Generation of Microglia-Containing APOE4 Cerebral Organoids as a Model of Down Syndrome-Associated Alzheimer's Disease

By

Rose Summers

Department of Integrative Physiology, University of Colorado Boulder

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Thesis Advisor:

Dr. Christopher Lowry, Department of Integrative Physiology

Defense Committee:

Dr. Christopher Lowry, Integrative Physiology

Dr. Lauren Chun, Psychology & Neuroscience

Dr. Tammy Maldonado, Integrative Physiology

Abstract

Interactions between microglia, the innate immune cells of the central nervous system, and the cholesterol carrier apolipoprotein E may drive neurodegeneration in Down syndrome-associated Alzheimer's disease and are promising potential therapeutic targets. However, much remains unknown about microglia in this context due to limitations of widely used animal and human monolayer cell culture models, necessitating the use of cerebral organoids as three-dimensional models of human brain tissue. Here, we hypothesized that cerebral organoids containing microglia could be generated from human induced pluripotent stem cells with trisomy 21 and the apolipoprotein E ε4 allele, the strongest genetic risk factor for Alzheimer's disease, and that these models have the potential to fill existing knowledge gaps on cellular neuropathology. We cultured neural and hematopoietic progenitor cells to generate microglia-containing cerebral organoids as models of Down syndrome-associated Alzheimer's disease. Immunohistochemistry revealed that hematopoietic progenitor cells failed to survive and mature into microglia in organoids, and that other methods should be tried for generating microglia-containing cerebral organoids with trisomy 21 and apolipoprotein E ε4 in the future. We hope that our findings will contribute to the eventual successful generation of these models for the advancement of medical care for people with Down syndrome-associated Alzheimer's disease.

Introduction

Alzheimer's disease (AD) is the most prevalent form of dementia in the United States and leads to progressive cognitive decline, diminished quality of life, and early mortality. People with Down syndrome (DS), the most common genetic cause of intellectual disability in the United States, have an increased risk of developing AD due to a triplication of the amyloid precursor protein (APP) gene on chromosome 21 which increases the production and accumulation of neurotoxic oligomers of amyloid-beta (Aβ). Importantly, inheritance of the ε4 allele of the apolipoprotein E gene (APOE4) is the strongest genetic risk factor for developing AD and increases the risk by approximately 15-fold (Kloske & Wilcock, 2020). Interactions between microglia and apoE may drive aspects of disease pathology in Down syndrome-associated Alzheimer's disease (DS-AD) (Chen et al., 2021), and may be a valuable therapeutic target for improving cognition and longevity in people with DS-AD. However, much remains unknown about the mechanisms underlying microglia-apoE interactions and their contributions to neuroinflammation and neurodegeneration in the human brain. In the context of studying the cellular neuropathology of AD and related disorders, cerebral organoids (CO) derived from human induced pluripotent stem cells (hiPSCs) offer several distinct advantages over other models. Furthermore, recent protocols for generating microglia-containing COs (MCOs) from co-culture have the potential to advance our scientific understanding and approaches to treatment of DS-AD.

The importance of studying DS-AD

AD is a complex, polygenic disease characterized by accumulation of neurotoxic Aβ oligomers, amyloid plaques, and neurofibrillary tau tangles. Recently, research has also revealed chronic neuroinflammation as a key aspect of AD pathology (Kinney et al., 2018). Even though no population is safe from potentially developing AD, certain populations of people have a significantly higher risk of developing AD relative to the general public. Although 40-80% of people with DS will develop AD by their 60s (Salehi et al., 2016), people with DS have historically been understudied due to discrimination against those with intellectual disability. Mechanisms underlying DS-AD are not yet fully understood, and no currently available drugs have proved capable of improving quality and length of life in people with DS-AD. Thus, it is essential that DS-AD be studied further, and that new therapeutic targets be identified to improve the treatment of this highly vulnerable population.

The role of microglia in DS-AD

Microglia, the resident immune cells of the brain, display altered phenotypes in both DS and AD independently, as well as in DS-AD. Brain tissue from people with DS shows upregulated levels of neuroinflammatory cytokines such as IL-1β and IL-6 throughout the lifespan (Flores-Aguilar et al., 2020), perhaps caused by glial dysfunction due to increased Aβ load resulting from APP triplication. DS microglia have larger cell bodies and shorter cell processes than healthy microglia (Martini et al., 2020), and this phenotype is associated with an activated, pro-inflammatory microglial state. While microglia in AD can play an important beneficial role in clearance of Aβ and tau from the brain, it appears that microglia

in AD may also become dysregulated and contribute to chronic neuroinflammation, which worsens Aβ and tau accumulation while driving neurodegeneration (Uddin & Lim, 2022). In support of the idea that microglia-apoE interactions may drive this neuroinflammation, APOE4 has been shown to induce a unique microglial response to Aβ (Fitz et al., 2021). Interestingly, one study concluded that depleting microglia in mice eliminated the effects of APOE allele on neurodegeneration (Shi et al., 2019). Ultimately, the study of microglia-apoE interactions in the context of DS-AD is essential for eventually improving patient care.

