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Tubular Human Brain Organoids to Model Microglia-Mediated Neuroinflammation

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Abstract

Human brain organoids, 3D brain tissue cultures derived from human pluripotent stem cells, hold promising potential in modeling neuroinflammation for a variety of neurological diseases. However, challenges remain in generating standardized human brain organoids that can recapitulate key physiological features of a human brain. Here, we present a tubular organoids-on-a-chip device to generate better organoids and model neuroinflammation. By employing 3D printed hollow mesh scaffolds, our device can be easily incorporated into multiwell-plates for reliable, scalable, and reproducible generation of tubular organoids. By introducing rocking flows through the tubular device channel, our device can perfuse nutrients and oxygen to minimize organoid necrosis and hypoxia, and incorporate immune cells into organoids to model neuro-immune interactions. Compared with conventional protocols, our method increased neural progenitor proliferation and reduced heterogeneity of human brain organoids. As a proof-of-concept application, we applied this method to model the microglia-mediated neuroinflammation after exposure to an opioid. We found isogenic microglia were activated after exposure to an opioid receptor agonist (DAMGO) and transformed back to the homeostatic status with further treatment by a cannabinoid receptor 2 agonist (LY2828360). Importantly, the activated microglia in tubular organoids had a stronger cytokine response compared to those in 2D microglial cultures. Our tubular organoid device is simple, versatile, inexpensive, easy-to-use, and compatible with multiwell-plates, so it can be widely used in common research and clinical laboratory settings. This technology can be broadly used for basic and translational applications in inflammatory diseases including substance use disorders, neural diseases, autoimmune disorders, and infectious diseases.

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⁶Conflict of Interest

The authors declare no conflict of interest.

Keywords

Microfluidics; Human Brain Organoid; Tubular Organoids; Neuroinflammation; Microglia

1. Introduction

Neuroinflammation plays a critical role in neurodegenerative diseases, autoimmune diseases as well as in substance abuse.¹ As the resident myeloid cells of the brain, microglia orchestrate brain inflammatory responses.² Homeostatic microglia survey the brain, perform synaptic pruning, facilitate synapse maturation, and phagocytize debris.³ Upon stimulation by factors such as toll-like receptor (TLR) ligands, β -amyloid (A β) aggregates, complement factors, or opioids, microglia can shift from homeostatic state to an activated state. Activated microglia can either secrete pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-1 β , and IL-18 or anti-inflammatory cytokines such as IL-4, IL-10, and transforming growth factor (TGF)- β depending on the dose, frequency, and context of the stimulation.^{4–7} Microglia can also shift between different states during disease progression.^{8,9} Microglia communicate extensively with astrocytes and neurons to shape the local immune environment.^{10–12} Particularly, during substance abuse, microglia-mediated neuroinflammation can exert direct neural toxicity and potentially exacerbate addiction via modulation of synapse plasticity.^{13,14} Despite the importance of microglia in neuroinflammation, the robust human model is in great need to interrogate how microglia mediate neuroinflammation in response to abused drugs and how they contribute to drug abuse-related pathology at mechanistic levels.

Current *in vitro* neuroinflammation studies are largely based on 2D cultures, which lack a complex brain microenvironment to preserve the native state of microglia, as well as the complex cross-talk between neurons and glial cells. Animal microglia are often inaccessible to real-time imaging, and cannot fully reflect human biology.^{15–17} Specifically, microglia modulate neuroinflammation differently in mice and humans in response to inflammatory cytokines. For example, mouse microglia respond to interferon- γ (IFN γ) by increasing the expression levels of class II major histocompatibility complex proteins (MHCII) whereas human microglia increase human leukocyte antigen (HLA). This IFN γ -induced response can be blocked by TGF β 1 in mice,¹⁸ but not in human microglia.¹⁹ Moreover, colony-stimulating factors have been shown to have opposite effects on MHCII, and subsequently, on neuroinflammation, in mouse and human microglia.^{20,21} Because increased MHCII and HLA levels are associated with neuroinflammatory response to disease and injury in mouse and human brains, mouse models can be limited in their usefulness in extrapolating the results of several therapeutic approaches into the clinic.

