3D tissue sample. The input parameters used for baseline fluorescence were F_0 = 10

sec of previous and 20th percentile. The input parameters for Ca^{++} event detection were template-based, threshold = 0.85, and minimum dF/F amplitude = 0.01. Descriptions of input parameters are detailed in (Patel et al., 2015). Samples with poor cell seeding efficiency (< 50% active ROI at baseline) were excluded.

% Active ROI and Ca^{++} event frequency of each 3D tissue was calculated as:

$$\%\ Active\ ROI = \% \frac{Number\ of\ ROIs\ containing\ at\ least\ one\ Ca^{++}event}{N} \quad \text{Eq. 1}$$

$$Ca^{++}event\ frequency_{Single\ ROI} = \frac{Number\ of\ events}{1\ min} \quad \text{Eq. 2}$$

$$Ca^{++}\ event\ frequeycy_{tissue} = \frac{1}{N}\sum Ca^{++}event\ frequency_{Single\ ROI}) \quad \text{Eq. 3}$$

where N is the total number of nodes, which is 37 in this study.

Global synchronization Index

Global synchronization index was calculated using FluoroSNNAP's *Synchronization* module with input parameters of method = Phase, #times to perform surrogate resampling = 20, minimum size of synchronization cluster = 3. The mathematical descriptions are detailed in (Patel et al., 2015). Briefly, this method separates the time-varying fluorescence signals into its amplitude and phase components and compares the similarity of timing of events (phase) of all the ROIs.

Functional connectivity and graph theory-based network analysis of 3D neural networks

Cross-correlation analysis

Cross-correlation analyses were done using FluoroSNNAP's *Estimate Functional Connectivity* module, with number of resampling = 100 and significance level = 0.5. The raw cross-correlation coefficient matrices of all node (ROI) pairs (i, j, whereas i≠j) were accessed in the output MATLAB file (located in the file 'processed analysis.mat/FC/C'), extracted, and saved as a numeric matrix MATLAB file. This file is then loaded into a single, user-friendly, MATLAB code, which is a compilation of the individual codes used to compute the graph theory-based network parameters.

Average edge weight calculation

Network analyses in our study were based on a weighted network (i.e. edges values, not binary). The cross-correlation coefficients between paired nodes i and j were used as edge weights in the graph. The average edge weight for each 3D culture sample was calculated by averaging all the edge weights.

Average clustering coefficient and path length

The local clustering coefficient for each node c_i in a weighted graph was computed as described by Onnela et al, using their published MATLAB script (Onnela et al., 2005). Briefly, clustering coefficient in a weighted graph reflects the intensity of triangle networks formed by triplets of nodes is calculated as follows:

$$c_i = \frac{1}{k_i(k_i-1)} \sum_{j,k} \frac{\sqrt[3]{w_{ij}w_{jk}w_{ik}}}{\max(w)}$$ Eq. 4

where k_i is the number edges connected to node *j* (36 in our case) and *w* is the edge weight. The average clustering coefficient for a network C is the average of all c_i:

$$C = \frac{1}{N} \sum c_i$$ Eq. 5

where N is the total number of nodes.

Average path length

Path length for the 3D culture network was computed as described by Muldoon et al., 2016 using their published MATLAB script (Muldoon et al., 2016). Briefly, the shortest distance *d* between node i to node j is inversely proportional to edge weight (w) and is defined as:

$$d_{ij} = \frac{1}{w_{ij}}$$ Eq 6.

The average path length (PL) for a network PL_N, with number of nodes N is calculated as:

$$PL_{i,j} = \frac{1}{N(N-1)} \sum_{i \neq j} d_{ij}$$ Eq 7.

Number of modules and modularity

Module assignment of nodes and modularity were computed as described in Newman, 2006 using their published MATLAB script (Newman, 2006). Briefly, modularity is the sum of edges falling into a module minus the sum of expected edges in an equivalent random network. Module assignment is based on maximizing modularity of the network.