Modeling DS-AD in microglia-containing cerebral organoids

Much of our understanding of DS-AD is based on rodent models or human monolayer cell culture, creating a formidable knowledge gap in the field regarding the human DS-AD brain. Animal and human neural cells differ substantially on a biological and physiological level, and animals do not develop the same neuropathologies as human patients. As a result, all animal models of DS and AD are approximations with many limitations, with one study finding that many animal models of AD were unable to recapitulate aspects of the disease with accuracy or reproducibility (Saito & Saido, 2018). While monolayer cell culture allows for the study of human cell types with mutations associated with DS and AD, it can lack biological relevance as it fails to recreate the complex three-dimensional structure of human brain tissue (Jorfi et al., 2018). COs offer distinct advantages in that they are three-dimensional structures derived from hiPSCs of patients with DS and AD, which can be altered with CRISPR/Ccas9 gene editing to model specific disease-associated genotypes; COs can then be cultured to contain multiple relevant cell types and capture biologically relevant aspects of neurological development such as the formation of neural rosettes.

Neural rosettes are radial structures of neural progenitor cells (NPCs) capable of differentiating into neurons and astrocytes and may be analogous to the formation of the spinal cord during secondary neurulation during *in-vivo* embryonic development (Fedorova et al., 2019). COs are generated through driving cells to the neuroectodermal lineage to generate only neurons and astrocytes, glial cells with complex supportive functions in the central nervous system. However, recent protocols for integrating microglia derived from the mesodermal lineage have been published. Specifically, a protocol for generation of MCOs through the co-culturing of NPCs and hematopoietic progenitor cells (HPCs) to mimic the migration of HPCs from the yolk sac to the CNS during embryonic development (Xu et al., 2021) can create an advantageous MCO model for DS-AD. Importantly, microglia in this model have been shown to function in synaptic pruning and phagocytic activity, suggesting that they may perform similar biological functions in MCOs as they do in the human brain.

Goal of the study

The goal of the study was to develop MCOs that would uniquely allow for the study of DS-AD pathology in a model of the human brain containing human neurons, astrocytes, and microglia. To optimize reproducibility, we implemented commercial kits for the generation of NPCs and HPCs. We predicted that this model would offer the benefits of replicating human-specific disease processes with enhanced biological relevance compared to MCOs generated through other protocols due to how co-culture mimics specific aspects of embryonic development. We hypothesized that differentiating hiPSCs with trisomy 21 and APOE4 into HPCs and NPCs and co-culturing them in media

containing neuron and microglial maturation factors would generate MCOs capable of modeling interactions between microglia and apoE in DS-AD.

Materials and Methods

hiPSC generation and characterization

One line of APOE3/3 cells with trisomy 21 and one line of APOE3/3 cells disomic for chromosome 21, named UWWC1-DS1 and UWWC1-DS2U respectively, were obtained from an APOE3/3 donor mosaic for trisomy 21 and were converted into hiPSCs by the WiCell research institute. APOE and chromosome 21 genotype for both cell lines was verified by karyotyping through G-band staining. Subsequently, CRISPR-Cas9 gene editing was utilized to create an APOE4/4 line with trisomy 21 from UWWC1-DS1. The resulting APOE4/4 line was validated through karyotyping, PCR, and gel electrophoresis. MCOs were also generated from hiPSCs disomic for chromosome 21 with APOE3/3 and with trisomy 21 and APOE3/3 simultaneously to control for any potential effects of trisomy 21 and APOE4 on the protocol.

hiPSC culture

All hiPSC lines were maintained in an incubator at 37 °C with 5% CO_2 and ~95% humidity. Cells were cultured on 6 well plates coated with Corning Matrigel (Thermofisher) in mTeSR medium (Stem Cell Technologies™) and were passaged weekly with ReLeSR passaging reagent (Stem Cell Technologies™) before being seeded into mTeSR supplemented with 10 μM Y-2762 Rock inhibitor (Thermofisher).

Differentiation of hiPSCs to neural and hematopoietic progenitors

Utilizing the STEMdiff Hematopoietic differentiation kit (Stem Cell Technologies™), all three lines of hiPSCs were differentiated into HPCs. Similarly, utilizing the STEMdiff SMADi Neural Induction Kit (Stem Cell Technologies™) and monolayer culture protocol, all three lines of hiPSCs were differentiated into NPCs. HPCs were used in co-culture EB formation on day 12 of the STEMdiff protocol, while NPCs underwent at least 3 passages and were used in co-culture EB formation between day 20 and day 28.