Human brain organoids are 3D cultures derived from human pluripotent stem cells and recapitulate native brain cellular components, cytoarchitecture, and exhibit neural activity.^{22–24} However, most current brain organoids lack mesoderm-derived microglia, prohibiting their application to model neuro-immune interactions. So far, several attempts have been reported to introduce microglia into brain organoids and 3D neural cultures. For example, a tri-culture system with neurons, astrocytes, and microglia was developed to study

their interactions in an Alzheimer's model.²⁵ The microglia derived from human induced pluripotent stem cells (iPSCs) carrying Alzheimer's disease-related APOE4 variant were co-cultured with human brain organoids to study their uptake of A β .²⁶ Moreover, iPSC derived microglia were integrated into brain organoids to study their responses to needle induced injury.²⁷ Functionalized brain-region specific organoids with isogenic microglia were established to study the transcriptome differences between microglia incorporated into dorsal and ventral brain regions.²⁸ These microglia/organoid systems may allow new insights that recapitulate key features of microglia, including phagocytosis, synaptic pruning, and responses to inflammatory cues.²³ More importantly, the patient iPSCs derived microglia/organoid systems may recapitulate disease-related signature phenotypes of an individual patient. However, these microglia/organoid models still suffer from (1) variation of organoid size and composition; (2) confounds stemming from organoid hypoxia/necrosis; and (3) physical damage from microglia injection.^{29–31} Thus, it is urgent and important to improve current microglia/organoid models to investigate neuro-glia interaction and neuroinflammatory diseases.

Recently, pioneering attempts have been made to improve current organoid cultures. For example, an air-liquid interface culture method was reported to reduce hypoxia and promote axon tract growth by slicing agarose embedded organoids and then culturing them on a transwell culture insert.³² Similarly, a sliced neocortical organoid system was developed to reduce hypoxic cores and promote the generation of distinct cortical layers via the repeated slicing of brain organoids every 4 weeks to limit the slice thickness to under 500 μ m.³³ Moreover, vascularized organoids were engineered to reduce apoptosis and promote neuron maturation by differentiating wild-type human embryonic stem cells (hESCs) and engineered hESCs expressing human ETS variant 2 (to form vascular-like networks) within a pumping culture system.³⁴ The results of these promising biotechnologies highlight the importance of nutrient/oxygen perfusion as well as the elimination of hypoxia/necrosis on organoid development and function. Recent advances in microfluidics offer significant potential benefits to improve organoid cultures due to advantages such as low reagent consumption, high throughput, parallelization, and automation.³⁵ A series of perfusable microfabricated devices have been developed to facilitate fabrication, promote perfusion, and reduce hypoxia/necrosis of organoids. For example, micropillar-based devices were fabricated using soft lithography and PDMS fabrication technique to provide continuous perfused cultures for improving the differentiation of human brain organoids and modeling prenatal nicotine exposure.^{36, 37} 3D printed mini-bioreactors have been employed to enhance medium perfusion and increase throughput.^{38, 39} Organ-on-a-chip devices have also been employed to enable better medium perfusion and vascularization of organoids.^{40–42} We have integrated perfusable microfluidic chambers and an air-liquid interface culture strategy to generate uniform-sized human brain organoids with significantly reduced hypoxia/necrosis, and demonstrated their application in modeling prenatal abused drug exposure.⁴³ However, the above methods are still not sufficient to fully support microglia-organoid interaction studies, due to reasons such as low throughput, poor reproducibility, labor intensiveness, complicated fabrication, or difficulty in immune cell introduction.

Here, we report an innovative tubular human brain organoid model. We integrated 3D printed hollow mesh scaffolds into multiwell-plates for the reliable, scalable, and

reproducible generation of tubular organoids. Moreover, we introduced rocking flows to perfuse nutrients, oxygen, and immune cells for improving organoid cultures as well as modeling neuro-immune interaction. Our technology has the following advantages: (1) Excellent perfusion: The device provides perfusion of medium and oxygen from both the outside and an embedded hollow lumen in the center, limiting the distance between the medium and all cells within the organoid culture to $< 500 \mu\text{m}$. (2) Noninvasive immune incorporation: The device allows simple and effective perfusion to introduce immunocytes such as microglia while avoiding invasive injection or manipulation and preserving the native, homeostatic status of microglia. (3) Compatibility with common laboratory settings: The device can be inserted into multiwell-plates (24, 96, 384, or higher), followed by culturing in an incubator. If using a standard rocking platform, the device can provide constant flows for further improving medium/oxygen perfusion. (4) Simple and low-cost devices: our devices fabricated by 3D printing technique are simple and cost-effective, as compared to current microfluidic devices fabricated by complicated lithography and PDMS fabrication. Our device will enable the generations of standardized organoids in a scalable, robust, and reproducible manner, highlighting the broad applications in other 3D organoids such as intestinal, liver, lung, and tumor organoids.

2. Results and Discussion

Tubular organoid device.

