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Supplemental Information

Extension of retinofugal projections in an assembled model of human pluripotent stem cell-derived organoids

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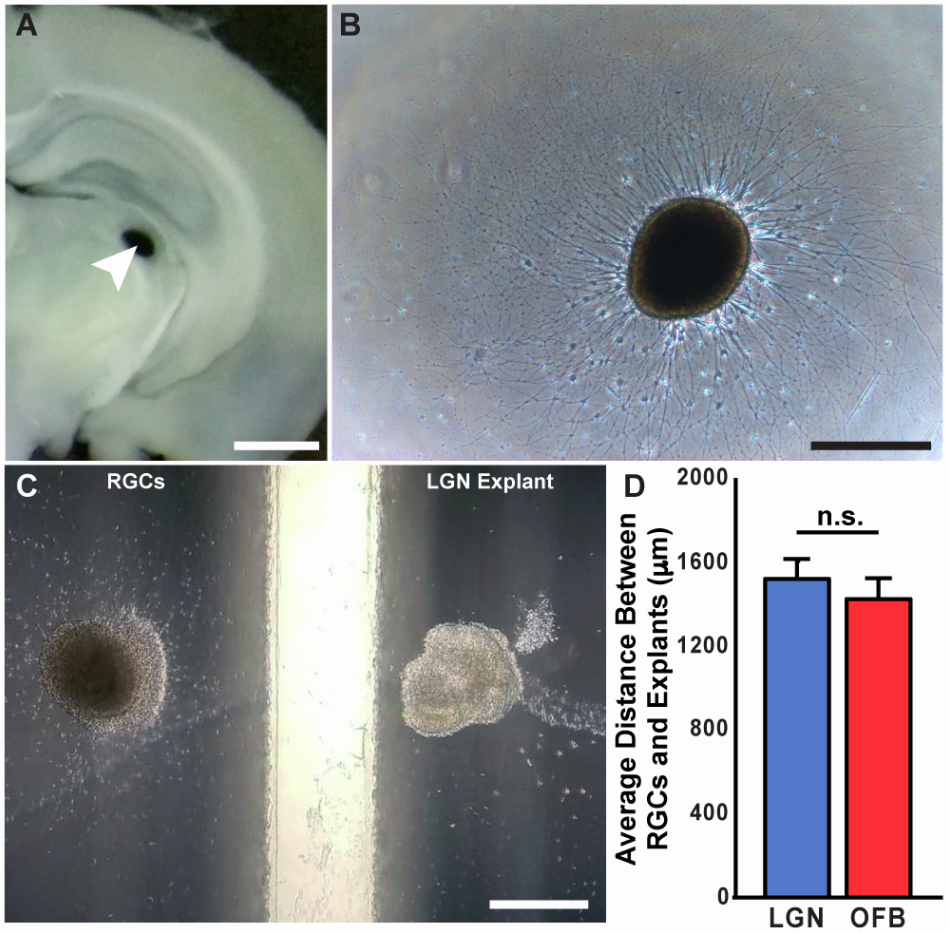
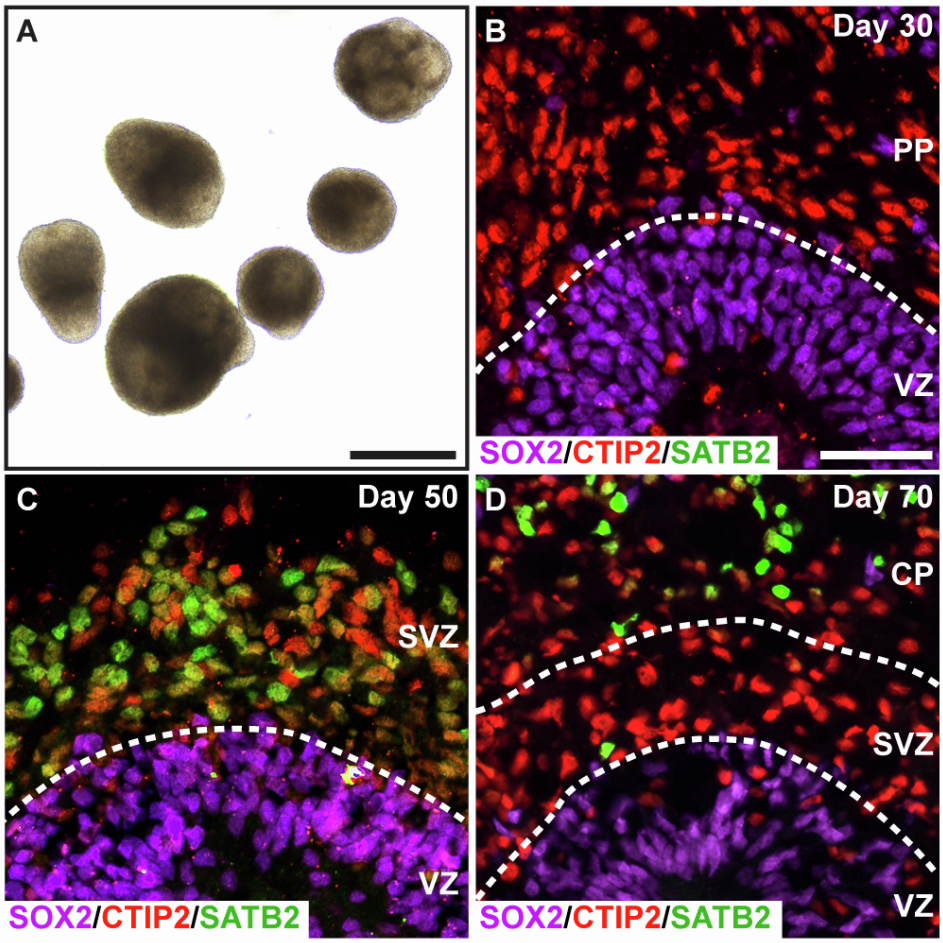
Supplemental Table 1: Primary Antibodies Used in the Study