Co-culture and organoid maintenance

On day 12 of progenitor differentiation, HPCs were collected from supernatant and NPCs were single-celled with accutase. 7,000 NPCs and 3,000 HPCs, as previously described by Xu et al. (2021), were then placed into each well of a non-adherence 96-well cell culture plate in a 1:1 combination of HPC and NPC induction medium described by Xu et al. (2021) and supplemented with 10 μM Y-2762 and left at 37°C for 3 days. This ratio was chosen to yield a microglial population of approximately 8%, which lies within the 5%-15% observed in human brain tissue (Zhang et al., 2023). Resulting embryoid bodies (EBs) were transferred through a wide-bore P200 pipette tip to a 6-well plate in 1:1 HPC and NPC medium for another 8 days, with media changes occurring every 2 to 3 days. On day 11, the organoids were moved to an orbital shaker and switched to the maturation medium described by Xu et al. (2021). Organoids were then kept in maturation medium, with full media changes every 3 to 4 days until collection of 3 organoids from each genotype group on day 41. This time point was chosen because Xu et al. (2021) had previously shown that

co-culture organoids contained cells expressing PU.1, a transcription factor for microglial differentiation, by day 35 following co-culture.

Preparing organoids for immunohistochemistry

Maturation medium was suctioned off at time of collection, and organoids were washed briefly in 1x PBS. Organoids were then fixed in 4% paraformaldehyde in 1x PBS for 45 minutes while rotating on an orbital shaker. After being washed in 1x PBS again briefly, organoids were incubated in 6.23% sucrose in H_2O for 4 hours at room temperature, 12.5% sucrose in H_2O for 12 hours at 4°C, and 25% sucrose in H_2O for 24 hours at 4°C to remove all water from the tissue. Organoids were then transferred to tissue-tek OCT (Sakura Finetek USA), frozen in liquid nitrogen, and then stored at -80°C until sectioned. Resulting blocks were sectioned at 20 µM thickness in a cryomicrotome at -25°C, and sections were mounted onto charged glass slides to be stored at -80°C.

Immunohistochemistry

Slides mounted with organoid sections were thawed to room temperature and lined with ImmEdge hydrophobic border pen (Vector Laboratories). Then, they were rinsed with 1x PBS to remove OCT and submerged in 3% BSA and 0.1% Triton X-100 for 1 hour at room temperature to increase permeability of the tissue and prevent non-specific binding. Primary antibodies in 3% BSA and 1x PBS were incubated for 12 hours at 4°C for 12 hours in darkness. The following day, slides were rinsed again in 1x PBS, and secondary antibodies were left to incubate for 45 minutes at room temperature in darkness.

Fluoromount-G (Southern Biotech) containing 4',6-diamidino-2-phenylindole (DAPI) was applied during cover slipping, and finished slides were stored at 4°C until imaged.

Results

HPCs fail to integrate into organoids and mature into microglia

We utilized antibodies against the purinergic receptor P2RY12 and the ionized calcium-binding adaptor molecule 1 (Iba1), expressed by microglia and macrophages, to test for the presence of microglia in organoids 41 days after co-culture (Figure 1). Co-culture organoids showed the same lack of Iba1 antibody binding as negative control organoids generated from NPCs alone, while positive controls of human brain tissue confirmed that the antibodies were capable of binding to Iba1 when present (Figure 2). P2RY12 antibody appeared to bind to cellular somata in organoids generated through co-culture and in human brain tissue, but the antibody could not have been binding to microglia as the signal was also observed in NPC-only organoids that could only possibly contain cells from the neuroectodermal lineage (Figure 3). In addition to these antibodies, numerous other microglial antibodies were tested in IHC experiments including those directed against Iba1, TMEM119, CD45, and CD11c (Table 1), and despite many of them binding well to microglia in human brain tissue, none of them bound specifically to cells in MCOs (data not shown).

NPCs integrate successfully into organoids, form neural rosettes, and mature into astrocytes and neurons

Cells in co-culture organoids express astrocyte marker glial fibrillary acidic protein (GFAP) and neuron marker microtubule-associated protein 2 (MAP2), as well as containing radially structured neural rosettes of neural progenitor cells with apical basal polarity (Figure 4). Utilizing human brain tissue as a positive control, we confirmed that our GFAP antibody could bind to human astrocytes (Figure 5). Utilizing cerebral organoids previously generated through use of the STEMdiff Cerebral Organoid Kit (Stemcell Technologies™) as a positive control, we confirmed that our MAP2 antibody was fully functional and able to bind to human MAP2+ neurons when present (Figure 6).

Discussion

The lack of Iba1+ and P2RY12+ microglia in 41 day-old cerebral organoids indicates that the methods utilized herein were not successful in generating MCOs. It is possible that the commercial kits used in the generation of NPCs and HPCs were not compatible with the co-culture protocol and resulted in the lack of microglial differentiation and/or survival. While our method of generating HPCs from hiPSCs took only twelve days, the original co-culture protocol utilized a method that generated primitive macrophage progenitors after a period of two to three weeks and allowed the cells to proliferate for approximately three months (Haenseler et al., 2017). If the HPCs generated here were at a different stage in development than the HPCs utilized in the original protocol by Xu et al. (2021), the supplements in the co-culture induction and maturation media may not have been sufficient for directing their survival and/or growth.