Small molecules such as lipopolysaccharide (LPS) and opioids activate microglia to elicit neuroinflammatory responses.^{44–46} The activated microglia release cytokines such as IL-1 β and IL-18 through inflammasome mediated pathways which further promotes inflammatory responses in neurons, neural progenitor cells, and astrocytes (Figure 1a).^{47–50} To model this process, we set out to design a tubular organoid device that allows for hypoxic-free brain organoid culture with constant medium/oxygen perfusion (Figure 1b). Additionally, we integrated isogenic microglia into the tubular organoid to recapitulate microglial responses inside an environment approximating the native brain microenvironment (Figure 1c). To minimize hypoxia and reduce necrosis inside these human brain organoid cultures, we designed a 3D printed device to fabricate tubular brain organoids comprised of 3 main design components: (i) a tubular scaffold lumen with a meshed structure that has $250 \mu\text{m}$ perfusable gaps to allow effective perfusion of oxygen and nutrients as well as an infusion of microglia; (ii) Two flanking medium reservoirs that hold culture medium allowing for easy medium change; (iii) a bottom embryonic body (EB) holder with raised interspaced skirts with $250 \mu\text{m}$ gaps allowing for gas perfusion, all attached to a thin glass coverslip bottom allowing for easy imaging. When the top and bottom pieces are assembled, a loading basket is formed in between parts (i) and (iii) (Figure 2a). This basket can then be loaded with EBs allowing for organoid formation. We designed the basket size so that the space between the inner lumen and outside skirt is $500 \mu\text{m}$ wide, limiting tubular organoid lumen growth to less than $500 \mu\text{m}$ in thickness. We designed the reservoir chamber to hold $60 \mu\text{L}$ medium on each side. When placed onto a rocking platform with a rocking speed of 1 rpm, this induces a constant flow of $2 \mu\text{L}/\text{second}$. We validated this flow with fluorescent beads (Supplementary Video S1).

Characterization of hypoxic conditions within tubular 3D cultures.

To characterize on-chip 3D culture and cell viability, we first cultured Neuro-2a (N2a) mouse neuroblast spheroids as control and loaded them into the loading basket of our tubular device. The spheroids were fabricated with a 300 μm average diameter so that the loading basket with 250 μm perfusable gaps could hold the spheroids without them leaking out of the device. Over 2 days in culture, the spheroids merged to form a tubular shape. We fixed and sectioned the tubular N2a culture to confirm the formation of a tubular-shaped structure (Figure 2b). To confirm that the tubular brain organoid device could maintain high cell viability, we then assessed the viability of these tubular N2a cultures (Figure 2c). We found that these tubular 3D cultures exhibited good viability over a 7-day culture (Figure 2d). Next, we characterized the hypoxic core formation inside our tubular 3D culture versus traditional organoid culture using a hypoxia indicator dye (Figure 2e). We found that the tubular 3D cultures of N2a cells showed no detectable signs of hypoxia. Suggesting that the perfusion from both the inner lumen and outside skirt gaps provides sufficient oxygen (Figure 2f). Additionally, we analyzed HIF1 α expression inside conventional and tubular cerebral organoid culture, the tubular culture showed significantly lower HIF1 α expression ($p < 0.005$), indicating reduction of hypoxia conditions (Figure 2g).

On-chip fabrication of tubular human brain organoids.

To fabricate tubular organoids using our device, we adapted a human forebrain organoid fabrication protocol (Figure 3a).⁵¹ Briefly, EBs were generated with WA01 human pluripotent stem cells (hPSC) to 300 μm in diameter and were loaded into the loading basket on day 3. Neural induction was induced with dual-SMAD inhibitors dorsomorphin and A83. These EBs were then cultured inside the tubular organoid device with a neural induction medium for 4 additional days to allow for EB aggregation to form a tubular culture surrounding the inner lumen. After the tubular culture was formed, we loaded the inner basket with Matrigel to embed the organoid (Figure 3b). The medium was then switched to CHIR-99021 and SB-431542 containing medium to activate the Wnt signaling pathway and guide its forebrain identity. The organoids were cultured for 7 additional days inside the inner basket for neural epithelium expansion. The device was finally loaded onto a rocking platform to create inner lumen fluid flow for medium perfusion and mechanical stimulation, with N2 and B27 supplemented medium to support neuron growth. Overall, we observe a success rate of on-chip tubular organoid formation around 60%, a rate comparable with the intrinsic success rate (70%) with the cerebral organoid protocol.⁵²

Characterization of organoid development.

