

Human cerebral organoids: Complex, versatile, and human-relevant models of neural development and brain diseases

Raquel Coronel^{1,*}, Rosa González-Sastre^{1,2,3,*}, Patricia Mateos-Martínez^{1,2,3,*}, Laura Maeso², Elena Llorente-Beneyto^{1,2,3}, Sabela Martín-Benito^{1,2,3}, Viviana S. Costa Gagosian⁴, Leonardo Foti⁴, M^a Carmen González-Caballero⁵, Victoria López-Alonso^{2,*}, Isabel Liste^{1,*}

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Abstract

The brain is the most complex human organ, and commonly used models, such as two-dimensional cell cultures and animal brains, often lack the sophistication needed to accurately use in research. In this context, human cerebral organoids have emerged as valuable tools offering a more complex, versatile, and human-relevant system than traditional animal models, which are often unable to replicate the intricate architecture and functionality of the human brain. Since human cerebral organoids are a state-of-the-art model for the study of neurodevelopment and different pathologies affecting the brain, this field is currently under constant development, and work in this area is abundant. In this review, we give a complete overview of human cerebral organoids technology, starting from the different types of protocols that exist to generate different human cerebral organoids. We continue with the use of brain organoids for the study of brain pathologies, highlighting neurodevelopmental, psychiatric, neurodegenerative, brain tumor, and infectious diseases. Because of the potential value of human cerebral organoids, we describe their use in transplantation, drug screening, and toxicology assays. We also discuss the technologies available to study cell diversity and physiological characteristics of organoids. Finally, we summarize the limitations that currently exist in the field, such as the development of vasculature and microglia, and highlight some of the novel approaches being pursued through bioengineering.

Key Words: assembloids; bioengineering; challenges; disease modeling; drug screening and toxicology; human brain organoids; human pluripotent stem cells; neurodegenerative diseases; neurodevelopment; vascularization

Introduction

The brain is the most complex organ in the human body, both architecturally and functionally. It is becoming increasingly evidence indicating that many neurological and psychiatric disorders, and even neurodegenerative diseases, could have their origin or be related to problems (abnormalities in the structure or function of the brain) during neurodevelopment (Sidhaye and Knoblich, 2021; Capizzi et al., 2022). Although neurological disorders are usually diagnosed between the ages of 40 and 60, depending on the specific condition, it is now broadly recognized that the clinical symptoms of these disorders emerge several decades after the neuronal circuits start to lose connectivity and cell function. Thus, a clinical diagnosis at the age of 60 years could still

indicate that the underlying cause or the onset of the condition may have occurred during early childhood or even in embryonic development (Shabani and Hassan, 2023), hence the importance of advancing in its study. However, understanding the mechanisms involved in neurodevelopment and comprehending the causes of these brain disorders remains a challenge, mainly due to the complexity of the human brain and limited access to brain samples (Sidhaye and Knoblich, 2021).

Animal systems such as *Drosophila melanogaster*, *Caenorhabditis elegans*, embryos of *Xenopus* and zebrafish, and even embryonic chickens, have been essential models in establishing our understanding of early embryogenesis, neural development, and the formation of different brain structures. Nevertheless, mouse models have

become the gold standard model system for the study of mammalian neural development, mainly due to its excellent genetic tool (Andrews and Nowakowski, 2019).

While the mouse has been a key tool for neuroscientific discovery, there are major differences between the mouse and the human brain (Eichmüller and Knoblich, 2022). The size, number of cells, different cell type composition, six-layered architecture, folding (called *gyrification*), and expansion of the cerebral cortex are several characteristics that differentiate the human brain from the mouse brain (Florio and Huttner, 2014; Eichmüller and Knoblich, 2022). Another key difference in neural development is the absence (or minimal presence) of outer radial glial cells (outer RGCs, oRGCs) in rodents

¹Unidad de Regeneración Neural, Unidad Funcional de Investigación de Enfermedades Crónicas (UFIEC), Instituto de Salud Carlos III (ISCIII), Majadahonda (Madrid), Spain; ²Unidad de Biología Computacional, Unidad Funcional de Investigación de Enfermedades Crónicas (UFIEC), Instituto de Salud Carlos III (ISCIII), Majadahonda (Madrid), Spain; ³Doctoranda en la Escuela Internacional de Doctorado de la Universidad Nacional de Educación a Distancia (UNED), Programa en Ciencias Biomédicas y Salud Pública, Madrid, Spain; ⁴Graduate Program in Biosciences and Biotechnology, Laboratory of Trypanosomatids Molecular and Systemic Biology, Carlos Chagas Institute (ICC) - Fiocruz Paraná, Curitiba, Brazil; ⁵Unidad de Evaluación de Riesgo, Centro Nacional de Sanidad Ambiental (CNSA), Instituto de Salud Carlos III (ISCIII), Majadahonda (Madrid), Spain

*Correspondence to: Isabel Liste, PhD, iliste@isciii.es; Victoria López-Alonso, PhD, victorialopez@isciii.es.

<https://orcid.org/0000-0002-3294-9558> (Isabel Liste); <https://orcid.org/0000-0001-8129-8352> (Victoria López-Alonso)

#These authors contributed equally to this work and share first authorship.

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compared to humans. oRGCs are specialized neural precursors that play a fundamental role in neuronal production, especially in the context of gyrencephalic brains (those with cortical sulci and gyri, as primate and human brains) (Hansen et al., 2010; Pollen et al., 2015). Comparing neocortex development across mammals shows that the proliferative potential of oRGCs and the duration of the neurogenic period are crucial factors in regulating neocortex complexity and size. These factors are believed to play a key role in the enhanced cognitive abilities observed in humans (Wang et al., 2011; Gilardi and Kalebic, 2021).

Given the ethical limitations presented using human embryos in research and the differences (both anatomical and physiological) that animal models present compared to humans, *in vitro* models based on human cerebral organoids (hCOs) are a good tool for delving into brain development and the complexity of the human brain. In addition, hCOs allow recapitulating *in vitro* the brain developmental timing (even the biology and development of oRGCs) (Walsh et al., 2024), giving us the opportunity to assay phenotypes unique to the human neurodevelopmental.

In this review, we discuss recent advancements in hCOs technology as a model for studying neurodevelopment and various brain pathologies. We begin by summarizing the protocols available for generating diverse brain organoids and assembloids, followed by an exploration of their application in investigating brain-related diseases, including key neurodevelopmental disorders, psychiatric conditions, neurodegenerative diseases, brain tumors, and infectious diseases. Additionally, we examine the use of brain organoids in transplantation studies, drug screening, toxicology assays, and brain evolution studies, as well as the technologies employed to assess cell diversity and the physiological characteristics of these organoids. Finally, we address the primary limitations currently facing the field, such as challenges in vascularization and microglial development, and highlight innovative bioengineering approaches aimed at overcoming these obstacles.

Search Strategy

Several databases, mainly PubMed (pubmed.ncbi.nlm.nih.gov) and Google Scholar (scholar.google.es), were used to search for published material. The keywords included in the search were “neurodevelopment AND human brain,” “types of cerebral organoids AND protocols,” “cerebral organoids AND disease models,” “applications AND brain OR cerebral organoids,” “molecular methods AND cerebral organoids,” “limitations OR challenges AND cerebral organoids.” The majority of selected references (> 80%) were published between 2019 and 2024.

The Need to Know the Human Neurodevelopmental Processes

Much of the current knowledge regarding human brain development comes from *in vitro* studies carried out in cell cultures, *in vivo* studies carried out in animal models and studies carried out using

human embryos. However, the great existing ethical limitations and the reduced access to human fetal tissues in research make the study of neurodevelopment and the complexity of the human brain difficult and limited (Sapir et al., 2022).

The nervous system development is a strictly orchestrated spatial and temporal process that generates an immense diversity of cell types. Understanding the basic mechanisms of human brain formation is important due to the impact on society and the health burden that neurological disorders generate (Li et al., 2023a). In recent years, improvements in conditions of *in vitro* culture for the maintenance of blastocysts and stem cells have provided very relevant information on early human embryogenesis. However, the mechanisms involved in lineage development, embryonic patterning, and the molecular pathways involved in its regulation are still not well understood (Rossant and Tam, 2022). Since a comprehensive understanding of the processes that govern human brain development will necessitate research on human brain development itself, the finding that brain-like structures, known as hCOs, can be created by differentiating stem cells presents an opportunity to examine several aspects of human brain development firsthand (Chan et al., 2021).

To create hCOs that accurately simulate the human brain, it is necessary to understand how neural cells differentiate, migrate, connect, and form functional circuits in the early stages of development. Furthermore, the brain is organized into specialized layers and regions during neurodevelopment. This organization is essential for cerebral function, and to create realistic hCOs, it is necessary to understand how these spatial organization processes occur. Below, we describe some known aspects of early embryonic development, neurodevelopment, and generation of different cell types in the brain.

Early embryogenesis and neural development

Human embryonic development begins with the formation of a totipotent, unicellular zygote during fertilization. Around day 5–6 after fertilization, the blastocyst is generated and subsequently, around day 14 after fertilization, the gastrulation process begins, which is considered a milestone in early embryonic development human because it lays the foundations for the generation of the three germ (or embryonic) layers and the body plan (Solnica-Krezel and Sepich, 2012; Zhai et al., 2022) (Figure 1A). It is important to mention that, at the level of human research, there is the 14-day rule, which is used as a limit in most of the ethical restrictions associated with studies of human embryonic development (Ghimire et al., 2021). The three primary germ layers are endoderm, mesoderm, and ectoderm, and their generation prepares the system for organogenesis. The ectoderm is the outermost layer of the embryo and, from it, the non-neural ectoderm (which gives rise to the epidermis) and the neural ectoderm or neuroectoderm (which gives rise to the neural tube) are generated (Muhr et al., 2023). Between the edges of the non-neural ectoderm and the neuroectoderm, there are border cells that, for the most part, form the neural crest (Gammill and Bronner-Fraser, 2003).