While the lack of microglia negates the utility of these organoids in the study of microglia-apoE interactions, the presence of mature neurons, astrocytes, and neural rosettes indicates that the organoids may still provide valuable insights into neuron-apoE and astrocyte-apoE interactions in DS-AD. Neurons can secrete Aβ and tau, modeling Aβ oligomer, plaque, and tau tangle accumulation in DS-AD. Neural rosettes indicate that neural progenitor cells are still capable of differentiating into new astrocytes and neurons at day 41, and thus the organoids will continue to survive and grow past this time point. Furthermore, astrocytes can secrete and respond to apoE to drive pathological neuroinflammation similarly to microglia (Lanfranco et al., 2021), so they are additional potential therapeutic targets and are valuable to study in the context of DS-AD.

One limitation of this experiment is the exclusive use of IHC, a purely qualitative assay, for the analysis of the cellular composition of organoids. Another limitation is that the data presented here is from only one time point, which does not allow for observation of changes in cellular composition across organoid development or the possibility of microglia being present in organoids at a later age. Future studies should utilize more quantitative assays such as flow cytometry or quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) for measuring relative ratios of astrocytes, neurons, and microglia in order to validate biological relevance of organoids, and organoids should be collected over a period of several months to observe cellular development and allow for age-related Aβ and tau accumulation.

Additionally, because the co-culture protocol described may have failed to generate MCOs for a variety of different reasons, future studies should not only repeat this method

with alternative protocols for NPC and HPC generation but should also attempt to generate MCOs from hiPSCs with trisomy 21 and APOE4 utilizing alternative published protocols. One study has found that microglia developed innately within cerebral organoids generated through the original Lancaster & Knoblich protocol (2014), and that microglial yield could be increased through simply reducing heparin concentration in media and delaying the time of Matrigel embedding (Ormel et al, 2018). Although this result has not been consistently replicated by other labs following the Lancaster protocol, it could offer a potentially affordable and efficient method for generating MCOs. Similarly, macrophage precursors have been co-cultured with pre-formed COs to generate MCOs (Sabate-Soler et al., 2022). These are several of the many protocols that have been developed in recent years for successfully generating MCOs. Ultimately, future studies should optimize and reattempt the progenitor co-culture method described herein, or try the various alternative protocols currently available, to generate MCOs for modeling DS-AD.

Once DS-AD MCOs have been successfully generated, an interesting future direction would be to use them to test drugs identified in blocking apoE and decreasing AD pathology, such as imipramine and olanzapine (Johnson et al., 2022) to determine if blocking microglia-apoE interactions decreases Aβ plaques and tau tangles over time. Additionally, anti-inflammatory drugs, such as *Mycobacterium vaccae* (*M. vaccae*), which has been indicated as a potential therapy for AD (Loupy et al., 2020), can also be applied to these MCOs. It has been hypothesized that *M. vaccae* may cross through the blood-brain barrier, which is increasingly permeable in inflammatory conditions such as AD (Haruwaka et al., 2019), in dendritic cells capable of then presenting the microbes to

microglia to exert an anti-inflammatory effect within the brain. To study this mechanism in MCOs, dendritic cells derived from genetically matched hiPSCs could be exposed to *M. vaccae* and then co-cultured with MCOs.

Conclusion

Although this study has failed to generate MCOs through NPC and HPC co-culture, we hope that our findings will contribute to the eventual successful generation of MCOs from hiPSCs with trisomy 21 and APOE4 and that these models will advance medical care for those with DS-AD. Additionally, the COs generated herein can be utilized in the study of neuron and astrocyte-related DS-AD pathology and treatment.

Table 1

Antibodies with manufacturer, catalog #, and dilution used in this study

Antibody	Manufacturer	Catalog #	Dilution
Iba1	Wako	019-19741	1:100
Iba1	Abcam	ab178846	1:100
Iba1	Abcam	ab178847	1:100
Iba1	Abcam	ab5076	1:100
P2RY12	Atlas Antibodies	HPA014518	1:100
Iba1	Synaptic Systems	234003	1:100
Iba1	PhosphoSolutions	91-AIF1	1:100
TMEM119	Abcam	ab209064	1:50
CD45	Thermo Scientific	14-9457-82	1:50
CD235a	Thermo Scientific	PA5-27154	1:50
MAP2	Millipore Sigma	M9942	1:500
GFAP	Millipore Sigma	AB5804	1:500

*Iba1 is the ionized calcium-binding adaptor molecule 1 expressed by microglia and macrophages.

*P2RY12 is a purinergic receptor expressed exclusively by microglia.

*TMEM119 is a transmembrane protein expressed exclusively by microglia.

*CD45 is a tyrosine phosphatase expressed by cells of hematopoietic origin.