After maturing inside the tubular device for 35 days, we sought to interrogate the differences between tubular human forebrain organoids (thFOs) and conventional forebrain organoids (hFOs). We first performed PAX6/MAP2 staining to identify the distribution of ventricular/subventricular zone (VZ/SVZ) within the organoids (Figure 4a). During brain maturation, neural progenitor cells (NPC) develop within the VZ/SVZ and give rise to mature neurons that migrate to the cortical plate. This process is detrimentally impacted by hypoxia. We found that our thFO culture system allows the growth of more uniformly distributed, size-consistent VZ/SVZ zones as compared with the more random and heterogeneous VZ/SVZ

zones inside the conventional hFO (Figure 4b). Additionally, we interrogated if the increased oxygen and nutrient perfusion inside thFO promoted NPC proliferation. We labeled newly divided cells within the organoids by the cell proliferation indicator EdU (Figure 4c). We found a higher number and more even distribution of newly divided cells inside thFOs as compared with conventional hFOs (Figure 4c). This suggests that better perfusion and constant passive flow stimulation increases cell division and guides the VZ/SVZ zone to form more uniformly.

Gene expression analysis of organoids.

We then further interrogated in detail the effect of tubular culture on brain organoid differentiation by qRT-PCR. We not only compared the neural fate commitment of the brain-region specific human forebrain organoids under conventional and tubular organoid culture but also interrogated the impact of tubular culture on a less-guided human cerebral organoid (hCO) protocol.⁵³ Organoids from 16 days (early neural differentiation stage) and 35 days (mature neuron stage) were collected and lysed for RNA-extraction followed by gene expression analysis. We discovered that (1) tubular culture for organoids derived from both hFO and hCO protocols have higher expression of dorsal forebrain neural progenitor cell markers PAX6 and SOX2 as well as the deep cortical neuron marker TBR1 ($p<0.05$), with the tubular culture showing less variation across organoids ($n=5$) (Figure 5a). (2) the hCO has a more heterogeneous differentiation with the expression of both dorsal and ventral forebrain differentiation markers as well as expression of some midbrain/hindbrain and even slight mesodermal and endodermal layer markers, with the diversity of differentiation increasing over time in culture (day 16 versus day 35). In comparison, under tubular organoid culture with constant flow perfusion, tubular human cerebral organoids (thCOs) showed higher dorsal forebrain fate commitment, with lower gene expression for other brain regions, consistent with other reports that cultured hCO with enhanced surface perfusion.⁵⁴ Additionally, as expected, the more guided human forebrain differentiation protocol limited organoid differentiation fate toward dorsal forebrain in both conventional protocols as well as the tubular culture protocol. With the tubular culture method, higher expression of PAX6, SOX2, and Tuj1 was detected in thFOs as compared with conventional hFOs, indicating an enhanced neural fate commitment as well as better neural differentiation (Figure 5b).

Incorporation of isogenic microglia into tubular organoids.

Brain organoids, especially region-specific forebrain organoids, lack microglia which are derived from embryonic mesoderm. To study microglia function and its relevance in neuroinflammation, we derived syngeneic induced microglia (iMG) from WA01 hPSC and introduced them into tubular hFOs. We confirmed iMG derived from WA01 by double staining with Iba1 and CD68 (Supplementary Figure S1). We discovered that iMG have lower baseline activation as compared with immortalized microglia like HMC3 and were significantly more activated upon LPS treatment followed by ATP, which facilitates NLRP3 inflammasome assembly (Supplementary Figure S2).⁵⁵ We then loaded the iMG into the medium reservoir of our tubular organoid device. Over a 3-day co-culture period, iMG migrated and integrated into tubular hFO and showed a homeostatic state ramified morphology (Figure 6a).

Characterization of induced microglial activation.

To test whether iMG incorporated inside thFO can respond to an inflammatory stimulus and recapitulate key aspects of neuroinflammation, we treated iMG infused thFO with LPS/ATP, which has been shown to activate microglia. Post LPS/ATP treatment, we observed the ramified microglia became activated and transformed into an amoeboid-like morphology (Figure 6b). To quantitatively analyze this activation, we adapted microglia morphometric analysis criteria including *fractal dimension*, which indicates the complexity of the branching pattern of microglia; *lacunarity*, which indicates heterogeneity and variance in microglia shape as well as *convex-hull circularity*, which indicates the shape circularity of microglia. (Figure 6c). We found that, upon LPS/ATP treatment, iMG infused into our tubular organoids displayed a lower complexity in patterns as well as a spherical shape, exemplified by lower fractal dimensions, lower lacunarity, and higher convex-hull circularity (Figure 6c).⁵⁶ We then tested if iMG infused thFO responded to opioid exposure. Opioids such as morphine were shown to activate microglia.⁵⁷ Here, we treated iMG infused thFO with DAMGO, a synthetic μ -opioid receptor agonist, at a concentration of 100 nM for 24 hours. We discovered that indeed, post-DAMGO treatment, iMG (identified by Iba1 staining) displayed an amoeboid morphology similar to that following LPS/ATP treatment (Figure 6b,c). Interestingly, this activated morphology can be partially reversed by treatment of cannabinoid receptor 2 (CB2) agonist LY2828360 (Figure 6b,c). CB2 is widely expressed in microglia in the central nervous system and has been interrogated as a potential target to treat neural inflammation.⁵⁸ This result demonstrated that CB2 agonist can partially rescue the activated microglia morphology by DAMGO in our 3D co-culture system.