The neural tube is the precursor structure of the central nervous system (CNS) and its formation occurs shortly after embryonic gastrulation, through a process called *neurulation*. The phenomenon of neurulation begins through the process of neural induction, in which the ectoderm is specified in a multicellular tissue with a spatial pattern, formed by the non-neural ectoderm, the neuroectoderm (which when thickened gives rise to the neural plate) and the edges of the neural plate (Xue et al., 2020). It is important to highlight that this neural induction process is carried out by an embryonic structure called *notochord*, which is a source of secreted signals capable of regulating the differentiation and maturation of surrounding tissues (Corallo et al., 2015; Ramesh et al., 2017). After neural induction, the neural plate begins to fold towards the dorsal side of the embryo and ends up fusing, forming the neural tube (Gammill and Bronner-Fraser, 2003; Figure 1B). Subsequently, the development of the neural tube continues through its differentiation towards the different regions of CNS, which occurs at several levels simultaneously. At the anatomical level, the neural tube and its cavity undergo a series of expansions and contractions along the anterior-posterior axis, giving rise to the three primary vesicles of the developing brain (forebrain, mesencephalon, and hindbrain), which will later form the five secondary vesicles (telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon) and the spinal cord. Furthermore, at cellular and tissue levels, the different populations of progenitor cells or stem cells of the neural tube are differentiated into numerous types of neuronal and glial cells, which migrate and reorganize forming the different functional regions of the developing brain and spinal cord (Yamashita, 2013).

The phenotypic specification of neural tube stem cells along the dorso-ventral and antero-posterior axes is directed by several morphogens and growth factors. Patterning molecules involved in the dorso-ventral mode include sonic hedgehog (Shh), bone morphogenetic proteins (BMPs), and wingless/integrated (Wnts), while those affecting antero-posterior patterning include fibroblast growth factors (FGFs), Wnts and retinoic acid (RA) (Nie et al., 2023). These morphogens and growth factors are generated and secreted by the notochord and surrounding tissues, generating several morphogen gradients that regulate the intrinsic signaling pathways and transcription of genes involved in the proliferation control, cellular survival, and cell fate determination (Goyal et al., 2020; Xue et al., 2020; Nie et al., 2023). The knowledge of morphogen gradients and patterns is essential for an effective and successful neural induction *in vitro*, and for the generation of diverse types of hCOs. In many stem cell *in vitro* systems, neural induction can be mimicked inhibiting the BMP and transforming growth factor- β signaling pathways, known as dual SMAD inhibitors (Chan et al., 2021). Recently, it has been possible to model *in vitro* the early development of the human neural tube from pluripotent stem cells (PSCs). This arises thanks to the research of Rifes and Kirkeby, who subjected stem cells to several morphogens (specifically Noggin, as an inhibitor of BMPs, and SB-431542,

as an inhibitor of transforming growth factor- β) and to a signaling gradient of Wnt activation (Rifes et al., 2020). Moreover recent studies show that human embryonic stem cells (ESCs) can be used to generate “gastruloids” (three-dimensional (3D) multicellular aggregates that differentiate to form derivatives of the three germ layers) that express markers of early neuroectoderm in the absence of SMAD inhibition (Moris et al., 2020). In this sense, more studies are necessary to deepen the understanding of the molecular underpinnings driving the processes of early embryogenesis and neural development in human, being the hCOs a successful model *in vitro* for closely mimic the developing the human brain.

Neurodevelopment and cell types

The CNS is one of the first organ systems in the human body to begin its development (during early embryogenesis) and is also one of the last to complete it (several brain regions and neural circuits continue to develop after birth, even into the 20 or 30 years old). This is mainly because the development of the human CNS requires very precise organization and coordination, which are associated with countless cellular and molecular processes (Silbereis et al., 2016). The cerebral cortex, which serves as a central controller for advanced cognitive functions and sensory processing, includes three main regions of the brain (neocortex, archicortex, and paleocortex). In this section, we focus on the neocortex development, which is the largest part of the cerebral cortex and a fundamental structure involved in human cognition and behavior (Moffat and Schuurmans, 2024).

During early CNS development, the neural tube is composed of a pseudostratified layer of neuroepithelial cells (acting as neural stem cells) that line the central cavity. These cells constitute the ventricular zone (VZ) of the neural tube, and they are the progenitor cells of all types of neurons and glial cells in adult CNS (Baggiani et al., 2020).

Initially, the neuroepithelial cells of the VZ undergo symmetric divisions, from which two daughter cells identical to the mother cell arise in each division. In this way, the VZ increases its thickness and surface area (Stiles and Jernigan, 2010). Subsequently, the neuroepithelial cells begin to divide asymmetrically, generating the radial glial cells (RGCs), which reside in both the VZ and the subventricular zone (SVZ). These RGC are capable of contacting, at least initially, both the ventricular surface and the pial surface of the developing brain, and are subjected to asymmetric divisions during neurodevelopment, from which they are generated, in each division, another RGCs and a nascent neuron (a process known as neurogenesis). Furthermore, RGCs act, on the one hand, as a scaffold to facilitate the migration of nascent neurons towards the pial surface and, on the other hand, as progenitor cells of glial cells (a process known as gliogenesis). It should be noted that, during neurodevelopment, the process of gliogenesis (generation of astrocytes and oligodendrocytes) occurs after the neurogenic stages, in which nascent neurons are generated and migrate to their final location in the cortical plate (Budday et al., 2015; Baggiani et al., 2020; Irie et al., 2022; **Figure 1C**). However, unlike what

happens in rodents, several studies indicate that gliogenesis and neurogenesis overlap extensively in humans (Malik et al., 2013). The case of hematopoietic lineage-derived microglia cells and endothelial cells lining blood vessels highlights that they migrate to the neuroectoderm-derived nervous system to support embryonic brain development (Zhou et al., 2024).

From a more detailed view, in the dorsal forebrain, the neural precursors (neuroepithelial cells and RGCs) of the VZ (in the neural tube) generate intermediate progenitor cells (IPCs), which are located in the SVZ. Apical RGCs divide in the VZ, whereas IPCs divide in the SVZ. Subsequently, IPCs and RGCs differentiate into postmitotic neurons that migrate radially (inside-out manner) to their

final location in the cortical plate. Throughout the advancement of this neurogenesis process, several batches (or cohorts) of neurons are generated, which migrate (in each batch) beyond their predecessors and settle in different regions of the cortical plate. This event causes a thickening of the cortical plate and a lamination in several cortical layers (numbered I to VI in humans). In this sense, the earliest nascent neurons reside in the deeper-layers (V–VI) and the later nascent neurons form the upper-layers (II–IV). Layer I is the most superficial of all and forms the marginal zone (Andrews and Nowakowski, 2019; Zhou et al., 2024; **Figure 1C**). While excitatory neurons originate exclusively in the dorsal forebrain, cortical interneurons are thought to originate

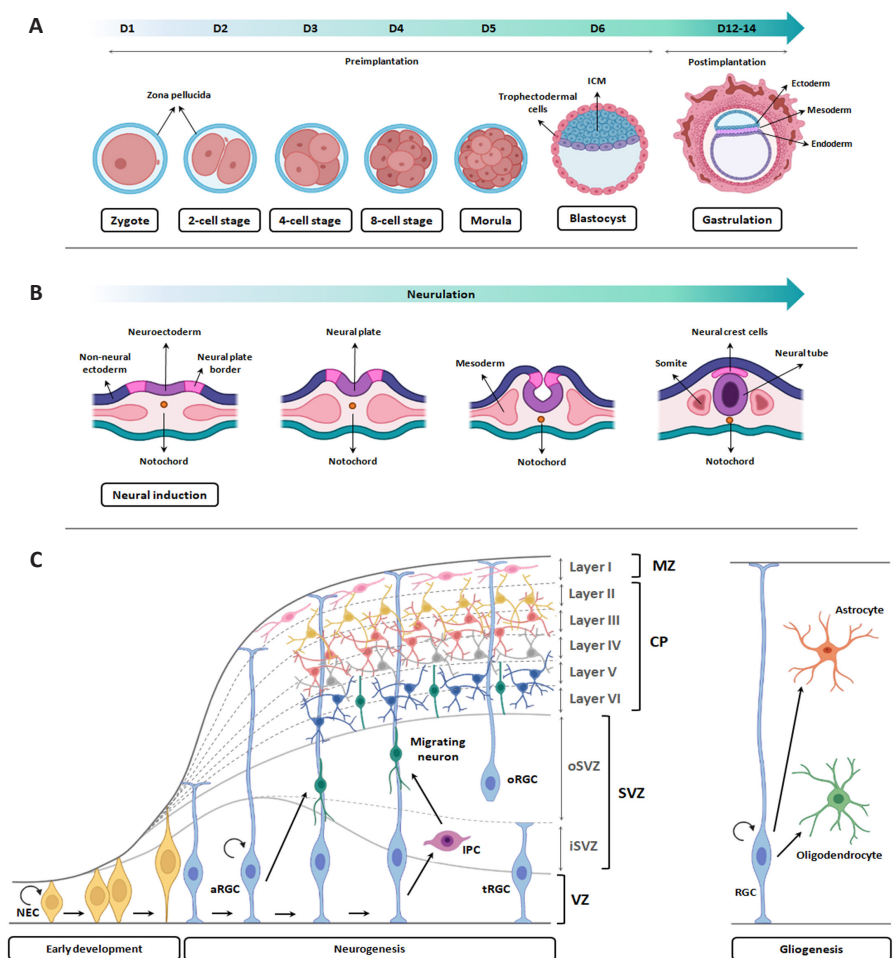


Figure 1 | Early embryogenesis and human neurodevelopment.