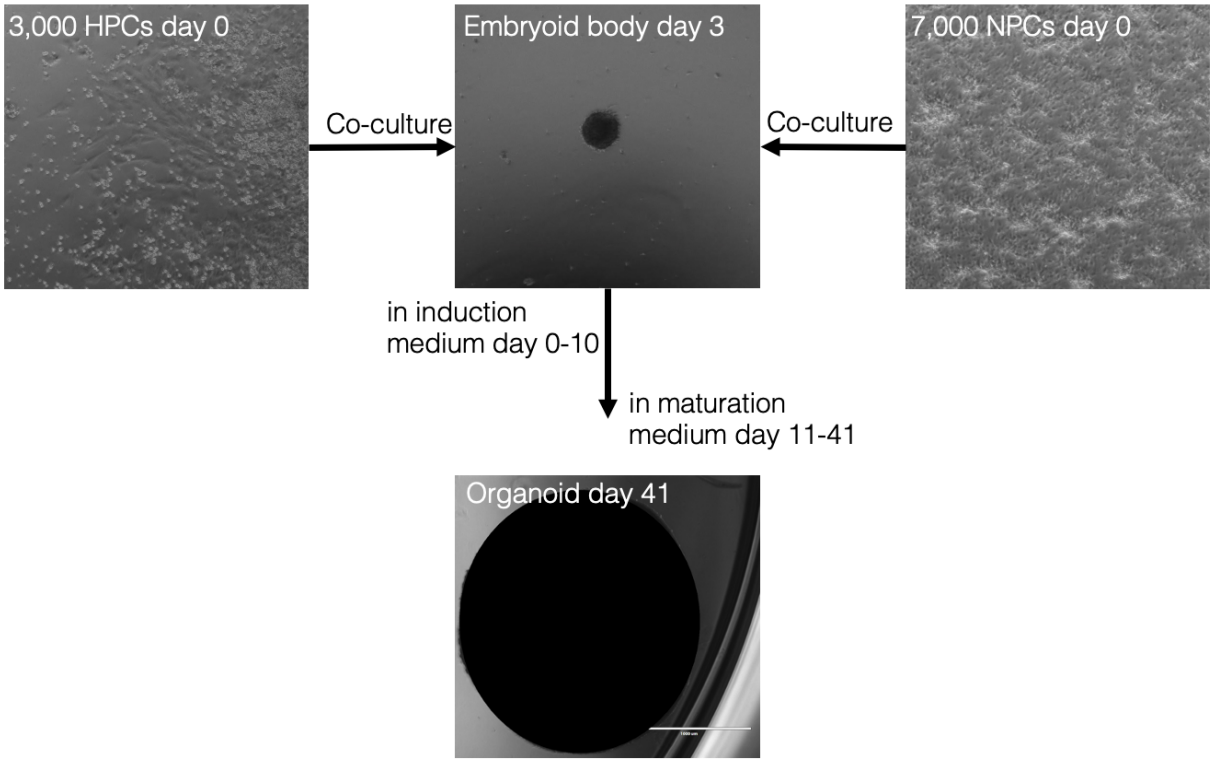
*CD235a is also known as glycophorin A and is expressed by cells of hematopoietic origin.

*MAP2 is a microtubule associated protein localized in the perikarya and dendrites of neurons.

*GFAP is glial fibrillary acidic protein expressed by astrocytes.