Characterization of inflammasome activation.

Microglia in vivo were shown to trigger neuroinflammation upon opioid exposure via NLRP3 inflammasome mediated pathways.⁵⁹ Upon opioid exposure, the TLR4 receptor on microglia can trigger colocalization of NLRP3 and ASC with activating caspase-1 to produce IL-1 β , IL-18, and TNF- α , which in turn triggers neurons and astrocytes to secrete pro-inflammatory cytokines, causing neuroinflammation (Figure 7a). To interrogate whether iMG infused into our tubular organoid culture also recapitulate this process, we analyzed NLRP3 inflammasome activation inside the infused iMG cells. After the treatment of each compound, we sectioned and stained the tubular organoid sections for iMG with an Iba1 marker. We quantified the colocalization of NLRP3 and ASC via proximity ligation assay. Colocalized NLRP3/ASC spots were identified by amplified dotted signals from the ligation assay. We quantified the NLRP3/ASC colocalization spots within the infused iMG post LPS/ATP, DAMGO, and DAMGO/LY2828360 treatment conditions. As we expected, NLRP3/ASC colocalization is significantly increased in LPS/ATP and DAMGO treatment conditions. Such elevated inflammasome activation was partially rescued by LY2828360 treatment (Figure 7b,c).

Characterization of cytokine levels.

We also analyzed IL-1 β and IL-18 secretion as downstream effectors of NLRP3 inflammasome activation. We found that, upon LPS/ATP treatment, activated iMG also secreted higher IL-1 β and IL-18, which indicates a functional output of inflammasome

activation (Figure 7d). Interestingly, TNF- α , a key mediator in neuroinflammation was not detected in high levels in 2D iMG cultures post LPS/ATP treatment. However, when we incorporated iMG into tubular brain organoids and then treated the culture with LPS/ATP, we observed a significant increase in TNF- α secretion. This could be explained by iMG cross-talk with neurons and astrocytes inside the tubular organoids, inducing a higher level of neuroinflammation and a significantly higher production of TNF- α , from not only iMG themselves but also potentially from neurons and astrocytes inside a tubular organoid culture. This is yet another advantage of our neural inflammation model. Finally, the elevated levels of proinflammatory cytokines post-opioid exposure were dampened by LY2828360 treatment (Figure 7d). Thus, we demonstrated that iMG infused to thFO could recapitulate key aspects of neuroinflammation including microglia morphology change, NLRP3 inflammasome activation as well as cytokine production. We also demonstrated that this model could be used to study opioid-induced neural inflammation and the efficacy of therapeutics.

3. Conclusion

In summary, we reported novel tubular organoids to model neuroinflammation. Our tubular organoid culture eliminated hypoxic core formation by scaffold guided growth and constant passive flow perfusion using a rocking-platform system. The tubular organoids showed reduced heterogeneity and improved neural development. Additionally, we demonstrated the incorporation of isogenic microglia introduced with the non-invasive flow, preserving their homeostatic morphology and function. Moreover, the microglia incorporated into our tubular organoid culture can respond to external stimulation such as LPS and DAMGO, recapitulating the *in vivo* phenotype and functions during neuroinflammation. Our neuroinflammation model can also be utilized for the screening of therapeutics as evidenced by its clear responses to LY2828360 treatment. In this study, our fabricated tubular organoid model using only human embryonic stem cells (hESC) derived culture. For future studies, we plan to incorporate induced pluripotent stem cell (iPSC) derived culture into our model to better represent patient-relevant genetics. Additionally, our current model has limited throughput with 2 mm $\times$ 2 mm inner basket for EB loading, we plan to adopt higher resolution 3D printing to print finer and smaller tubular structure to scale our model to fit within a 96 or 384 well plate format. To conclude, our method is scalable, robust, and cost-effective. We envision this technology to be broadly applicable for 3D culture systems such as the brain, intestinal, and tumor organoid cultures to improve the standardization, throughput, and reduce heterogeneity.

4. Experimental Section

Tubular brain organoid device design and fabrication.

The tubular brain organoid device was designed in AutoCAD software. The dimension of the device was designed as 20 $\times$ 4 $\times$ 10 mm (L $\times$ W $\times$ H) with a tubular layer thickness of 25 μ m. The device was then printed using a stereolithography 3D printer (Form 3B, Formlabs) and printing material of FormLabs Clear Resin V4 (FormLabs).