(A) The blastocyst is formed around day 5–6 (D5–D6) after fertilization and is composed of the ICM and trophectodermal cells. Around day 14 (D14) gastrulation begins, which generates the three germ layers (ectoderm, mesoderm, and endoderm). (B) Generation of the neural tube through the neurulation process. During this stage, the neural plate folds and ends up fusing, creating the neural tube. (C) Progenitor types in the human brain development. Initially, the NECs undergo symmetric divisions and the VZ increases in thickness. Subsequently, NECs divide asymmetrically and transition to RGCs, which reside in both the VZ and the SVZ. During the neurogenic period, RGCs undergo asymmetric divisions, generating RGCs and nascent neurons directly or producing neurons indirectly through IPCs. These neurons are capable of migrating to their final location in the CP. aRGCs are defined by residing in the VZ and oRGCs by residing in the oSVZ. tRGCs are defined by their basal process terminating in the border between the iSVZ and the oSVZ. After the neurogenic stages, RGCs can also differentiate into astrocytes and oligodendrocytes. Created with BioRender.com. aRGCs: Apical radial glial cells; CP: cortical plate; ICM: inner cell mass; IPCs: intermediate progenitor cells; iSVZ: inner subventricular zone; MZ: marginal zone; NECs: neuroepithelial cells; oRGCs: outer radial glial cells; oSVZ: outer subventricular zone; RGCs: radial glial cells; SVZ: subventricular zone; tRGCs: truncated radial glial cells; VZ: ventricular zone.

primarily from ganglionic eminences located in the ventral forebrain. Unlike excitatory neurons, interneurons do not migrate following the RGCs fibers, but instead move tangentially (orthogonal to the RGCs scaffold) towards the cortex, distributing themselves throughout all cortical layers and areas (Ma et al., 2013; Eichmüller and Knoblich, 2022). Recent studies demonstrate that cortical organoids model critical stages of early embryogenesis and neural development with high developmental fidelity and reproducible cellular diversification processes that closely mirror those observed in human fetal corticogenesis. Specifically, these organoids recapitulate cell type-specific transcriptional signatures and epigenetic states corresponding to endogenous fetal cortical cells, serving as a powerful platform to study neurodevelopmental dynamics and lineage specification across key developmental timelines (Uzquiano et al., 2022).

While the neocortex is found in all mammals, its size has varied due to evolutionary differences across different mammalian orders. In species with gyrencephalic brains (such as humans and primates), the SVZ is extensively expanded and subdivided into the inner SVZ and outer SVZ (oSVZ). As mentioned above, outer RGCs (oRGCs, also known as basal RGCs) are specialized neural precursors that reside in the oSVZ and play a fundamental role in neuronal production (Hansen et al., 2010; Pollen et al., 2015; **Figure 1C**). These oRGCs, mainly absent in the developing cerebral cortex of lissencephalic species (such as mice and rabbits), have an enhanced proliferative capacity and produce the majority of neurons in the upper layers, coinciding with expansion in size and surface area of the primate cortex (Sousa et al., 2017). In this sense, the enlargement of the SVZ and the emergence of oRGCs could have been crucial evolutionary developments that contributed to the transition from a lissencephalic to a gyrencephalic neocortex (Wang et al., 2011). Recently, a new protocol for the generation of hCOs with a structured oSVZ has been described by activating the STAT3 signaling pathway using leukemia inhibitory factor. In this way, the authors have managed to recapitulate *in vitro* key and specific aspects of human neocortical development such as the expansion of progenitor pool oRGCs into the oSVZ (Walsh et al., 2024). Another specific cell type that has recently been reported in gyrencephalic brains is truncated RGCs. These cells, as their name suggests, have a “truncated process,” which means that their morphology is altered and they lack the full extension (basal attachment) that apical RGCs normally have (Bilgic et al., 2023). However, it is still unclear how widely truncated RGCs are present in the development of gyrencephalic mammals, what mechanisms drive their formation, and what role they play.

Since the hCOs are crucial study models for replicating *in vitro* several processes of brain development (including the human fetal corticogenesis and the generation and biology of oRGCs), their use offers us a valuable tool to investigate the cellular and molecular mechanisms involved in neurodevelopmental and neurodegenerative diseases, being a more realistic approach (compared to traditional models) to improve our understanding of human neurobiology.

Human Cerebral Organoids: Generation Methods and Types

hCOs are 3D cell culture models that can be generated from PSCs and recapitulate the architecture and function of the developing the human brain. Two types of human PSCs can be used for the generation of hCOs: ESCs, which are derived from the inner cell mass of the blastocyst, and induced pluripotent stem cells (iPSCs), which are generated from the reprogramming of somatic cells through the administration of transcription factors. Currently, two types of protocols can be established for the generation of hCOs: *non-guided protocols* employ a series of standard media from which hCOs containing different brain regions are generated; *guided protocols* employ patterning factors to direct the hCOs differentiation to a particular brain region (Eichmüller and Knoblich, 2022; **Figure 2**).

Non-guided protocols

hCOs were first generated using a non-guided protocol, from which the same organoid developed several interdependent brain regions (Lancaster et al., 2013; Lancaster and Knoblich, 2014). The procedure consisted of dissociating PSCs and posterior aggregating them to form embryoid bodies (EBs). EBs later underwent

neural induction to promote the development of the neuroectoderm, neural induction medium contained DMEM/F12, N2 supplement, Glutamax, MEM-NEAA, and heparin. Once the tissues developed neuroepithelium, they were transferred to Matrigel droplets that were gelled and subsequently cultured in differentiation media (1:1 DMEM/F12 and Neurobasal supplemented with N2, B27 without vitamin A, mercaptoethanol, insulin, Glutamax, and MEM-NEAA). Finally, tissue droplets were transferred to a spinning bioreactor containing differentiation media with some modifications (B27 supplement with vitamin A and RA).

Within the different cerebral regions developed in the organoid, the generation of a cerebral cortex containing progenitor populations that organize and produce subtypes of mature cortical neurons was noteworthy.

Guided protocols

Since then, guided protocols have been established to generate region-specific organoids employing different patterning factors.

Forebrain organoids

Forebrain-specific organoids were developed from human iPSCs, which consisted of treating Matrigel-embedded EBs with GSK3 β inhibitors and dual

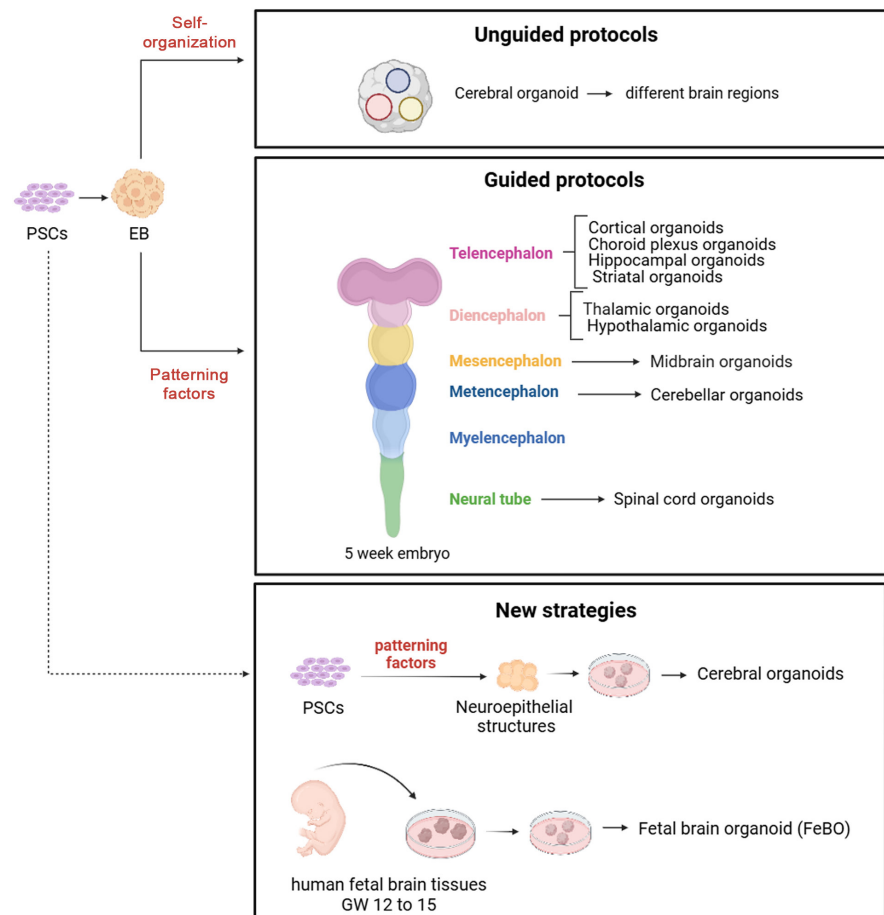


Figure 2 | Overview of the different hCOs that have been developed.

Schematic of the different types of protocols (non-guided and guided) and the new strategies available to generate hCOs. Created with BioRender.com. EB: Embryoid bodies; FeBO: fetal brain organoid; GW: gestational week; hCOs: human cerebral organoids; PSCs: pluripotent stem cells.

SMAD inhibitors and, subsequently, maturing them in a rotary reactor (Qian et al., 2016, 2018). These organoids managed to recapitulate key features of human cortical development, such as progenitor zone organization, neurogenesis, and gene expression. An important result was the ability to generate the human-specific oRGs layer.

Midbrain organoids

To drive EBs differentiation toward a mesencephalic fate, they were supplied with the patterning factors Shh and fibroblast growth factor 8 (FGF8) thus obtaining 3D midbrain-like organoids that contained distinct layers of neuronal cells expressing markers characteristic of the human midbrain. In addition, electrically active and functionally mature dopamine-producing midbrain dopaminergic neurons were detected. Moreover, the human midbrain-like organoids produced neuromelanin-like granules that were structurally similar to those isolated from human *substantia nigra* tissues (Jo et al., 2016).

Subsequently, a different approach for the generation of midbrain organoids was performed by culturing human neuroepithelial stem cells in the presence of the GSK3 β inhibitor CHIR99021 to stimulate the canonical Wnt signaling pathway, and purmorphamine to activate the Shh pathway. This resulted in organoids that exhibited neuronal, astroglial, and oligodendrocyte differentiation and that also exhibited synaptic connections, electrophysiological activity, and myelination of neurites (Monzel et al., 2017). Lately, protocols are more focused on developing midbrain-striatal assembloids due to their greater utility in studying Parkinson's disease (PD) (Reumann et al., 2023).

Hindbrain organoids

To obtain hindbrain-like 5-HT organoids, purmorphamine and RA were administered at 5–13 days of the EBs stage. This protocol demonstrated the importance of fine-tuning the timing of RA administration, as earlier administration causes rapid induction resulting in the absence of adequate organoid structures (Valiulahi et al., 2021).

Cortical organoids

Paşca et al. (2015) generated human cortical spheroids from PSCs containing electrophysiologically mature neurons from both deep and superficial cortical layers. The neurons were surrounded by nonreactive astrocytes and formed functional synapses (Paşca et al., 2015). Recently, cortical organoids generated with a “semi-guided” protocol, recapitulated complex neural oscillations observed in the developing human brain and yielded the cell type diversity of unguided approaches. The protocol had shorter induction and differentiation steps and employed less-specific patterning molecules than most guided protocols; however, it maintained the use of some neurotrophic factors, unlike unguided approaches (Fitzgerald et al., 2024).