Figure 1

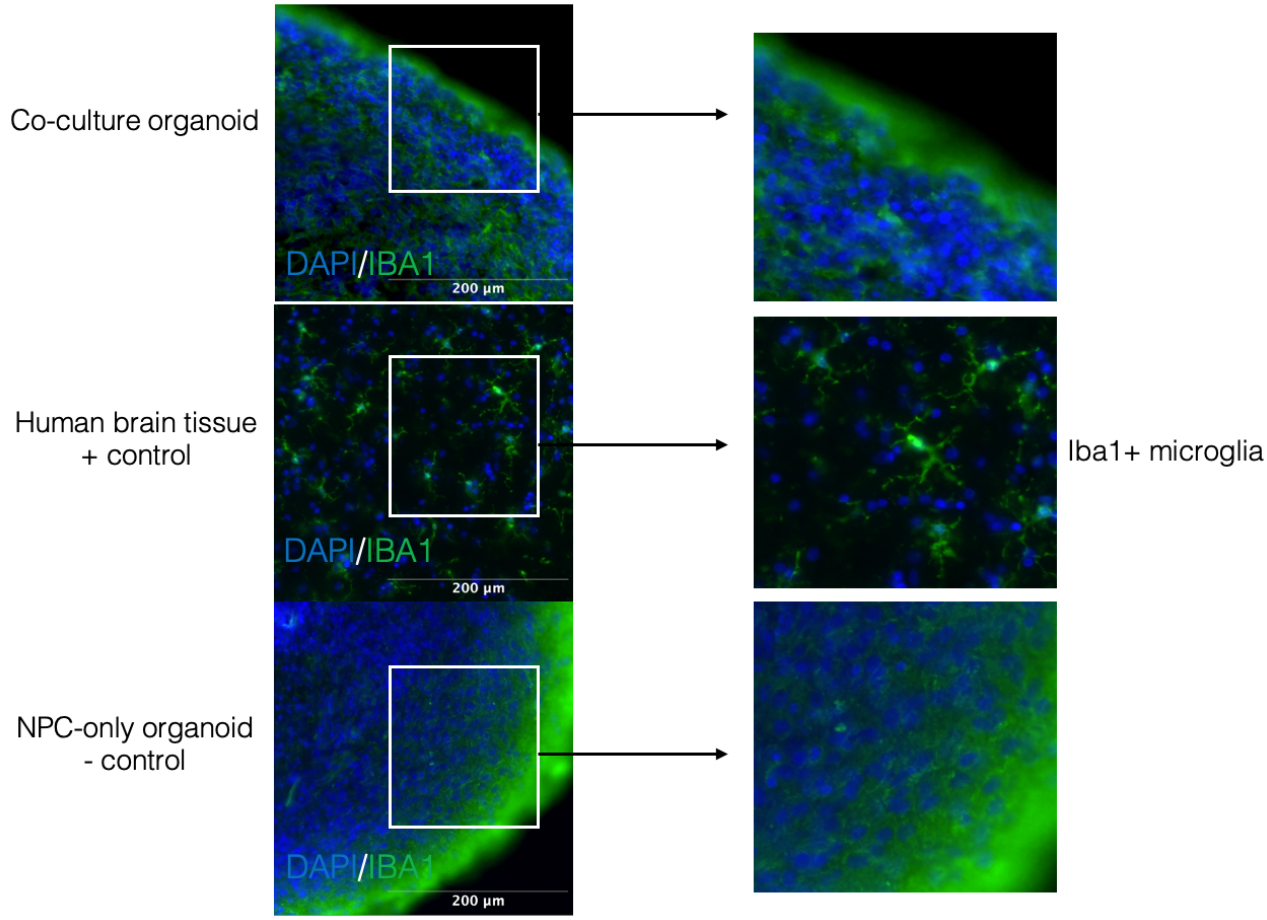
Co-culture diagram



HPC and NPC co-culture on day 0 lead to EB formation by day 3, with the organoids growing in size until collection on day 41. From day 0 to day 10, the cells and EBs were maintained in induction medium described by Xu et al. (2021). From day 11 to collection on day 41, the organoids were maintained in maturation medium described by Xu et al. (2021).

Figure 2

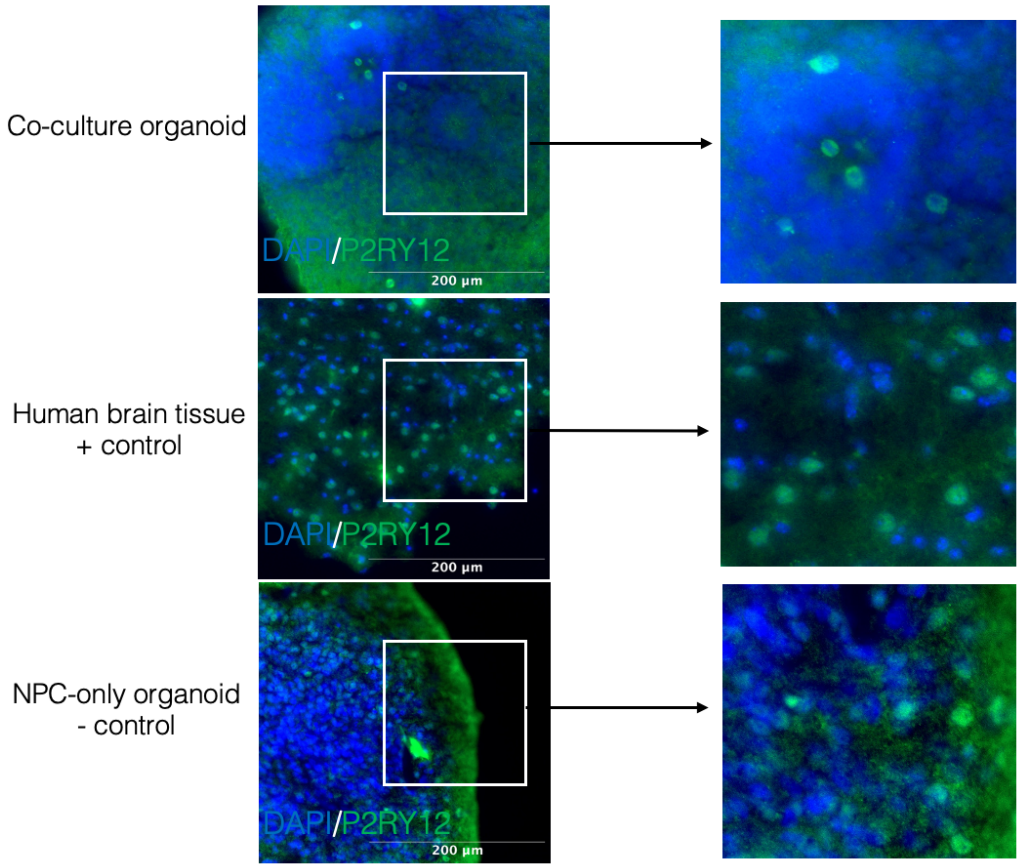
Iba1+ cells in a co-culture organoid, human brain tissue and an NPC-only organoid



Binding of anti-Iba1 antibody (Abcam) in a co-culture organoid section, human brain tissue, and an NPC-only organoid section, in green. DAPI, in blue, stained the location of individual cell nuclei.