Human embryonic stem cell culture.

Human embryonic stem cell WA01 was purchased from WiCell, and used following the relevant laws and guidelines approved by the Indiana University and the WiCell institute. WA01 cells were maintained on Matrigel (Corning) coated 6 well plates in mTESR plus medium (Stemcell Technologies) with a medium change every other day. The WA01 cells were passaged every 7 days using ReLeSR (Stemcell Technologies). WA01 cells under the passage of 42 were used to fabricate brain organoids.

Fabrication of human forebrain and cerebral organoids.

Human forebrain and cerebral organoids were fabricated as we previously reported.^{60, 61} Briefly, 9,000 WA01 cells were harvested from culture and aggregated into embryonic bodies using Aggrewell-800 plates (Stemcell Technologies) in EB formation medium (Stemcell Technologies) supplemented with 10 μ M Y-27632 (SelleckChem). The aggregated WA01 cells form EBs in one day. The EBs were then derived into human cerebral organoids using the STEMdiff Cerebral organoid kit (Stemcell Technologies) or derived into human forebrain organoids by dual-SMAD inhibition (Dorsomorphin and A83-01) followed by Wnt activation (CHIR99021) and TGF-beta inhibition (SB431542) as previously described.⁵¹ Detailed forebrain protocol medium composition can be found in Table S3.

N2a and HMC3 cell culture.

Neuro-2a neuroblast cells (N2a) (ATCC) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 100 U/mL Penicillin-Streptomycin (Gibco). HMC3 human microglia (ATCC) were maintained in DMEM supplemented with 10% FBS and 1X GlutaMAX (Gibco) and 100 U/mL Penicillin-Streptomycin (Gibco). Cells were maintained in a 37 °C, 5% CO₂ supplemented humidified incubator (Thermo Fisher) and passaged with trypsin-EDTA (Gibco) at 70% confluency.

Tubular organoid fabrication.

To fabricate tubular brain organoids, EBs of 3 days were removed from Aggrewell and sedimented by gravity in a 15 ml centrifuge tube. Once aggregated at the bottom of the tube, EBs were carefully aspirated using a P1000 pipette with a cupped tip and slowly loaded onto a tubular device. Once EBs fully sedimented inside the device, 50 μ L of the medium was carefully loaded into both side of the chambers, the device was then transferred to a 6-well plate and carefully topped with an additional 2 mL of the corresponding medium. After EB merge to form a tubular organoid, the culture medium was removed from the device. 50 μ L of Matrigel (Corning) was loaded into the center chamber and allowed to solidify in a 37°C incubator for 30 minutes before loaded with the pre-warmed medium. The device was then cultured for additional 7 days with a medium change every other day before it was placed onto a rocking platform (Thermo Scientific) set at a 7° rocking angle and speed of 1 rpm for continuous rocking inside an incubator. Medium change was performed every other day to maintain the tubular organoids. All medium composition and medium change timelines followed the exact protocols used to generate conventional cerebral organoids or forebrain organoids.^{51, 53}

Live/dead staining.

To visualize cell viability, tubular/conventional brain organoids were subjected to staining using a live/dead viability kit (Invitrogen). Briefly, samples were incubated with a live/dead staining mix containing CFSE and ethidium homodimer-1. After incubation at 37 °C for 1 hour, the samples were washed twice with the fresh medium before visualization on an inverted fluorescence microscope (Olympus IX-83).

Hypoxia staining.

To visualize hypoxic core formation within tubular or conventional brain organoids, samples were subjected to staining by the Image-iT red hypoxia kit (Invitrogen). Briefly, samples were incubated with the Image-iT red hypoxia dye for 4 hours before imaging on an inverted fluorescence microscope (Olympus IX-83).

Cryosection of organoids.

To section tubular and conventional organoids, the cultures were first washed twice with 1XPBS (Gibco) and then submerged in 4% paraformaldehyde in PBS (Thermo Scientific) at 4 °C overnight. The samples were then washed twice with 1XPBS and then submerged in 15% sucrose (w/v) for 8 hours followed by 30% sucrose (w/v) overnight at 4 °C to cryoprotect. The organoids were then incubated with 7.5% gelatin (w/v) and 10% sucrose (w/v) in the PBS solution at 37 °C for 1 hour. Finally, the samples were transferred to a cryomold (Sakura Finetek) and snap-frozen in a dry ice/ethanol slurry. The frozen block was then sectioned on a cryostat (Leica) at 30 µm thickness.

Immunofluorescence staining.