Antibody	Species	Company	Catalog Number	Dilution
Caspase-3	Rabbit	Promega	G7481	1:200
CHX10	Goat	Santa Cruz	SC-21690	1:200
CTIP2	Rat	Abcam	ab18465	1:500
Ki-67	Mouse	BD Biosciences	550609	1:500
OTX2	Goat	R&D Systems	AF1979	1:000
PAX6	Mouse	DSHB	PAX6	1:50
Recoverin	Rabbit	Millipore	AB5585	1:2000
S100β	Mouse	Abcam	ab11178	1:200
SATB2	Rabbit	Abcam	Ab34735	1:500
SOX2	Goat	R&D Systems	AF2018	1:1000
TCF7L2	Rabbit	Cell Signaling Technology	2569	1:200

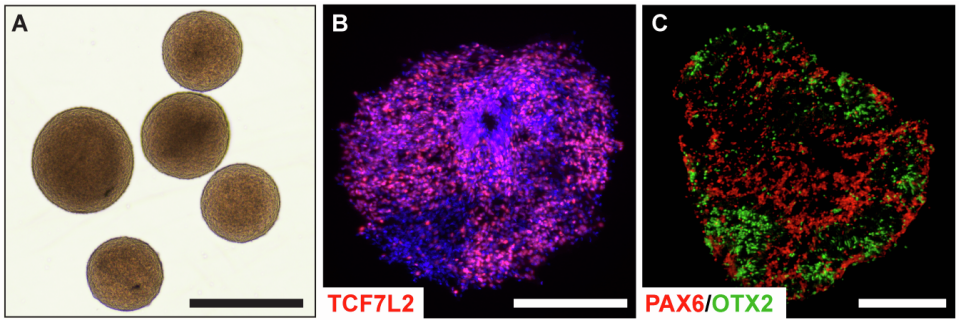
Supplemental Figures



Supplemental Figure 1: **Establishment of co-cultures between retinal organoids and LGN or olfactory bulb explants.** (A) Explants were obtained from coronal sections of P1-P3 mouse brains, with explants from either LGN (pictured, arrowhead) or olfactory bulb extracted with a tissue punch. (B) Explant cultures of mouse LGN survived and extended lengthy neurites over the first week of growth. (C) Co-cultures were initially established between retinal organoids and explant cultures with a culture insert separating the two populations. (D) The distance between retinal organoids and either LGN or olfactory bulb explants was not significantly different across experiments. Scale bars equal 800 um in A, 200 um in B and C.



Supplemental Figure 2: **Differentiation of cortical organoids from hPSCs**. (A) Brightfield image of cortical organoids. (B-D) Sections through cortical organoids from 30-70 days of differentiation demonstrate primitive layering of cell types. PP = Preplate, VZ = Ventricular zone, SVZ = Subventricular zone, CP = Cortical plate. Scale bars = 1 mm in A, 25 um in B. Scale bar in B applies to C and D.



Supplemental Figure 3. Differentiation of thalamic organoids from hPSCs. (A) Brightfield image of thalamic organoids. (B-C) hPSC-derived thalamic organoids broadly express the thalamic-associated marker TCF7L2, as well as associated markers including PAX6 and OTX2.