Node degree and determination of hubs

To calculate the node degree for each node, the sum of edge weights incident to the node was calculated. The frequency distribution (%) of individual weight node degree in all samples under the same condition was calculated with bin size = 0.05 using GraphPad Prism.

$$Weighted\ node\ degree_i = \frac{1}{A} \sum_{i \neq j} w_{ij}$$ Eq. 8

To test if the frequency distribution of weighted node degree followed a normal distribution, a Kolmogorov-Smirnov normality test was performed.

Statistical analysis

Statistical tests were performed using GraphPad Prism. Statistical significance in changes in neurite density (Fig. 2C) and mRNA expression (Fig. 2D and 3A) were tested with ANOVA with post-hoc Tukey tests. For changes in neuronal activity (Fig. 4E, F) between different cultures at 2 and 3 weeks, unpaired t-tests were used. Differences in neuronal activity (Fig. 5B-D) and network properties (Fig. 6C-F) between baseline and post-treatments of each individual 3D tissue were determined with paired t-tests. A p-value of 0.05 was used for significance level.

Supplementary Figures

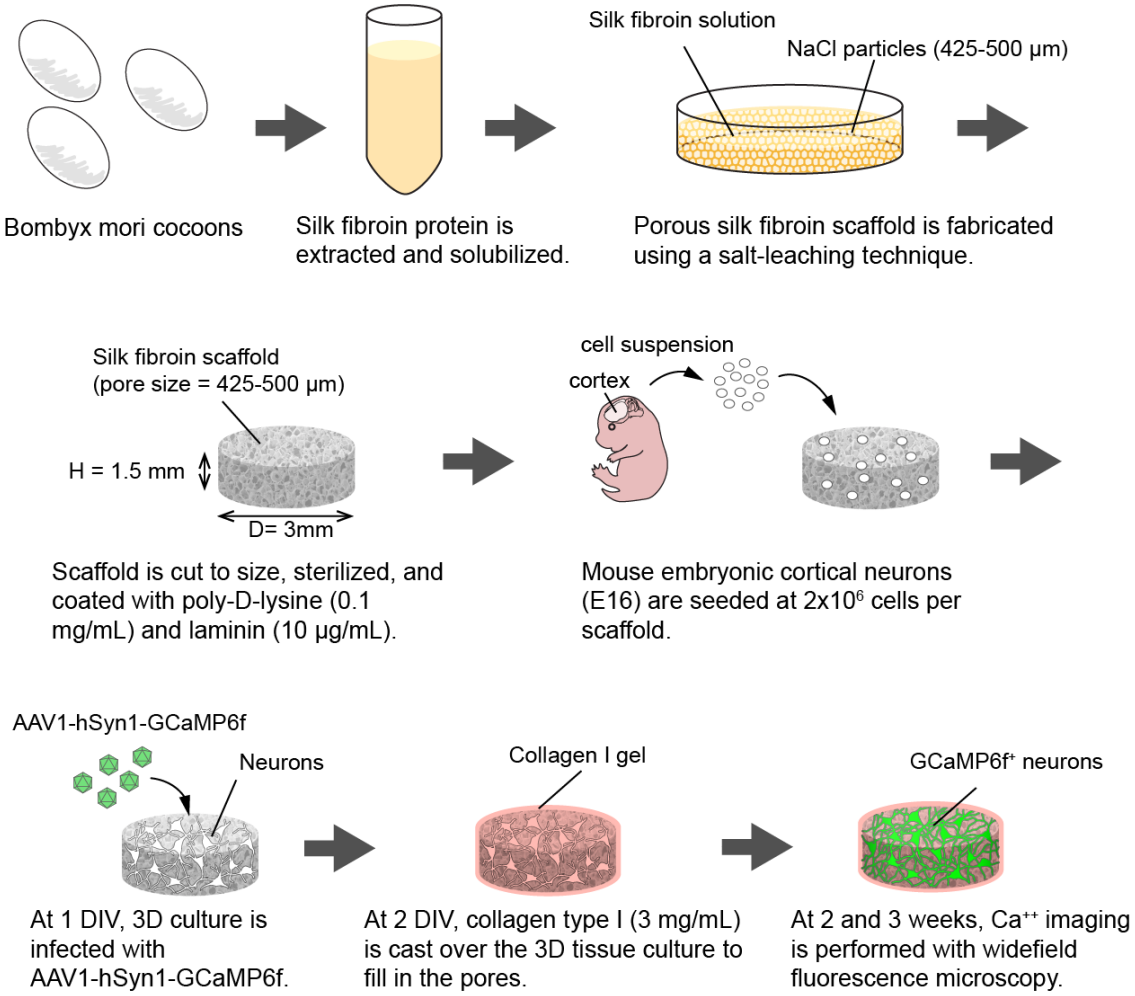

Figure S1. Schematic of the 3D *in vitro* cortical culture set up for cellular and network activity recording. Related to Figure 1.

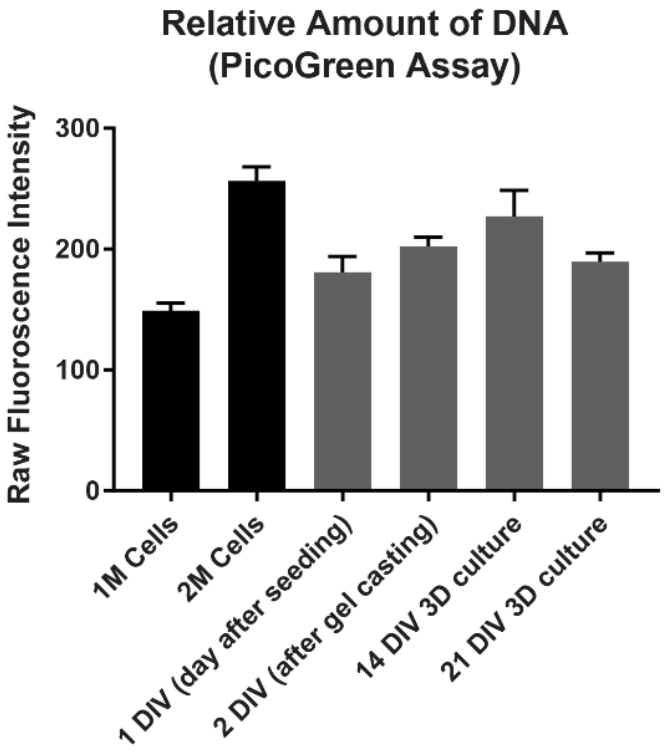

Figure S2. Relative cell numbers in 3D culture. DNA quantity in cortical cell pellets (1 and 2 million) and in 3D cultures were analyzed with PicoGreen DNA Quantification Kit. n = 3-5 samples. Related to Figure 1.