To characterize the tubular/conventional brain organoid cultures, sectioned samples were placed onto a charged glass slide and washed twice with 1XPBS. The samples were then treated with 3N hydrochloric acid (HCl) for 15 minutes for antigen retrieval. Following HCl treatment, the samples were washed again twice with 1XPBS and subjected to blocking (0.3% Triton-X100, 5% normal goat serum in 1XPBS) for 1 hour, followed by primary antibody incubation in a humidified chamber at 4 °C overnight. The samples were then washed 3 times with 1XPBS followed by secondary antibody at room temperature for 1 hour before being washed and coverslipped with gold anti-fade mounting medium with DAPI (Invitrogen). Detailed antibody information and dilution factors can be found in Supplementary Table S1.

EdU Staining.

To characterize cell proliferation, tubular/conventional organoids were stained by Click-iT cell proliferation kit, Alexa 647 (Thermo Fisher). Briefly, organoids were incubated with EdU substrate for 4 hours at 37 °C. The samples were then fixed and stained for EdU detection. Following the EdU staining, immunofluorescence staining for MAP2 was performed to label neurons as described above.

qPCR analysis.

To analyze the gene expression profile of EBs, brain organoids, and tubular organoids, samples were first washed twice with 1XPBS and lysed for RNA extraction using RNeasy Plus Mini Kit (Qiagen). RNA was then reverse transcribed into cDNA using qScript cDNA synthesis kit (Quantabio). Then cDNA was analyzed by real-time qPCR using SYBR Green real-time PCR master mix (Thermo Fisher). qPCR primer sequences can be found in Supplementary Table S2. Relative expression ($-\Delta\Delta C_t$) was calculated as the delta C_t value between target gene C_t value in organoid and the target gene C_t value in day-1 EB, both normalized against housekeeping gene GAPDH C_t value ($-\Delta C_t$ method). Each qPCR reaction was repeated in triplicates and 5 organoids/EBs were analyzed for each group. Mean C_t values are shown. Samples that did not amplify for a certain gene were denoted with C_t values of 40.

Microglia differentiation from WA01 cells.

To differentiate WA01 cells into microglia, WA01 cells were plated onto Matrigel (Corning) coated 6-well plate at a density of 30–40 colonies per well. The WA01 cells were then differentiated into hematopoietic progenitor cells (HPC) using STEMdiff hematopoietic kit (Stemcell Technologies). The differentiated WA01 derived HPC were then further differentiated into induced microglia (iMG) cells using STEMdiff Microglia Differentiation kit (Stemcell Technologies).

Microglia treatment with drugs.

To treat microglia cultured in well plates or microglia embedded in hFO, lipopolysaccharide (LPS) (Sigma) was dosed at 100 ng/mL for 24 hours followed by Adenosine 5'-triphosphate (ATP) (Sigma) at a concentration of 2mM for 30 minutes. Similarly, DAMGO was given at a concentration of 100 nM for 24 hours. For LY-2828360 (Cayman Chemicals) treatment, microglia were first treated with DAMGO for 24 hours. The medium containing DAMGO was then removed and replaced with a medium containing 100 nM LY-2828360 for an additional 24 hours.

Microglia morphology analysis.

Cryosectioned tubular organoids slides stained with Iba-1 was imaged using an inverted microscope (Olympus IX-83). Images were taken using 20X objective. Raw TIFF images were imported into Image J to extract Iba-1 channel (Cy5). The extracted images were then thresholded and analyzed by FracLac plugin (v2.5) for fractal dimensions, lacunarity and convex-hull circularity.⁶²

Inflammasome NLRP3/ASC colocalization proximity ligation assay.

To visualize inflammasome activation inside microglia cells, we analyzed the colocalization of NLRP3 and ASC by proximity ligation assay (PLA). Samples were subjected to NLRP3 and ASC staining following by colocalization PLA using the Duolink in situ red kit (Sigma). After the PLA labeling, samples were then additionally labeled by Iba1 and MAP2 before coverslipped with Prolong gold anti-fade DAPI mounting medium (Thermo Fisher) and visualized under an inverted fluorescence microscope (Olympus IX-83).

ELISA assays.

Conditioned medium of 24 hours (total volume 500 μ L) from control or drug-treated conditions were collected from microglia cultured in 24 well plates or incorporated inside tubular organoids. The medium was centrifuged at 2,000 g for 10 minutes to remove cell debris. 400 μ L of supernatant was then aspirated and used for ELISA assays. Samples were assayed using ELISA plates for IL-1 β (Tribioscience), IL-18 (Tribioscience), and TNF- α (Abcam). Each sample was tested in triplicate and read at 450 nm for absorbance.

Statistical analysis.