Supplemental Experimental Procedures

Maintenance and Expansion of hPSCs

Undifferentiated cells were maintained and expanded as previously described (Fligor et al., 2020; Meyer et al., 2011), using Matrigel-coated 6-well plates and mTeSR1 medium (StemCell Technologies) until reaching approximately 70% confluency. Subsequently, cells were passaged using Dispase (2 mg/mL, Life Technologies) and split at a ratio of 1:6 every 4-5 days. Multiple lines of hPSCs were utilized in this study, including the H7, H9 and tiPS5 cell lines. Additionally, some of these lines were genetically modified to contain either a RGC-specific BRN3b:tdTomato reporter or a constitutive GFP reporter, as previously described (Sluch et al., 2017; VanderWall et al., 2020).

Differentiation of regional organoids

For retinal organoids, differentiation was initiated by lifting hPSCs from Matrigel-coated wells using Dispase (2 mg/mL). Cellular aggregates were maintained in suspension and gradually transitioned to a chemically defined neural induction medium (NIM), which consisted of DMEM/F12 (1:1), N2 supplement, MEM non-essential amino acids, heparin (2 ug/mL) and antibiotics. After 6 days, BMP4 (50 ng/ml) was added to induce retinal lineage differentiation. 2 days later, cellular aggregates were plated onto 6-well plates with 10% FBS to induce adhesion. Half media changes were performed on days 9 and 12 with a full media change occurring on day 15. After a total of 16 days of differentiation, cell aggregates were mechanically lifted and kept in suspension in Retinal Differentiation Medium (RDM), which consisted of DMEM/F12 (3:1), B27 supplement, MEM non-essential amino acids, and antibiotics. Retinal organoids were maintained in this medium until experimental time points indicated, and were screened for the expression of the BRN3:tdTomato reporter before use in subsequent analyses or to generate assembloids. Prosencephalic brain organoids were differentiated from hPSCs by excluding BMP4 from the medium at day 6 differentiation and EBs were plated on laminin-coated plates 2 days later, with all subsequent conditions maintained the same.

For differentiation of thalamic organoids, hPSCs were dissociated to a single cell suspension using Accutase for approximately 5 minutes. Single cells were resuspended in induction media (DMEM-F12, 15%

KSR, 1% MEM-NEAA, 1% Glutamax, 1% PSA and 100 μM β-Mercaptoethanol, 100 nM LDN-193189, 10 μM SB-431542, 4 μg/ml Insulin, 5% heat-inactivated FBS, and 50 μM Y27632) and aggregated in ultra-low-attachment 96-well plates (Nunc) at a density of 7,000 cells/well. Half media changes were performed every other day. After 8 days, aggregates were transferred to spinning culture (80 rpm/min) in 24-well low attachment plates (Corning) and maintained in patterning media (DMEM-F12, 0.15% Dextrose, 100 mM β-mercaptoethanol, 1% N2 supplement, 1% PSA, 2% B27 supplement minus vitamin A, 30 ng/ml BMP7 and 1 mM PD325901). Media was changed every other day until day 16, when differentiation media (1:1 mixture of DMEM-F12 and Neurobasal media, 0.5% N2 supplement, 1% B27 supplement, 0.5% MEM-NEAA, 1% Glutamax, 0.025% Insulin, 50 mM β-Mercaptoethanol, 1% PSA, 20 ng/ml BDNF and 200 mM ascorbic acid) with media changes every other day until day 25, and every four days thereafter.

Growth of hPSC-derived RGCs in microfluidic platforms

Retinal organoids were differentiated from hPSCs with an RGC-specific BRN3:tdTomato reporter, as previously described (Sluch et al., 2017; VanderWall et al., 2020). Within 45 days of differentiation, tdTomato-positive organoids were dissociated to single cells with Accutase and magnetically sorted with beads specific to the Thy1.2 cell surface antigen to yield a purified population of RGCs. These RGCs were then plated within microfluidic devices (Xona Microfluidics, XC450) at a density of 250,000 cells/device in BrainPhys medium (StemCell Technologies). To induce axonal recruitment into the contralateral chamber of each device, a volume differential between chambers was established, with the soma chamber containing 150 μl of BrainPhys medium, while the axonal compartment contained 100 μl, creating a net flow of medium toward the axonal chamber. Additionally, extra BDNF was added to the axonal chamber (total concentration 50 ng/ml) to recruit axons to cross over. Cultures were maintained in this state for up to 3 weeks, with media changes occurring every 2-3 days.