Figure 3

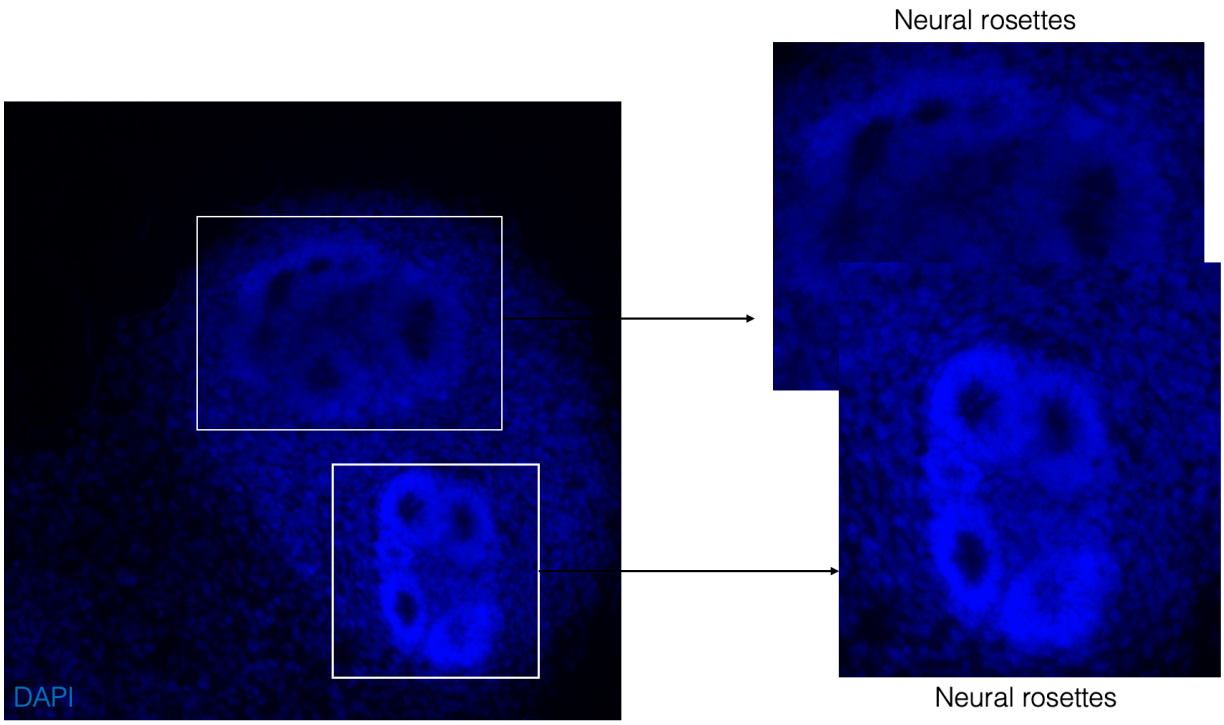
P2RY12+ cells in a co-culture organoid, human brain tissue and an NPC-only organoid



Non-microglial soma-localized binding of anti-P2RY12 antibody (Atlas Antibodies) in a co-culture organoid section, human brain tissue, and an NPC-only organoid section, in green. DAPI, in blue, stained the location of individual cell nuclei.

Figure 4

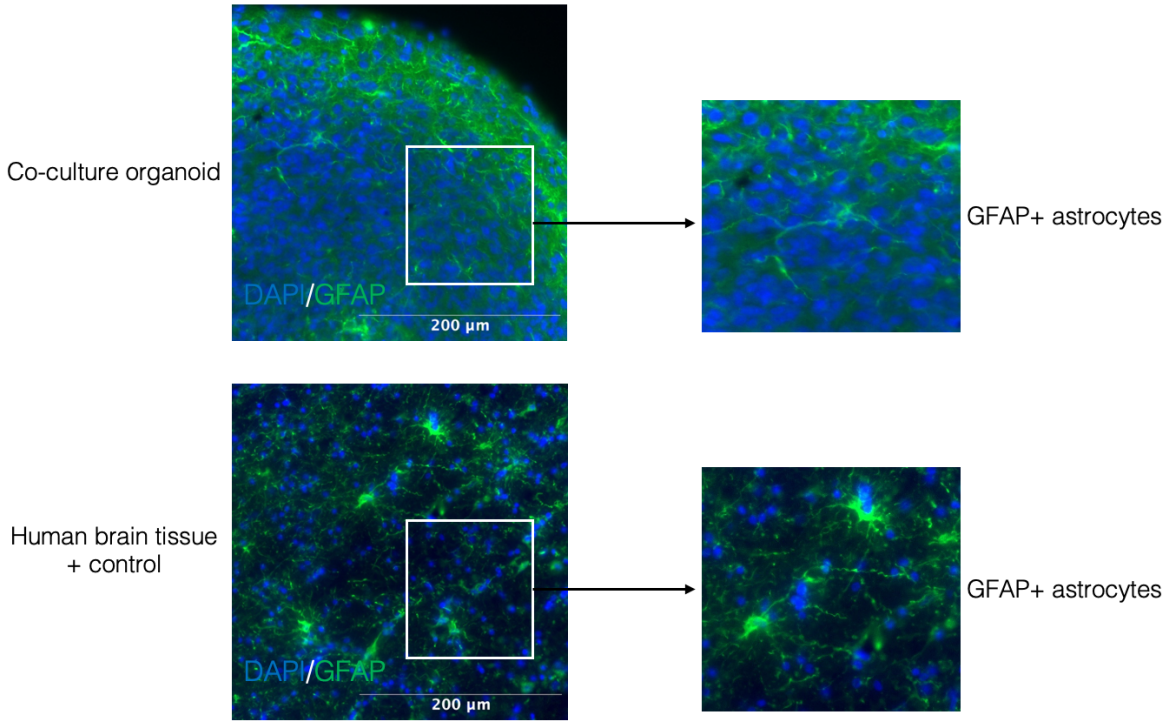
Neural rosettes in a co-culture organoid