Choroidal plexus and hippocampal organoids

Since the hippocampus comes from the medial pallium, which is included in the developing dorsomedial telencephalon, an approach followed

to generate hippocampal organoids was first inducing choroid plexus, the most dorsomedial portion of the telencephalon, by addition of BMPs and Wnt signaling. Following long-term dissociation culture, these tissues gave rise to functional hippocampal granule- and pyramidal-like neurons (Sakaguchi et al., 2015).

Later, a work achieved the development of a selective barrier and cerebrospinal fluid-like secretion in human choroid plexus organoids. Furthermore, the *in vitro* barrier exhibited the same selectivity to small molecules as the choroid plexus *in vivo* and transcriptomic and proteomic analysis showed that the organoids also presented a high degree of similarity to the choroid plexus *in vivo* (Pellegrini et al., 2020).

Striatal organoids

Another study developed human striatum organoids with electrically active medium spiny neurons by using the patterning factors Wnt pathway inhibitor (IWP-2), activin A, and retinoid X receptor (RXR) agonist (SR11237) (Miura et al., 2020).

Thalamic organoids

To generate thalamic organoids a work reported the addition of BMP7 combined with MEK/ERK inhibition which revealed the formation of distinct thalamic lineages diverging from telencephalic fate, as shown by single-cell RNA sequencing (scRNA-seq) analysis. Moreover, the thalamic organoids were fused with cortical organoids forming reciprocal projections (Xiang et al., 2019).

To better recapitulate the subregional identity of the thalamus, a study reported a method for obtaining ventral thalamic organoids with transcriptionally diverse nuclei identities, highlighting the thalamic reticular nucleus, which is a GABAergic nucleus previously unachieved (Kiral et al., 2023).

Hypothalamic organoids

Qian et al. (2018) described how to obtain hypothalamic organoids by dual SMAD inhibition, Wnt inhibition, and Shh pathway activation. Later, a work modeled the development of the human hypothalamic arcuate nucleus by generating arcuate organoids with different nuclei in the hypothalamus (Huang et al., 2021a).

Pituitary organoids

Since the pituitary primordium emerges from the oral ectoderm under the influence of the ventral hypothalamic neuroepithelia, a group followed the co-induction of hypothalamic and oral ectoderm by adding BMP4 and Shh activators. Functional pituitary placode tissues self-formed from oral ectoderm via local interactions with hypothalamic neuroepithelia. Pituitary placode was subsequently differentiated into pituitary hormone-producing cells and after transplantation into hypopituitary mice, the *in vitro*-generated corticotrophs rescued physical activity levels and survival of the hosts (Ozone et al., 2016). The same group described in a posterior study the simultaneous maturation of the pituitary with hypothalamic neurons within the same aggregates which resulted in the

secretion of the adrenocorticotrophic hormone (ACTH). Corticotropin-releasing hormone from the hypothalamic area regulated ACTH cells since the hybrid organoids responded to environmental hypoglycemic conditions *in vitro* through the corticotropin-releasing hormone-ACTH pathway, increasing ACTH secretion (Kasai et al., 2020).

Brainstem organoids

Since the brainstem is composed of the midbrain, pons, and medulla oblongata, a group generated human brainstem organoids containing midbrain/hindbrain progenitors, noradrenergic and cholinergic neurons, dopaminergic neurons, and neural crest lineage cells. The human brainstem organoids were found to be similar to the human brain stem after performing different analyses such as scRNA-seq, proteomics, and electrophysiology (Eura et al., 2020).

Cerebellar organoids

In the work of Muguruma et al. (2015), cerebellar structures with electrophysiologically functional Purkinje cells were generated. They also found that the addition of fibroblast growth factor 19 (FGF19) resulted in dorsoventrally polarized neural tube-like structures and that the addition of stromal cell-derived factor 1 (SDF1) and FGF19 promoted the formation of a cerebellar plate neuroepithelium similar to the embryonic cerebellum (Muguruma et al., 2015).

Recently, another work refined a protocol to generate human cerebellar organoids by adding FGF8b and mimicking the complex cell diversity of the fetal cerebellum. The novelty was the generation of a human-specific rhombic lip progenitor population. In addition, human cerebellar organoids formed organized laminar layering and demonstrated that inhibitory and excitatory neurons showed coordinated network activity. The human cerebellar organoids also exhibited functional Purkinje cells over a longer culture period (Atamian et al., 2024).

Spinal cord organoids

Dorsal, intermediate, and ventral spinal cord tissues have also been generated from human PSCs. First, 3D spinal cord induction recapitulated patterning of the developing dorsal spinal cord presenting four types of dorsal interneurons. Second, intermediate and ventral spinal cord-like tissues were induced activating Shh signaling (Ogura et al., 2018).

Human spinal cord-like organoids were improved by recapitulating the neurulation-like tube-forming morphogenesis of the early spinal cord. Additionally, they presented not only the major types of spinal cord neurons but also glial cells and mature synaptic functional activities (Lee et al., 2022a).

New strategies

Generation of human cerebral organoids directly from two-dimensional cultures of pluripotent stem cells

As described above, most protocols start with EBs that subsequently undergo neural induction to generate hCOs, but this procedure is tedious and time-consuming, and the efficiency to form

neuroepithelial structures could be increased. To this end, our group developed a new method to obtain hCOs directly from two-dimensional (2D) cultures of PSCs without aggregation of EBs. It is a simple and reproducible approach that consists of subjecting PSC colonies to a neural induction medium enriched with a strategic combination of patterning factors. This protocol not only has a neuroepithelium formation efficiency of almost 100% but also generates hCOs with VZs in which neural precursors and RGCs differentiate into mature neurons and glial cells. hCOs present important non-neuronal cell types such as oligodendrocyte precursors, astrocytes, microglia-like cells, and endothelial-like cells, a crucial aspect when recapitulating human brain development (Gonzalez-Sastre et al., 2024; **Figure 3**).

Fetal brain organoids

It has recently been shown that the healthy human fetal brain *in vitro* is capable of self-organizing into organoids (called fetal brain organoids, FeBOs), recapitulating aspects of the cellular heterogeneity and complex organization of the brain *in vivo* (Hendriks et al., 2024).

Assembloids

Assembloids are self-organized cellular systems that are generated by combining two types of organoids or one organoid with different types of specialized cells that eventually integrate (Paşca, 2019).

Forebrain-eye assembloids

A protocol has been described for generating brain organoids containing optic vesicles (called OVB organoids), whose long-term culture results in photosensitive organoids that constitute complementary cell types, including primitive corneal epithelial and lens-like cells, retinal pigment epithelia, retinal progenitor cells, axon-like projections, and electrically active neuronal networks (Gabriel et al., 2023).

Cortico-striatal assembloids

In the work described before by Miura et al. (2020), human striatum organoids were assembled with cerebral cortical organoids to form cortico-striatal assembloids and showed that cortical neurons sent axonal projections into striatal organoids and formed synaptic connections. They also proved the electrophysiological maturation of medium spiny neurons which displayed calcium activity after optogenetic stimulation of cortical neurons.

Ventral midbrain-striatum-cortex assembloids

Ventral midbrain dopaminergic neurons project to both the striatum and cortex and are implicated in PD, addiction, and neuropsychiatric disorders. However, an appropriate model of the human dopaminergic system was lacking. Therefore, an *in vitro* human model was developed based on ventral mesencephalic-striatal-cortical organoids in which functional long-range dopaminergic connections with striatal and cortical tissues were formed and ventral midbrain patterned injected progenitors matured and innervated the tissue (Reumann et al., 2023).

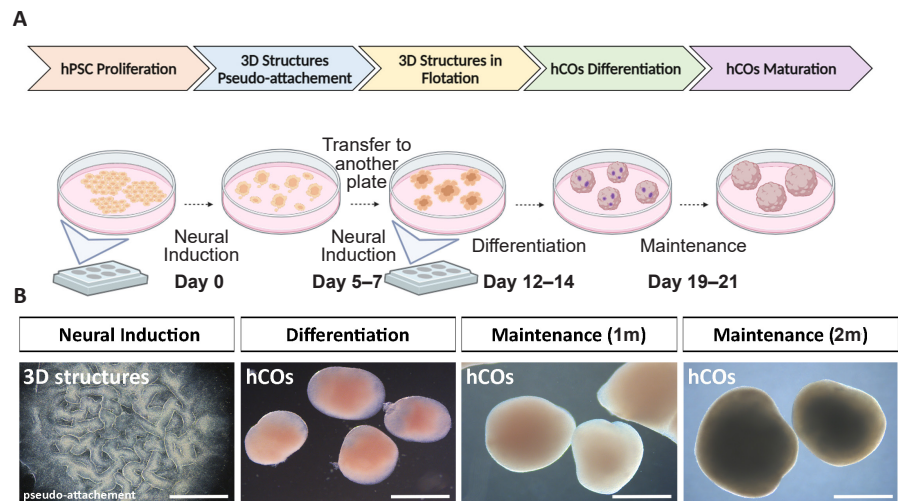


Figure 3 | Generation of hCOs directly from 2D cultures of PSCs.

(A) Schematic representation of the protocol described in González-Sastre et al., 2024. Image created in BioRender.com. (B) Representative phase contrast images of the hCOs generated directly from 2D cultures of PSCs. Images are shown in the stages of neural induction, differentiation, and 1 month (1m) and 2 months (2m) in maintenance. Scale bars: 500 µm. Unpublished data. 2D: Two-dimensional; hCOs: human cerebral organoids; hPSC: human pluripotent stem cell.

Cortico-motor assembloids

One study developed cortico-motor assembloids from organoids resembling cerebral cortex or hindbrain/spinal cord which were assembled with human skeletal muscle spheroids. In addition, corticofugal neurons were shown to project and connect to spinal spheroids, whereas spinal cord-derived motor neurons connected to muscle. Another important finding was that glutamate release or optogenetic stimulation of cortical spheroids triggered muscle contraction (Andersen et al., 2020).