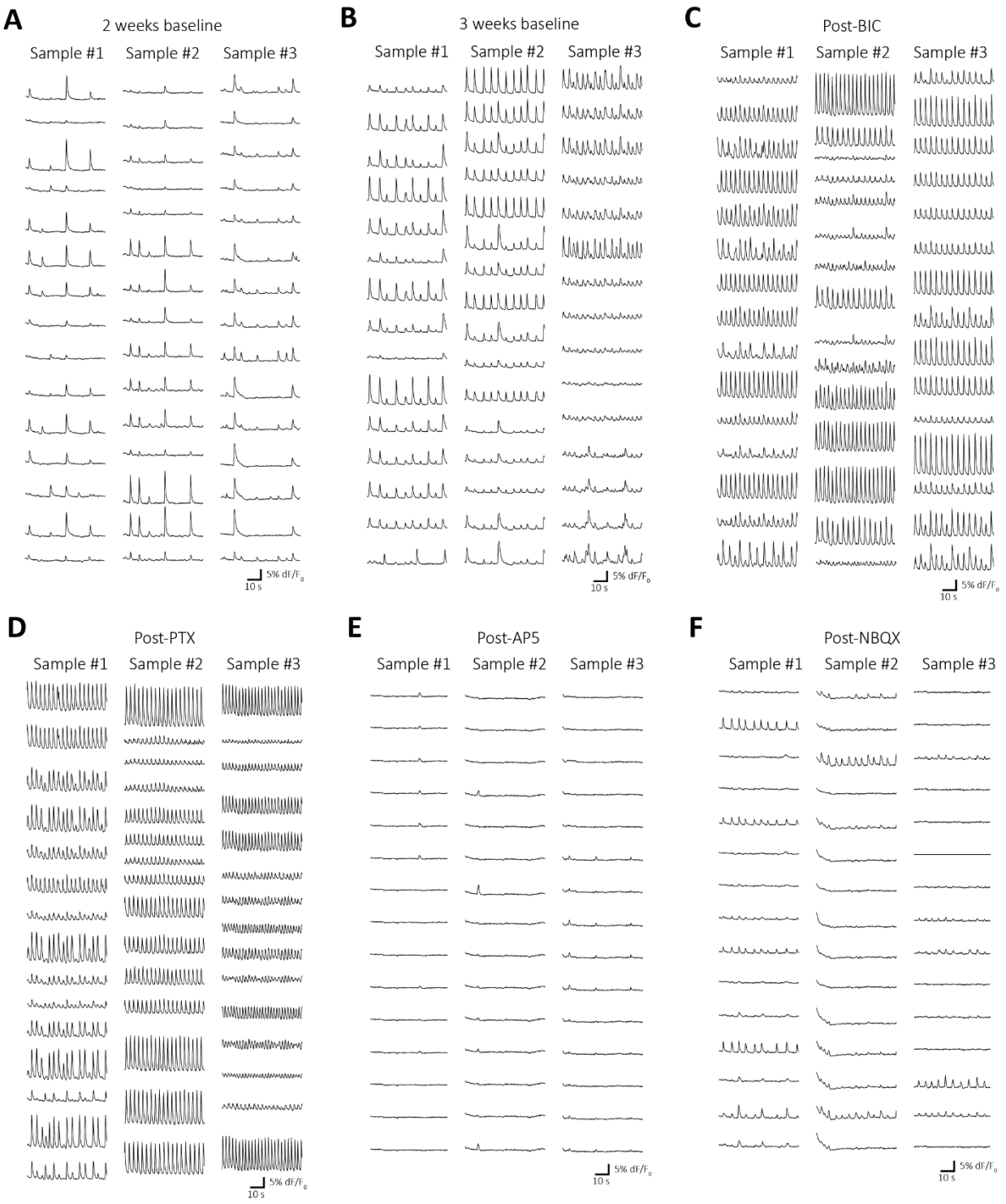

Figure S3. Example traces of Ca^{++} transients from three independent 3D *in vitro* biomimetic cortical cultures at (A) 2 weeks and (B) 3 week at baseline and post-drug treatments (3 wks only) of (C) bicuculline (BCC, 10 µM), (D) picrotoxin (PTX, 50 µM) (E) AP5 (50 µM), and (F) NBQX (5 µM). Each trace represents the average fluorescence intensity over time normalized to its basal fluorescence level (dF/F_0) from an individual hexagonal ROI (side-to-side distance 500 µm). Related to Figures 4-5.