Nuclear DAPI staining, in blue, of a co-culture organoid section revealing presence of multiple neural rosettes.

Figure 5

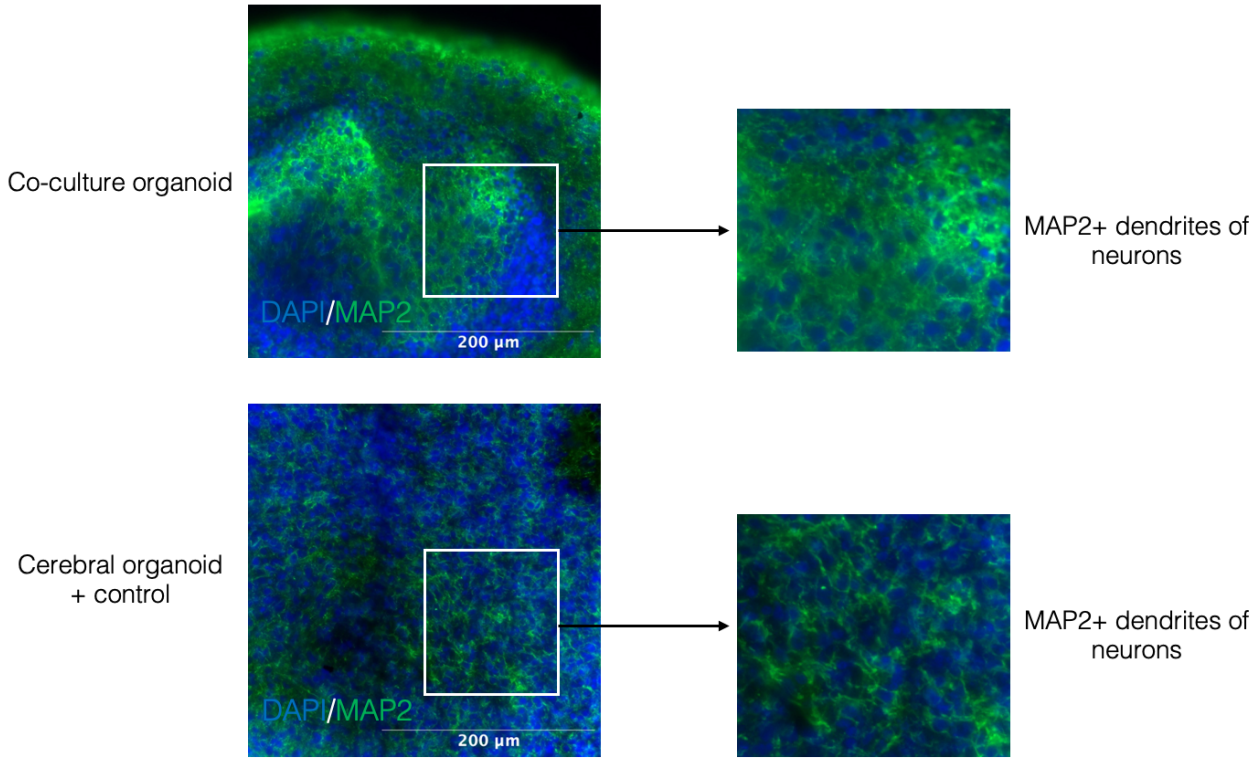
GFAP+ cells in a co-culture organoid, human brain tissue and an NPC-only organoid



Binding of anti-GFAP antibody (Millipore Sigma) to astrocytes in a co-culture organoid section, human brain tissue, and an NPC-only organoid section, in green. DAPI, in blue, stained the location of individual cell nuclei.

Figure 6

MAP2+ cells in a co-culture organoid and cerebral organoid



Binding of anti-MAP2 antibody (Millipore Sigma) to dendrites of neurons in a co-culture organoid section and a positive control cerebral organoid section, in green. DAPI, in blue, stained the location of individual cell nuclei. The cerebral organoid positive control was known to contain neurons and astrocytes as their presence had been validated through previous IHC assays.

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