Animal Care and Brain Dissection

Pregnant female C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME, http://www.jax.org) and housed within the animal care facility at the School of Science at Indiana University-Purdue University Indianapolis. Within the first 2 days after birth, mouse pups were anesthetized with

isofluourane and sacrificed by decapitation. Brains were then immediately dissected out and placed in a solution of artificial cerebrospinal fluid (aCSF). Coronal sections were then cut on a vibratome at a thickness of 300 μm and collected for analysis on a Leica A60 stereomicroscope to anatomically identify the lateral geniculate nuclei. Upon identification, the dorsal LGN was isolated with a 0.5 mm tissue punch (Electron Microscopy Sciences) and explant cultures were subsequently established. All experiments involving mice were approved by the Institutional Animal Care and Use Committee within the School of Science at Indiana University Purdue University Indianapolis and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Co-culture of RGCs and Mouse Brain Explants

To establish co-cultures between mouse LGN explants and hPSC-derived RGCs, a two-chambered silicone culture insert (Ibidi) allowed for the LGN and RGCs to be separated by at least 500 um. Any samples in which the LGN and RGC aggregates were more than 10 mm apart were excluded. Explants were plated into the left chamber of two-chamber culture inserts and allowed to adhere for 48 hours in RDM. hPSCs with a BRN3:tdTomato fluorescent reporter were differentiated as described above and after 40 days of differentiation, fluorescing organoids were chopped to a reproducible size of approximately 200 um with a tissue chopper (McIlwain) and plated in the adjacent chamber. Following adhesion of LGN and RGCs, culture inserts were manually removed to allow the growth of RGC neurites towards LGN explants. RGCs and LGN explants were co-cultured for one week before fixation in 4% paraformaldehyde, with tdTomato expression allowing for the identification of RGC neurites. As controls, some cultures consisted of only RGC-containing retinal organoid cultures, while other control experiments used RGCs in co-culture with mouse olfactory bulb explants, in which the olfactory bulb was dissected out of the brain and explants were cultures similarly to LGN explants.

Immunocytochemistry

For cryostat sectioning, organoids and assembloids were fixed with 4% paraformaldehyde, washed 3x in PBS, and then equilibrated in 20% and 30% sucrose solutions overnight at 4°C. After reaching equilibrium, organoids and/or assembloids were embedded in OCT and frozen on dry ice, and sections were then cut

at 90 um thickness on a cryostat. Similarly, co-cultures grown on coverslips were fixed in 4% paraformaldehyde and washed 3x in PBS before staining. Immunocytochemical staining of all samples was performed as previously described. Briefly, samples were permeabilized in 0.2% Triton X-100 for 10 minutes and subsequently blocked in 10% donkey serum for one hour at RT. Primary antibodies (Supplemental Table 1) were diluted in 0.1% Triton X-100 and 5% donkey serum. Following overnight incubation at 4°C, samples were washed in PBS and blocked with 10% donkey serum for 10 minutes. Secondary antibodies were diluted 1:1000 in 0.1% Triton X-100 and 5% donkey serum and incubated for 1 hour at RT. Finally, cells were washed with PBS and mounted onto slides for analysis.

Measurement of axonal outgrowth in assembloid cultures

Axonal outgrowth was examined in sections of assembloids cut at 90 um thickness at 3, 5, and 7 days post-assembly (dpa). RGC axons were identified by the expression of tdTomato within assembloids. The degree of axonal outgrowth was quantified following methods previously described for thalamic organoids (Xiang et al., 2019). Briefly, the ranges of axonal outgrowth were classified into 3 categories: not approaching the midline of targeted organoid (r1), already crossing the midline of targeted organoid (r2), and already approaching the opposite tip of the targeted organoid (r3). 30 assembloids were generated and examined for each experiment, with 10 assembloids analyzed per timepoint. The percentage of axons extending to various ranges at specified timepoints were quantified for each experiment.

Growth Factor Array

Growth Factor Array analysis was performed using the C-Series Human Cytokine Antibody Array C1000 (RayBiotech #AAh-CYT-1000-2) following manufacturer's instructions. Briefly, brain organoids were lysed in the provided lysis buffer and diluted to a concentration of 500 ug/mL using the provided blocking buffer. Antibody membranes were subsequently blocked for 30 minutes at RT. After removal of blocking buffer, 1 mL of diluted sample was added to antibody membranes and incubated overnight at 4°C. The following day, membranes were washed with provided buffers and incubated with appropriate biotinylated antibody cocktails overnight at 4°C. The following day, membranes were once again washed and incubated in 1X HRP-Streptavidin for 2 hours at RT. Following a final wash, membranes were transferred onto blotting

paper and incubated in the provided detection buffer for 2 minutes at RT. Finally, dot blots were imaged using a chemiluminescence imaging system (Odyssey CLX, LiCor) and analyzed using the gel analysis function in Image J.