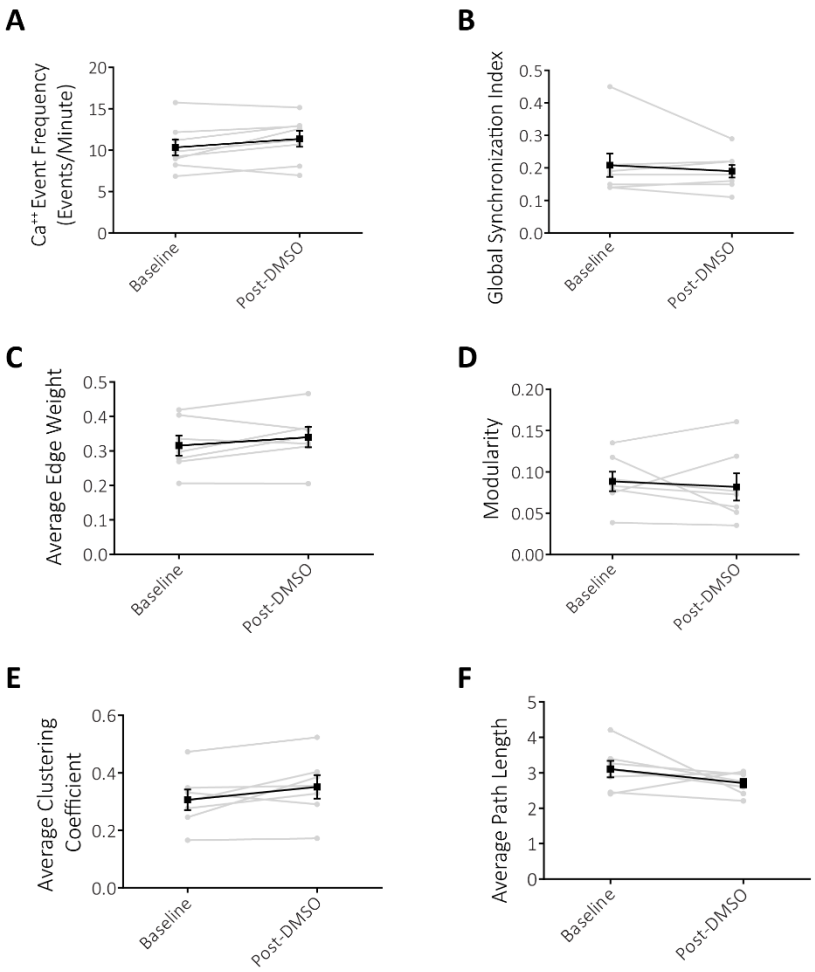

Figure S4. Control experiments with DMSO treatment, at the equivalent concentration (0.5%) as for BCC and PTX treatments, did not induce changes in (A) Ca^{++} event frequency, (B) global synchronization index, (C) average edge weight, (D) modularity, (E) average clustering coefficient, and (F) average path length. Grey data points and lines indicate baseline and post-treatment measurements of the same 3D tissue culture samples, and color lines show Mean ± SEM. n = 7 from two independent experiments. Related to Figure 5.