Applications of Human Cerebral Organoids

Neurological diseases cover a broad and complicated spectrum, especially in pathologies that are difficult to replicate for research, such as neurodegenerative diseases, neurodevelopmental disorders, and psychiatric disorders. Furthermore, as discussed above, *in vivo* models have certain limitations, since there are interspecies differences with respect to humans, such as the absence of the oSVZ progenitors in mice compared to humans. In the following section, we present how hCOs are increasingly used for modeling neurological diseases, brain tumors, infection studies, drug screening, toxicology assays, brain evolution studies, and gene-environment interaction research (**Figure 4**).

Neurodevelopmental diseases

Neurodevelopmental dysfunctions include a range of disorders such as autism spectrum disorder (ASD) or microcephaly (Zamora-Moratalla et al., 2021).

Autism spectrum disorder

ASD can be classified into two types based on risk factors and the cells that are affected in the cerebral cortex: syndromic and idiopathic. Within the syndromic one, it is found the Rett syndrome (*MECP2*), Fragile X syndrome (*FRM1*) and Tuberous

Sclerosis Complex syndrome (*TSC1*, *TSC2*), Angelman syndrome (*UBE3A*), among others. In the case of idiopathic syndrome, it is related to common (*PTEN*, *CHD8*, *SUV420H1*, *RAB39b*) and rare genetic modifications, including some medications and infections during pregnancy (Yang and Shcheglovitov, 2020; Rabeling and Goolam, 2023).

Genetic association studies have identified genes such as *SHANK3*, *NEGR1*, and *PTBP2* as contributors to ASD, particularly due to their high expression during fetal corticogenesis (Grove et al., 2019). Although 80% of ASD cases have ambiguous causes, research using hCOs highlights disrupted signaling pathways influenced by environmental, genetic, and epigenetic factors. These disruptions lead to abnormal neurodevelopment, such as altered synaptic plasticity or imbalances between inhibitory and excitatory neurons. Additionally, mutations in genes related to chromatin remodeling (*MECP2*, *CHD8*), protein synthesis (*NF1*, *PTEN*), protein degradation (*UBE3A*), and synaptic function (*SHANK3*, *CNTNAP2*) are significant risk factors for ASD development (Santos et al., 2023).

De Jong et al. (2021) studied prosencephalon organoids derived from human iPSCs of patients with syndromic ASD caused by homozygous loss-of-function mutations in *CNTNAP2*. The mutated organoids exhibited an increased number of cells, attributed to heightened proliferation of neural progenitors. For *CHD8* gene haploinsufficiency hCOs, a significant increase in the size of hCOs was found with accelerated generation of inhibitory neurons and cortical interneurons and decreased production of excitatory neurons (Astorkia et al., 2024).

The generation of *SHANK3*-deficient hCOs applying the CRISPR/Cas9 technique was used to study the molecular, cellular, and functional effects of *SHANK3* associated with intellectual disability and ASD (Wang et al., 2022).

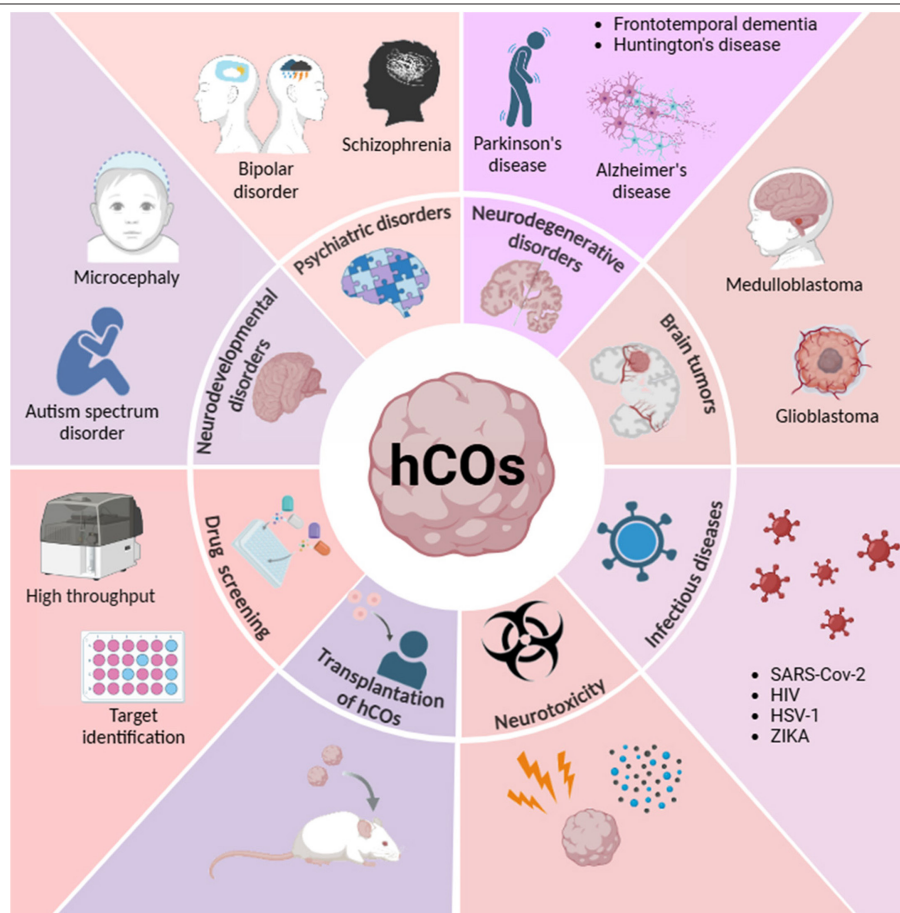


Figure 4 | Applications of the hCOs in human disease research.

Created with BioRender.com. hCOs: Human cerebral organoids; HIV: human immunodeficiency virus; HSV-1: herpes simplex virus 1.

Microcephaly

Another neurodevelopmental pathology that can be further investigated using hCOs is microcephaly. Studies have reported a reduction in the hCOs size linked to microcephaly mutations that down-regulate proteins altering the neural precursor fate. Mutations in *WDR62* or *CPAP* result in longer, more numerous cilia, and promote premature neuronal differentiation. hCOs with mutations in *NARS1* reduce asparagine tRNA, causing radial glial cell cycle arrest. Similarly, mutations in *CDKSRAP2* reduce glial cell numbers while increasing neurons (Alic et al., 2021; Wang et al., 2023).

Apart from the various mutations for microcephaly, it has been observed that the disease can be studied by inducing it through the use of external factors such as ZIKA virus infection. In a study of a Brazilian population, a link was established between microcephaly and ZIKA infection during pregnancy. This is explained by the affinity of the ZIKA virus for neuronal progenitor cells, specifically a set of membrane receptors. Its infection causes defects in specific populations of the cortex as well as cell death, promoting the onset of acquired microcephaly (Trujillo and Muotri, 2018).

Psychiatric disorders

hCOs are a promising tool for the study of brain disorders such as bipolar disorder (BD), schizophrenia (SCZ), and major mental disorders (Villanueva, 2023).

Bipolar disorder

The use of hCOs in BD research has grown, helping to explore both clinical treatments and altered pathways of this pathology. BD-hCOs exhibit reduced size, smaller ventricular areas, abnormal progenitor positioning, and proliferation defects (Hewitt et al., 2023; Phalnikar et al., 2024). Transcriptomic studies highlight changes in proliferation-related pathways (Kathuria et al., 2020a), including the transcriptional repressor REST (Meyer et al., 2024), ion-binding genes, and disrupted mitochondrial-to-endoplasmic reticulum signaling in cortical neurons (Kathuria et al., 2023). Astrocytes in BD-hCOs show reduced neuronal support linked to elevated interleukin-6, although this cytokine increase is not BD-specific (Couch et al., 2023). Immune imbalances with increased cytokines and decreased neuronal activity are commonly observed. Prolonged lithium treatment regulates this altered phenotype by modulating over 100 genes (Osete et al., 2023).

Schizophrenia

Studies using hCOs in SCZ have provided valuable insights into the altered pathways underlying the pathology. Similar to findings in BD, SCZ-hCOs show disruptions in neurogenesis and synaptic function pathways (Kathuria et al., 2020b), confirmed by proteomic comparisons with postmortem tissue patients (Nascimento et al., 2022). Transcriptomic studies reveal a reduction

in neuronal factors such as *MAP2*, *TUBB3*, and *NCAM1*, leading to decreased progenitor survival in VZs and reduced neurogenesis, linked to *BRN2* (*POU3F2*) downregulation. Axonogenesis, axon guidance, and neuronal differentiation pathways are also impaired (Notaras et al., 2021, 2022).

Genome-wide association studies carried out in SCZ-hCOs have identified key neurodevelopmental genes affected in SCZ. Alterations in *FEZ1* disrupt oRGs organization, reducing *HOPX*⁺ cells and causing ectopic neuroprogenitors in cortical layers (Qu et al., 2023). *MAD1L1* mutations impair neuronal migration and cell polarity (Goo et al., 2023). Additionally, genes such as *NRX1* and *PCCB*, which regulate neuronal networks, are associated with reduced GABA levels, further contributing to the disorder (Sebastian et al., 2023; Zhang et al., 2023b).

In addition to altered neurodevelopmental genes, SCZ-hCOs exhibit mitochondrial dysfunction and immune system abnormalities, including upregulation of cytokine- and antigen-binding genes (Kathuria et al., 2020, 2023). Mitophagic gene alterations have been linked to neutrophil infiltration and active dendritic cells (Yang et al., 2024). SCZ-hCOs also show increased expression of inflammatory response genes in endothelial cells, along with genes involved in angiogenesis and vascular regulation. Notably, these changes are associated with the development of *PECAM1*⁺ vessel-like structures within SCZ-hCOs (Stankovic et al., 2024).

Neurodegenerative diseases

The use of hCOs opens a range of possibilities to better understand the mechanisms associated with neurodegeneration, making it possible to recapitulate the genetic identity of the pathology by using patient-derived iPSCs.

Alzheimer's disease

Since Raja et al. (2016) developed the first hCOs from iPSCs of patients with familial Alzheimer's disease (fAD), numerous studies have described the usefulness of these models in studying the development of the main histopathological features of fAD. These features are increased amyloid- β (A β) peptide and A β _{42/40} ratio, which contributes to amyloid plaque formation, and increased hyperphosphorylated Tau protein, which leads to neurofibrillary tangles (Pomeshchik et al., 2023; Kim et al., 2024a).