The statistics comparing two sample groups were conducted using the Students' t-test in GraphPad Prism 7. Statistical significance was denoted as following: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Tubular human brain organoids to model neuroinflammation.

(a) Schematics of microglia-mediated neuroinflammation under opioid treatment. (b) Illustration of a microfluidic device for the formation and culture of tubular human brain organoids. (c) Schematics of tubular human brain organoids that incorporate microglia through flow and recapitulate microglia responses inside the brain microenvironment.

Figure 2. Tubular organoid device design and validation.

(a) The tubular organoid device comprises a tubular scaffold (i), a medium reservoir (ii), and an embryonic body (EB) bottom holder (iii). The EB holder holds EBs to enable them to fuse and form tubular organoids while allowing medium and gas perfusion. The tubular scaffold has an inner mesh-structured lumen to introduce medium flow, oxygen, and microglia. The medium reservoir holds the culture medium and creates constant flow once placed on a rocking platform. (b) Immunofluorescence staining of tubular cultures of N2a to show tubular structure formation. (c) High viability of tubular 3D cultures (N2a cells). (d) Viability of tubular 3D culture over a prolonged culture period. (e) Hypoxia dye staining of the conventional organoid culture and the tubular 3D culture (N2a cells). (f) Quantification of hypoxia dye distribution from the organoid surface of the conventional organoid and the tubular 3D culture (N2a cells). (g) qRT-PCR analysis of relative HIF1a expression (– Ct) inside conventional and tubular cerebral organoid (n=3). Scale bar: 500 μm.

Figure 3. On-chip fabrication of tubular human brain organoids.

(a) Illustration of tubular forebrain organoid fabrication process. (b) Top-down view of tubular device and the formation of the tubular human forebrain organoid. Scale bar: 500 μm .

Figure 4. Characterization of tubular human brain organoids.

(a) Cross-section staining indicating PAX6 neural progenitor cells and MAP2 neuron distribution inside human forebrain organoid (hFO) and tubular human forebrain organoid (thFO). VZ/SVZ distribution is indicated by dotted circles. (b) Quantification of VZ/SVZ zone size variation inside a conventional forebrain and tubular forebrain organoid. (c) Proliferation dye EdU staining of human forebrain organoid and tubular organoid. (d) Quantification of EdU positive cell percentage inside forebrain organoids (n=5). (e) GFAP staining to identify astrocyte presence in 3 months old conventional and tubular hFO. (f) Quantification of GFAP+ cells in conventional and tubular hFO (n=3). Scale bar: a. 500 μm , c. e. 200 μm .

Figure 5. Gene expression analysis of tubular human brain organoids.

(a) Comparison of PAX6, MAP2, and TBR1 gene expression in day 35 conventional human cerebral organoids (hCOs), tubular human cerebral organoids (thCOs), conventional human forebrain organoids (hFOs), and tubular human forebrain organoids (thFOs). Log₂FC calculated as delta Ct of GAPDH and target gene normalized against day 1 embryonic bodies (EBs) (n=5). (b) The plot of gene expression fold change (Log₂FC) heatmap of hCOs, thCOs, hFOs, and thFOs on day 16 and day 35 respectively as compared with day 1 EBs.

Figure 6. Microglia activation by LPS/ATP or DAMGO.

(a) The illustration shows our experimental procedure. The tubular organoids and microglia were differentiated separately from human embryonic stem cells (hESCs) WA01 and then cocultured to model the neuroinflammation during opioid exposure. Schematics show the activation of homeostatic microglia by DAMGO and restoration to their homeostatic status by LY-2828360 treatment. (b) Immunofluorescence (Iba1) staining of microglia infused into tubular organoids under control and LPS/ATP, DAMGO, and DAMGO/LY2828360 treatment conditions. (c) Morphological analysis of microglia within tubular organoids under different treatment conditions. The activated microglia showed a lower fractal dimension and lacunarity, as well as higher convex hull circularity than the resting microglia (n=20). Scale bar: 50 μ m.

Figure 7. Modeling microglia-mediated neuroinflammation.

(a) Mechanism of opioid initiated neuroinflammation through the activation of inflammasomes and microglia. (b) Immunofluorescence staining of microglia and inflammasomes within engineered tubular organoids. The microglia were identified by Iba1 positive staining (in white), and the inflammasomes were characterized by NLRP3/ASC colocalization spots (in pink). (c) Quantification of inflammasomes per microglia under control and LPS/ATP, DAMGO, and DAMGO/LY2828360 treatment conditions (n=20). (d) Secreted cytokine levels from tubular forebrain organoids and 2D cultures under control and LPS/ATP, DAMGO, and DAMGO/LY2828360 treatment conditions (n=3). Scale bar: 50 μ m.