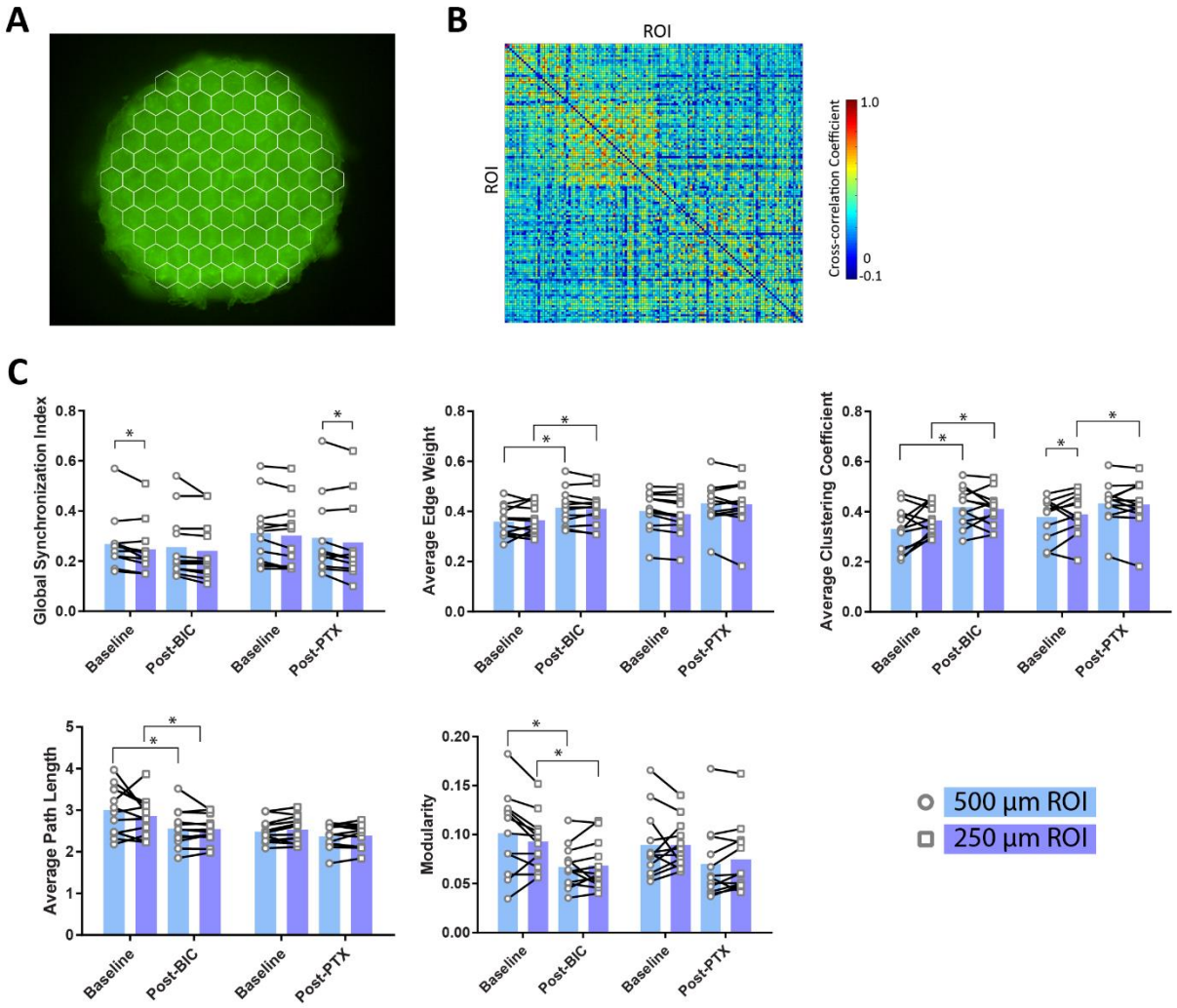

Figure S5. Network analysis with 500 µm vs 250 µm ROIs. (A) Time-lapse image stacks were applied with 127 hexagonal ROIs with side-to-side distance of 250 µm. (B) Representative cross-correlation matrix of neuronal activities in each ROI at baseline condition. (C) Network descriptors of global synchronization index, average edge weight, average clustering coefficient, average path length, and modularity, calculated based on using 500 µm or 250 µm ROI mask. Data are induvial values and Mean ± SD (n = 12 samples each group). Paired t-tests were used for statistical significance comparing the same condition with 500 µm or 250 µm ROI and for comparing baseline and post-treatment of the same sample with same ROI size. *p < 0.05. Related to Figure 6.