In addition to these hallmark characteristics, the use of hCOs has allowed the detection and analysis of other alterations that occur during fAD progression. For instance, in neurons, changes have been observed in dendritic size and the expression of synaptic proteins (Ghatak et al., 2021; Kuehner et al., 2021; Park et al., 2023) as well as their receptors. One example is the increase in VGLUT levels accompanied by a decrease in VGAT (Ghatak et al., 2019). Elevated expression of proinflammatory cytokines, such as interleukin-6 and tumor necrosis factor- α , along with evidence of apoptosis and synaptic degeneration, has also been reported (Yan et al., 2018).

Although sporadic Alzheimer's disease (sAD) cases are more prevalent than fAD cases, sAD-hCOs have been shown to take longer to develop AD-associated histopathological features (Meyer et al., 2019; Huang et al., 2022). Despite this time limitation, several interesting studies on sAD-hCOs have been conducted. Among the risk factors for sAD, the *APOE4* allelic variation of the *APOE* gene is associated in AD-hCOs with synapse loss by acting on α -synuclein protein, dysfunctional lipid metabolism, impaired myelination and neuroinflammation (Park et al., 2021; Zhao et al., 2021). Recently, it was observed that certain allelic variants of *APOE*, such as *APOE3* Crischurch (*APOE3ch*), may have a protective role, reducing symptomatology in hCOs (Liu et al., 2024).

Other genes have also been identified as risk factors for sAD. *BIN1*, the second risk factor after *APOE4* has been shown in hCOs to alter the size of primary endosomes (Lambert et al., 2022), and the proportion of glutamatergic neurons (Saha et al., 2024). Mutations in *PITRM1* have been associated with disruptions in mitochondrial membrane potential, leading to impaired A β degradation in hCOs (Perez et al., 2021).

Beyond studying AD using hCOs derived from patients with either fAD or sAD, alternative approaches have been developed to investigate the pathology. For example, it is possible to study AD by generating hCOs with pathologies such as Down syndrome, since they share alterations in *APP* (Gonzalez et al., 2018; Ferten et al., 2024). AD-like pathology can also be induced in hCOs through various methods, such as exposure Aftin-5 (Pavoni et al., 2018), stimulation with A β peptides (Zhang et al., 2023a), or treatment with serum from AD patients (Chen et al., 2021), obtaining hCOs that exhibit AD-like pathology. Additionally, hCOs provide a platform to explore the impact of environmental risk factors, including infections. Studies have shown that infecting hCOs derived from healthy lines with pathogens such as herpes simplex virus (Abrahamson et al., 2021), Zika virus (Lee et al., 2022b), or SARS-CoV-2 (Wang et al., 2021) can induce an AD-like phenotype which can be mitigated using antiviral treatments.

Frontotemporal dementia

Seo et al. (2017) carried out the first frontotemporal dementia (FTD) hCOs using cells from FTD patients, with Tau-mutated, studying the reduction of hyperphosphorylated Tau protein by inhibiting p25 (that leads to aberrant Cdk5 activation) (Seo et al., 2017). Further alterations produced by taupathies have been observed in hCOs, such as a reduction of glutamatergic neurons (Bowles et al., 2021) or up-regulation of astrocytic cholesterol biosynthesis (Glasauer et al., 2022).

Other studies have developed hCOs as models of amyotrophic lateral sclerosis (ALS) and FTD to study the alterations associated with the repeats of *C9orf72* (Szebenyi et al., 2021), causing alterations in neurogenesis: abnormal rosettes, late differentiation, and alterations of cortical neurons (Ferguson et al., 2024; van der Geest et al., 2024). FTD-hCOs are interesting models to understand neurodegeneration because of

the heterogeneity of the FTD and the features common with AD and ALS.

Parkinson's disease

In recent years, studies with midbrain organoids have advanced the understanding of PD. Not only do they reflect hallmarks, such as α -synuclein aggregation, but they also allow the testing of drugs with potential therapeutic effects (Jin et al., 2024; Zhang et al., 2024a). For example, it has been observed how disruption of mitochondrial complex I is affected by mutations in *GBA1* would alter mitochondrial metabolism facilitating neurodegeneration (Baden et al., 2023). Associated with the lack of energy support, it was observed that α -synuclein aggregation is favored by astrocytic senescence by mutations in *SNCA* (Muwanigwa et al., 2024), or by liposomal proteolysis due to mutations in *DJ1* (Morrone Parfitt et al., 2024).

Huntington's disease

Huntington's disease is an autosomal dominant neurodegenerative disorder. By studying striatal Huntington's disease-hCOs, smaller rosettes and fewer neural precursors were observed (Chen et al., 2022). This change in neuronal maturation may be driven by Golgi apparatus alterations, leading to smaller vesicles due to insufficient ADP-ribosylation factor 1, which is essential for proper vesicle development (Liu et al., 2024). Other studies have pointed to neurodegeneration due to mitochondrial damage caused by fission defects (Liu et al., 2022), or by failures in the cellular stress response (Lisowski et al., 2024). These developmental abnormalities in cytoarchitecture are associated with the reduction of GABAergic neurons (Galimberti et al., 2024), and disrupted connectivity of medial striatal spiny neurons to the *substantia nigra*. To study this connectivity between brain regions, striatum-like organoids, and midbrain assembloids replicating the *substantia nigra* have been developed (Wu et al., 2024).

Cerebral tumors

hCOs are not only instrumental in studying neural diseases and disorders but have also significantly advanced our understanding of malignant brain tumors. Since the development of the first glioblastoma (GBM) model using hCOs (GBOs) by Hubert et al. (2016), numerous studies have focused on GBM as the most common malignant brain tumor (Hubert et al., 2016).

Currently, GBO models can be classified into four main types based on their generation method: *Neoplastic Cerebral Organoids* (neCORs) are derived from PSCs modified using CRISPR/Cas9 technology (Bian et al., 2018). Recent studies using these models produced an atlas summarizing frequent GBM mutations through transcriptomic, metabolomic, lipidomic, proteomic, and phosphoproteomic analyses (Wang et al., 2024). *Glioma Brain Organoids* (GLICO) are created by combining patient-derived GBM cells with healthy hCOs, and this model facilitates the study of interactions between healthy and tumor tissues (Kim et al., 2024b). *Direct Patient-Derived GBOs* is a method for creating biobanks due to its high reproducibility and rapid production that involves

the generation of GBOs directly from patient-derived GBM cells, bypassing GBM cell lines. These models are highly reproducible, rapidly generated, and retain the intrinsic characteristics of the parental tumor, whether primary or metastatic (Jacob et al., 2020), as well as they also mimic treatment resistance (Majc et al., 2024). *Bioprinted Models* closely mimic *in vivo* conditions by incorporating endothelial cells for oxygen gradients (Yi et al., 2019) or biodegradable hydrogels can be added to complement the tumor microenvironment (Tang et al., 2022). In the last year, such models have been used to combine glioblastomas with dorsal forebrain organoids and/or ventral forebrain organoids. This has made it possible to detect the regional affinity of GBM for the dorsal region in terms of invasion and the hyperactivity caused by VGAT downregulation (Fan et al., 2024).

Infectious diseases

As previously described, the use of hCOs in conjunction with several infections has led to a better understanding of how these infections interrelate with some neurodevelopmental and neurodegenerative diseases. Beyond this, hCOs are valuable for studying the brain-specific effects of various pathogens.

In the context of SARS-CoV-2, multiple studies utilizing hCOs have elucidated its impact on neuronal function. Key findings include the formation of syncytia mediated by viral fusogens (Martinez-Marmol et al., 2023), disruptions in action potentials accompanied by alterations in synaptic fields (Partiot et al., 2024), and neurodegeneration driven by cellular senescence (Aguado et al., 2023).

Other viruses with pronounced neural effects include cytomegalovirus, HIV (human immunodeficiency virus), and herpes simplex virus 1. For example, cytomegalovirus has been shown to disrupt rosette formation and impair neuronal differentiation (Ijezie et al., 2023; Egilmez et al., 2024). HIV studies have identified significant disruptions in microglial immune responses and Type I interferon pathways (Kong et al., 2024). Herpes simplex virus 1 has been observed to induce both structural and functional neuronal damage (Rybak-Wolf et al., 2023).

In addition, hCOs have been instrumental in modeling parasitic infections. For instance, studies on amoebic pathogens have advanced our understanding of their neuropathological effects (Tongkrajang et al., 2024). These findings highlight the versatility and utility of hCOs in investigating the mechanisms of infectious brain diseases.

Transplantation of human cerebral organoids

One of the most innovative and promising applications of hCOs is their use as a tool in cell-based therapies for various neurological pathologies. Recent studies have demonstrated the ability of hCOs to survive, integrate, and differentiate within mouse brain tissue (Dong et al., 2021). This has allowed progress to be made in the study of the effects of the transplantation of healthy hCOs in several murine models of brain damage or neurodegenerative diseases.

In mouse models of traumatic brain injury, for example, hCOs not only integrate into the tissue, but also increase neurogenesis and angiogenesis, as well as the cognitive capacity of the animal (Kim et al., 2022a). Similar effects have also been shown in murine stroke models, repairing damaged areas (Cao et al., 2023a) or in rats with lesions in the visual cortex, where transplanted hCOs react to visual stimuli from the host (Jgamadze et al., 2023).

In the case of neurodegenerative pathologies, the transplantation of healthy midbrain organoids (or the transplantation of cells derived from these midbrains) has been studied in murine models of PD. In these models, not only survival and correct differentiation to dopaminergic neurons has been observed, but these neurons release dopamine that interacts with the host, integrating into its neuronal network. In addition, improved motor function has been detected in these murine models of PD with midbrain transplantation (Zheng et al., 2023; Fu et al., 2024).