Statistical Analyses

For measurements of axonal outgrowth within microfluidic platforms, the number of axons entering the axonal compartment was quantified at 3, 5 and 7 days post plating. Multiple replicates (n=5) were obtained at each time point. A student's two-tailed t-test using the Holm-Sidak method determined significance between + and – BDNF, with *p* values less than 0.05 considered significant.

In order to analyze and quantify the effects of co-culturing RGCs with or without explant targets, RGC neurites were identified by tdTomato expression and neurites were traced using a semi-automatic ImageJ plug-in, NeuronJ. The mean length of neurites in each condition were calculated along with the standard error of the mean. One-way ANOVA statistical analyses at 95% confidence (post-hoc Tukey) was performed to determine significance compared to controls. Statistical significance was determined based on a *p* value less than 0.05. Sholl analysis was performed to determine complexity of neurite outgrowth. The number of neurites crossing each ring was quantified for each condition indicated (n=9). Grubb's test was used to remove outliers with an alpha of 0.05. One-way ANOVA followed by Tukey's post hoc or student's two-tailed t-test using the Holm-Sidak method determined significance between samples, with *p* values less than 0.05 considered significant. To determine specificity of outgrowth, the longest neurite in 10 degree sections was measured and compared to average neurite lengths of controls. One-way ANOVA statistical analyses at 95% confidence (post hoc Tukey) was performed, excluding outliers, to determine significance compared to the control. Statistical significance was determined based on a *p* value less than 0.05.

Within assembloid cultures, range index examination was calculated and significance was determined with a one-way ANOVA to compare each range at the time points indicated. Statistical significance was determined based on a *p* value less than 0.05.To determine the long-term effects of assembloids upon organoid growth, the retinal area and TdTomato intensity of retinal organoids was measure using FIJI ImageJ. Grubb's test was used to remove outliers with an alpha of 0.05. One-way

ANOVA followed by Tukey's post hoc determined significance compared to control. For proliferation and apoptosis studies, the overall area of Ki67 or Caspase-3 was measured and compared to controls using a student's two-tailed t-test using the Holm-Sidak method. A one-way ANOVA was once again used to determine the effectiveness of BDNF treatment on retinal organoids. For all tests, a p value of 0.05 was used was used to determine statistical significance. In order to analyze outgrowth within thalamic organoids, the number of axons crossing after just 3 days of fusion was quantified. A student's two-tailed t-test using the Holm-Sidak method determined significance between samples, with p values less than 0.05 considered significant. All statistical analyses were performed using Graphpad Prism software.

Supplemental References:

Fligor, C.M., Huang, K.C., Lavekar, S.S., VanderWall, K.B., and Meyer, J.S. (2020). Differentiation of retinal organoids from human pluripotent stem cells. Methods Cell Biol *159*, 279-302.

Meyer, J.S., Howden, S.E., Wallace, K.A., Verhoeven, A.D., Wright, L.S., Capowski, E.E., Pinilla, I., Martin, J.M., Tian, S., Stewart, R.*, et al.* (2011). Optic vesicle-like structures derived from human pluripotent stem cells facilitate a customized approach to retinal disease treatment. Stem Cells *29*, 1206-1218.

Sluch, V.M., Chamling, X., Liu, M.M., Berlinicke, C.A., Cheng, J., Mitchell, K.L., Welsbie, D.S., and Zack, D.J. (2017). Enhanced Stem Cell Differentiation and Immunopurification of Genome Engineered Human Retinal Ganglion Cells. Stem Cells Transl Med *6*, 1972-1986.

VanderWall, K.B., Huang, K.C., Pan, Y., Lavekar, S.S., Fligor, C.M., Allsop, A.R., Lentsch, K.A., Dang, P., Zhang, C., Tseng, H.C.*, et al.* (2020). Retinal Ganglion Cells With a Glaucoma OPTN(E50K) Mutation Exhibit Neurodegenerative Phenotypes when Derived from Three-Dimensional Retinal Organoids. Stem Cell Reports *15*, 52-66.

Xiang, Y., Tanaka, Y., Cakir, B., Patterson, B., Kim, K.Y., Sun, P., Kang, Y.J., Zhong, M., Liu, X., Patra, P.*, et al.* (2019). hESC-Derived Thalamic Organoids Form Reciprocal Projections When Fused with Cortical Organoids. Cell Stem Cell *24*, 487-497.e487.