Drug		**Bicuculline**		**Picrotoxin**		**NBQX**		**AP5**	
Concentration		10 μM		50 μM		5 μM		50 μM	
Target and mechanism of action		Competitive $GABA_A$ receptor antagonist		Non-competitive $GABA_A$ and GABAρ receptors antagonist		Competitive AMPA receptor antagonist		Competitive NMDA receptor antagonist	
Sample Number		12		12		9		11	
Condition		Baseline	Post-Treatment	Baseline	Post-Treatment	Baseline	Post-Treatment	Baseline	Post-Treatment
% ROI	Min	94.59	100	89.18	100	94.59	16.22	89.19	0
	Median	100	100	100	100	98.65	48.65	100	32.43
	Max	100	100	100	100	100	100	100	100
Unpaired t-test		p = 0.3388		p = 0.3388		p = 0.0068		p < 0.0001	
Ca^{++} Event Frequency (events/min) (Mean ± SEM)		10.88 ± 1.10	15.41 ± 0.72	12.30 ± 1.10	17.80 ± 1.08	10.47 ± 1.05	2.68 ± 0.94	11.48 ± 1.61	0.35 ± 0.12
paired t-test		p = 0.0002		p = 0.0013		p < 0.0001		p < 0.0001	
Global Synchronization Index (Mean ± SEM)		0.269 ± 0.031	0.256 ± 0.037	0.312 ± 0.038	0.293 ± 0.045	0.268 ± 0.027	0.142 ± 0.035	n.a.	
paired t-test		p = 0.4105		p = 0.6981		p = 0.0273			
Average Edge Weight (Mean ± SEM)		0.360 ± 0.018	0.414 ± 0.021	0.402 ± 0.023	0.433 ± 0.025	0.394 ± 0.032	0.118 ± 0.051	n.a.	
paired t-test		p = 0.0134		p = 0.147		p = 0.0009			
Average Clustering Coefficient (Mean ± SEM)		0.331 ± 0.025	0.419 ± 0.023	0.380 ± 0.024	0.433 ± 0.025	0.358 ± 0.036	0.117 ± 0.043	n.a.	
paired t-test		p = 0.0057		p = 0.0711		p = 0.0005			
Average Path Length (Mean ± SEM)		3.01 ± 0.168	2.56 ± 0.13	2.49 ± 0.09	2.37 ± 0.08	n.a.		n.a.	
paired t-test		p = 0.0331		p = 0.2740					
n. of Modules (n. of samples)	with 3 modules	4	3	3	2	2	0	n.a.	
	with 2 modules	8	9	9	10	7	6		
	with 1 module	0	0	0	0	0	3		
Modularity (Mean ± SEM)		0.102 ± 0.012	0.067 ± 0.006	0.090 ± 0.010	0.070 ± 0.011	0.083 ± 0.018	0.083 ± 0.184 (N. Mod > 1)	n.a.	
paired t-test		p = 0.0173		p = 0.2043		p = 0.0202 (N. Module >1)			

Table S1. Results summary of neuronal activity measurements and functional network descriptors of the 3D *in vitro* biomimetic cultures at baseline and post-bicuculline, picrotoxin, NBQX, and AP5 treatments at 3 weeks. Related to Figures 4-6.

Gene	Encoded Protein	ThermoFisher Taqman Assay ID
Dcx	Doublecortin	Mm00438400_m1
Gabra1	Gamma-aminobutyric acid type A receptor alpha1 subunit	Mm00439046_m1
Gad2	Glutamate Decarboxylase 2	Mm00484623_m1
Gria1	Glutamate Ionotropic Receptor AMPA Type Subunit 1	Mm00433753_m1
Grin1	Glutamate Ionotropic Receptor NMDA Type Subunit 1	Mm00433790_m1
Pax6	Paired Box Protein Pax-6	Mm00443081_m1
Rn18s	18s Ribosomal RNA, 45s Ribosomal RNA	Mm03928990_g1
Shank3	SH3/ankyrin domain gene 3	Mm00498775_m1
Slc17a7	Vesicular glutamate transporter 1	Mm00812886_m1
Slc1a3	Excitatory amino acid transporter 1	Mm00600697_m1
Syn1	Synapsin I	Mm00449772_m1

Table S2. Taqman assays used in qRT-PCR in this study. Related to Figure 2.

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