Drug screening and evaluation

The use of hCOs in drug screening and evaluation is diverse. Drug screening traditionally involves multiple stages: design, *in vitro* testing, animal model testing, and clinical trials. hCOs improve this process by bridging the gap between animal models and human patients and could offer greater accuracy and scalability through automation, offering new tools for pharmacological research, and better replicating human biology (Fagiani et al., 2024; Nishimura et al., 2024; Yao et al., 2024). The integration of automated high-throughput technologies has revolutionized hCOs-based drug screening allowing large-scale compound testing, and increasing efficiency and precision (Renner et al., 2020; Nguyen, 2022).

hCOs provide a platform for the evaluation of potential drugs for ASD (Villa et al., 2021; Yang et al., 2022; Zhu et al., 2023). In the case of AD, hCOs models that express AD phenotypes have enhanced drug screening (Kim et al., 2024a). Examples include drugs to reduce the A β aggregation (Raja et al., 2016; Kim et al., 2023), to decrease pro-inflammatory markers (Yan et al., 2018), to address mitochondrial alterations via NeuroD1 (Pomeshchik et al., 2023), or to reduce synaptic receptor disruption, as in the case of NitroSynapsin (Ghatak et al., 2021). Scalable hCOs platforms allow evaluation of A β and hyperphosphorylated Tau protein accumulation using techniques such as RNA sequencing (RNA-seq), protein quantification, and automated imaging (Park et al., 2021), showing promising results in reducing pathological protein levels. These also allow to identify of protective factors such as oestrogens (Kim et al., 2022b), compounds that reduce dysfunctional mitochondria and mitophagy as the flavonoid galangin (Zhang et al., 2023a), and evaluation of genes such as *BACE2* (Alic et al., 2021; Luo et al., 2022).

Glia-enriched hCOs were used to identify treatment for Multiple Sclerosis (Fagiani et al., 2024). In Creutzfeldt-Jakob disease research with hCOs has shown the efficacy of pentosan polysulfate in reducing prion activity. Niemann-Pick type C disease models using iPSCs with NPC1

mutations have evaluated compounds such as valproic acid and hydroxypropyl- β -cyclodextrin, demonstrating phenotype rescue (Giorgi et al., 2024). In mitochondrial diseases, hCOs enable drug testing to improve mitochondrial function, offering viable treatment strategies for complex conditions (Lei et al., 2024; Liang, 2024).

In the case of gliomas, a combined system of murine xenografts and hCOs has been developed to test treatments such as temozolomide, demonstrating the potential of hCOs in personalized medicine (Giorgi et al., 2024). In pediatric tumors such as medulloblastoma, hCOs have advanced the understanding of tumor biology and enabled targeted therapy development, reducing invasiveness and toxicity (Lago et al., 2023; Xu et al., 2023).

Toxicology assays

Developmental neurotoxicity, which involves damage to the developing nervous system caused by chemical exposures during critical stages of development, is a significant concern. hCOs offer a promising solution for assessing developmental neurotoxicity by modeling early human brain development and the effects of chemical substances on neurogenesis and neuronal function (Acharya et al., 2024).

To date, a number of researchers have used hCOs as a model to determine how chemical substances (such as recreational drugs, heavy metals, pesticides, nanomaterials, or air pollutants) affect human nervous system development and brain development (Yan et al., 2024).

Cadmium (Cd) exposure causes neuroinflammation in hCOs, characterized by excessive expression of GFAP, release of interleukin-6, and inhibition of cell cilia-related genes was observed (Huang et al., 2021b). In addition, chronic Cd exposure alters the neurodevelopment of hCOs, impairing growth, neuronal differentiation, and cortical laminar structure, disrupting zinc and copper homeostasis through over synthesis of metallothionein and interference with synaptogenesis, which may affect nervous system function (Huang et al., 2024). Most recently, an improved human ESCs (hESCs)-derived hCOs showed that Cd exposure markedly inhibited organoid growth through mechanisms involving extensive cell death, neural progenitor cell activation, premature differentiation, and eventual depletion of the progenitor population (Hu et al., 2025). Arsenic and lead exposure is significantly neurotoxic to hCOs with optic vesicles (OVb organoids), leading to disturbances in cell proliferation and differentiation, cell cycle disruption, and effects on autophagy and Wnt signaling pathways, which may increase the risk of neurodevelopmental disorders and carcinogenesis (Chen et al., 2024).

ZnO nanoparticles, which are widely used in commercial products, have been shown to increase the ratio of autophagy protein LC3B-II/I and chromosomal aberrations (Liu et al., 2023). Other nanomaterials have been tested in hCOs, revealing various effects on brain development. Hua et al. (2022) investigated the impact of polystyrene microplastics and found that short-term exposure promoted gene expression

associated with cell proliferation and neural progenitors. However, long-term exposure reduced cell viability and the expression of markers in mature neurons and cortical layers. Similarly, Chen et al. (2023) demonstrated that nanoplastics could affect cell viability, damage the structure of mitochondria, and regulate the expression of Wnt signaling pathway genes. Notably, although particle sizes differed, the results showed a dose-dependent effect. In contrast, multi-walled carbon nanotubes, which are inhalable particles, disrupt the multilayered laminar structure of cerebral organoids, impairing development (Jiang et al., 2020). However, multi-walled carbon nanotubes exposure did not show dose-dependent cytotoxicity. Decreased expression of neuronal NO synthase protein was observed both on the surface and within cerebral organoids, likely due to oxidative stress and regulation of the NF- κ B-KLF4 pathway.

To investigate PM2.5-induced neurotoxicity, hCOs demonstrate that exposure to diesel particulate matter has been shown to affect oxidative phosphorylation, leading to abnormalities in mitochondrial function and cellular respiration in brain organoids, which are linked to ASD (Bilinovich et al., 2020).

hCOs have also already been used to test the effect of pesticides on neuronal differentiation, maturation and brain function in general. *Rotenone* causes an increase in reactive oxygen species and mitochondrial dysfunction. It alters key neuronal processes such as calcium reuptake, synaptogenesis, and peroxisome proliferator-activated receptor signaling. Ultimately, different cell populations within hCOs are affected (Huang et al., 2022). Neonicotinoids, which interact with acetylcholine receptors in the brain, can impair neurogenesis, neuronal differentiation, and synaptic function, contributing to neurodevelopmental disorders, in the context of prenatal exposure (Mariani et al., 2024). Chlorpyrifos has been shown to exacerbate neurodevelopmental alterations, particularly in hCOs with CHD8 mutations linked to ASD. This highlights genetic predispositions that may amplify the toxic effects of environmental exposures, underscoring the relevance of incorporating genetic factors in neurotoxicity studies (Modafferi et al., 2021).

Bisphenol A (BPA) and di(2-ethylhexyl) phthalate (DEHP) are recognized endocrine disruptors that adversely affect neurodevelopment. Both BPA and DEHP exposures result in decreased surface area of hCOs, reduced thickness of VZs and SVZs, and impaired cortical morphology (Yang et al., 2023). BPA exposure has been shown to inhibit progenitor cell proliferation, disrupt cortical structure development, and promote premature neuronal differentiation. Notably, T3 supplementation has been found to mitigate these BPA-induced cortical abnormalities (Cao et al., 2023b). Similarly, DEHP exposure reduces the surface area of hCOs, impairs neurogenesis, and disrupts RGC development and neural progenitor migration. RNA-seq suggests these effects are linked to disturbances in extracellular matrix interactions (Yang et al., 2023).

hCOs have also been used to study ethanol toxicity in brain development (Arzua et al., 2020). These organoids reveal that ethanol exposure induces apoptosis, mitochondrial dysfunction, and ultrastructural changes, providing valuable insights into fetal alcohol spectrum disorders by demonstrating dose-dependent effects on neural pathology and gene expression. Zhu et al. (2017) demonstrated that ethanol exposure attenuates neurite outgrowth, and alters neuronal maturation and expression of key genes involved in organogenesis, synaptic plasticity, neuronal transmission, and stem cell proliferation and differentiation. In particular, their study was the first to link ethanol-induced impairment of neurogenesis to the *GSX2* and *RSPO2* genes, as well as to alterations in the Hippo signaling pathway.

Dang et al. (2021) utilized hCOs combined with scRNA-seq to investigate the impact of prenatal methamphetamine exposure on brain development. Their findings revealed a pronounced transcriptional response in astroglial cells, characterized by disrupted cAMP signaling and impaired glutamate regulation. These alterations contributed to gliosis, neuroinflammation, and neurotoxicity, highlighting the detrimental effects of methamphetamine on neural development.

Wang et al. (2018) studied the effects of nicotine on hCOs and found that nicotine exposure led to early neural differentiation (with enhanced expression of the neuron marker *TUJ1*), disrupted cortical development (as evidenced by altered expressions of forebrain (*PAX6* and *FOXG1*), hindbrain (*PAX2* and *KROX20*), and cortical neural layer markers (preplate *TBR1* and deep-layer *CTIP2*), and reduced neurite outgrowth. Furthermore, abnormal neuronal differentiation and migration were observed, suggesting that nicotine exposure impairs neurogenesis during early fetal brain development. In contrast, cannabis, particularly its psychoactive component *THC*, produces opposite effects in hCOs. Ao et al. (2020) demonstrated that cannabis exposure resulted in reduced neuronal maturation and increased neurite outgrowth, which differs from the effects of nicotine. Despite this, both substances affect similar cell populations in the brain, including neurons, astrocytes, and other glial cells, and alter signaling pathways, metabolites, and cellular mechanisms.

Brain evolution studies

Another novel application of hCOs is its usefulness for evolutionary studies. In this type of research, human brain development is usually compared with that of other species, mainly primates, through the generation of brain organoids. To identify human-specific features of cortical development, Pollen et al. (2019) generated pluripotent stem cell-derived cerebral organoids from chimpanzees and compared them with hCOs. The authors identified several differentially expressed genes (including multiple regulators of *PI3K/AKT/mTOR* signaling) in hCOs compared to chimpanzee organoids. Another transcriptomic study of scRNA-seq and accessible chromatin profiling revealed specific features of human brain development. Specifically, the neuronal

development occurred at a slower pace in hCOs relative to chimpanzee and macaque cerebral organoids. In addition, human-specific gene expression showed distinct cellular states along progenitor-to-neuron lineages and divergences in chromatin accessibility in the cortex (Kanton et al., 2019).

Loss-of-function assays in hCOs and chimpanzee cerebral organoids revealed that depletion of *CDK2*, *CDK4*, or *CCNE1* genes did not affect the size of hCOs, while chimpanzee organoids were substantially smaller. The authors confirmed that chimpanzee brain organoids with depletion of *CDK2* presented a large fraction of neural precursors in the G1 phase, suggesting that hCOs were more robust to the depletion of regulators of G1/S progression (She et al., 2023). Furthermore, these evolutionary investigations also allow learning more about the functions of specific genes in the human brain. For example, expression of the human *ARHGAP11B* gene in chimpanzee cerebral organoids doubles the levels of basal progenitors, which are key in the expansion of the neocortex. In contrast, interference of *ARHGAP11B* function in hCOs decreases basal progenitor levels to the levels observed in chimpanzee organoids (Fischer et al., 2022).

Advanced Technologies to Study Cellular Diversity and Physiological Features of Human Cerebral Organoids

Currently, the characterization of hCOs includes the use of techniques of imaging, molecular, and electrophysiology analysis (Figure 5A). Below, we provide an overview of the cellular composition and physiological features found in hCOs using molecular methods (transcriptomics and proteomics) and new electrophysiological technologies, crucial aspects to increase our understanding of the mechanisms underlying brain development under both, physiological and pathological conditions.

Using histological and electron microscopy techniques, hCOs have been shown to present the major cell lineages found in the human brain (Gonzalez-Sastre et al., 2024; Mateos-Martinez et al., 2024).

However, the standardization of quantitative cellular data remains a significant challenge. Data from scRNA-seq have revealed substantial variability in cell populations, influenced by cell lines, generation protocols, and technical methodologies (Tanaka et al., 2020; Fitzgerald et al., 2024; Glass et al., 2024; He et al., 2024). In general, unguided protocols generate greater neural cell variability (Velasco et al., 2019) than guided ones (Yoon et al., 2019). Recently, a Human Neural Organoid Cell Atlas was developed to integrate scRNA-seq data from 26 differentiation protocols, both guided and unguided (He et al., 2024). This atlas revealed that over half of the protocols shared one-third of the gene expression patterns, indicating that many neural cell types are protocol-independent.

Mapping the Human Neural Organoid Cell Atlas to a single-cell atlas of the developing human

brain (Braun et al., 2023) demonstrated that hCOs reproduce the complexity of neuronal, glial, and non-neuronal cell types present during early neurodevelopment. Nevertheless, hCOs lack non-neuroectodermal cells, such as immune and endothelial cells, limiting their ability to mimic the neuronal maturation and cellular diversification observed in the adult human brain (He et al., 2024).

Studies utilizing scRNA-seq have advanced our understanding of neurodevelopmental processes (Werner and Gillis, 2023), and provided disease-specific insights. Datasets from hCOs derived from diseased and healthy controls have been used to study cellular functions, identify key genes, and uncover pathways involved in neurological disorders such as ALS (Szebenyi et al., 2021), AD (Vanova et al., 2023), ASD (Li et al., 2023b), SCZ (Notaras et al., 2022) and GBM (Jacob et al., 2020).

Integration of scRNA-seq data from hCOs with primary brain datasets remains a crucial challenge for elucidating cellular dynamics in neurodevelopment and disease. Prominent examples include analyses of RGCs differentiation into mature excitatory or inhibitory neurons (Velasco et al., 2019), exploration of neuronal communication networks (Zhao et al., 2023), and identification of common or distinct developmental cellular trajectories (He et al., 2023).

The majority of protein-level readouts of hCOs patterning have been focused on immunofluorescence microscopy, however during the last years global proteomic approaches have been adopted. Proteomics has demonstrated its feasibility in exploring the human neural proteome (Nascimento et al., 2019), characterizing specific cell populations (Dezonne et al., 2017), identifying proteins implicated in disease processes (Chen et al., 2018), discovering therapeutic candidates, and assessing drug efficacy.

Electrophysiological analyses, including measurements of neuronal and glial activity and the formation of network oscillations, remain technically challenging. However, such studies hold significant potential for modeling the electrophysiological changes associated with neurodegenerative diseases (Sharf et al., 2022). Calcium imaging-based technologies have enabled the visualization of global neuronal activity in human cortical organoids. These techniques face significant limitations, including poor penetration of fluorescent dyes and an inability to monitor activity over extended culture periods. Similarly, patch-clamp electrophysiology, while effective for detailed single-cell recordings, struggles to capture the complexity of neural networks and oscillatory patterns. The inherent 3D structure of hCOs further limits the accessibility of neurons for both imaging and electrophysiological techniques.

A promising solution to these challenges is the implementation of multi-electrode arrays, which allow for the non-invasive monitoring of neuronal activity across large networks (Tasnim and Liu, 2022). Multi-electrode arrays provide a valuable platform for assessing functional connectivity and synchronized network activity within hCOs, overcoming some of the limitations imposed by their 3D architecture.

Several studies have demonstrated that hCOs exhibit oscillatory activity that mirrors brain signals observed in human brains. For instance, Trujillo et al. (2019) reported robust patterns of network activity characterized by alternating periods of quiescence and short bursts of spontaneous, synchronized spiking. Despite these promising findings, the neuronal activity and inter-regional connectivity within hCOs remain immature, reflecting the early developmental stages of the human brain.

To address the limitations of current methods and improve the physiological relevance of hCOs, new approaches are being developed. Advances such as bioprinting and microfluidic techniques are emerging as transformative tools to enhance the structural and functional mimicry of human brain activity (Saglam-Metiner et al., 2024). These technologies offer the potential to create highly sophisticated organoid models that better replicate the complex dynamics and connectivity of the human brain.

Current Challenges of Human Cerebral Organoids

hCOs represent one of the most advanced tools for biomedical research, offering a more accurate model than traditional cell cultures and animal models for studying the human brain itself and its pathologies. However, they still face significant challenges in terms of structural complexity, scalability, lack of maturity, lack of cell diversity and connections, and variability in their generation. (Figure 6).

Some approaches to reducing hCOs heterogeneity and for improving neuronal development and maturation have been developed, for example, with the application of spinning bioreactors to overcome diffusion limitation in hCOs (Acharya et al., 2024; Lancaster and Knoblich, 2014) and the use of microfluidic devices for increasing hCOs maturity and physiological relevance (Cho et al., 2021). As technology advances, some of these barriers can be overcome, allowing cerebral organoids to play a more central role in personalized medicine and the study of neurological diseases (Acharya et al., 2024).

Vascularization of human cerebral organoids

One of the major challenges today in organoid research is the vascularization of hCOs, as organoids do not possess a vascular system of their own. This lack of a vascular network limits their size and functional development.

Additionally, the lack of vasculature leads to hypoxia, lack of oxygen and nutrients, and the accumulation of toxic metabolites inside the organoid, which causes cellular stress and, finally, the death of these cells. Consistent with this, cells in hCOs without vasculature express high levels of gene markers associated with cellular stress. Therefore, the absence of vasculature leads to the presence of defective cells, which limits the size of hCOs. Lack of vasculature also limits blood vessel endothelial cell signaling, which is necessary for proper brain development (Ye, 2023). In fact, the vascularization of the proliferative zones is important for late development since it helps

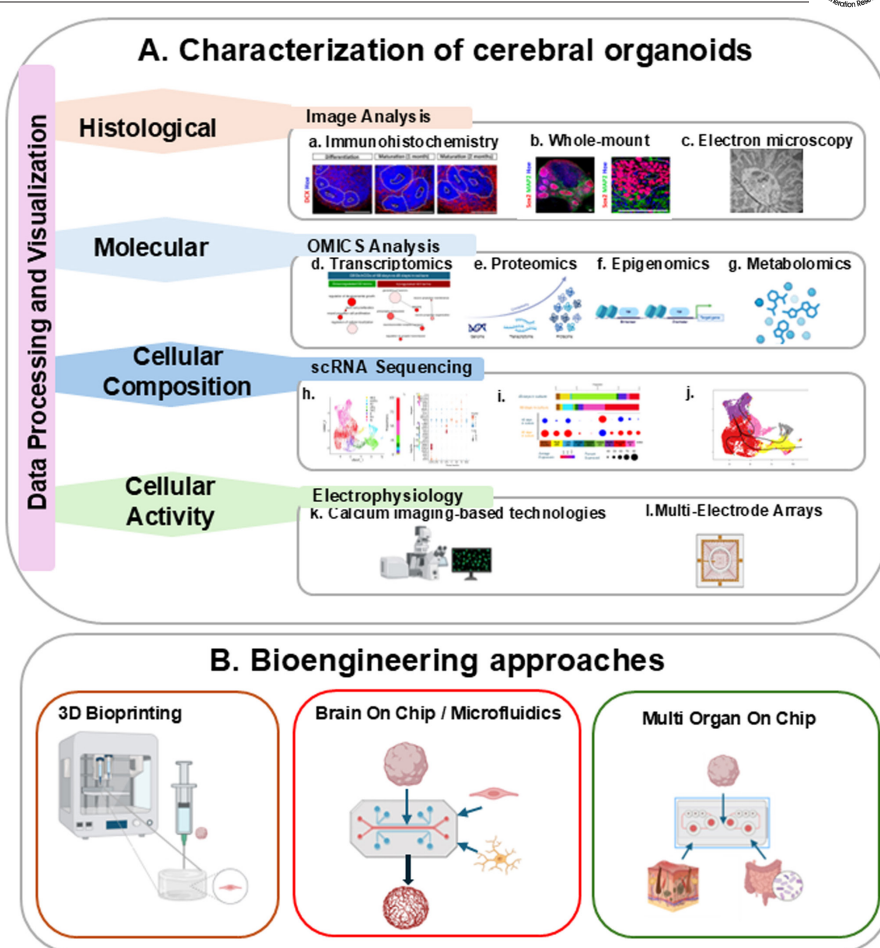


Figure 5 | Advanced technologies to study hCOs and emergent bioengineering approaches.

(A) Schema of technologies used to advance in the histological and molecular characterization and the knowledge of the cellular composition and activity of the hCOs. Images obtained from hCOs generated with the protocol described in González-Sastre et al., 2024: a. Immunohistochemistry; b. Confocal microscopy image (González-Sastre et al., 2025); c. Electron microscopy image (Mateos-Martínez et al., 2024); d. Representation of transcriptional pathways altered in hCOs generated at 30 and 45 days (Mateos-Martínez et al., 2024); e. Proteomics; f. Epigenomics; g. Metabolomics; h. UMAP of scRNA-Seq and biomarker dot plot (González-Sastre et al., 2024); i. Graphical representation of cell types (Mateos-Martínez et al., 2024); j. Cellular trajectory representation using UMAP; k. Calcium imaging-based technologies; l. Multi-electrode arrays. (B) Emergent bioengineering approaches with hCOs. Created with BioRender.com. 3D: Three-dimensional; hCOs: human cerebral organoids; UMAP: uniform manifold approximation and projection; scRNA-seq: single-cell RNA sequencing.

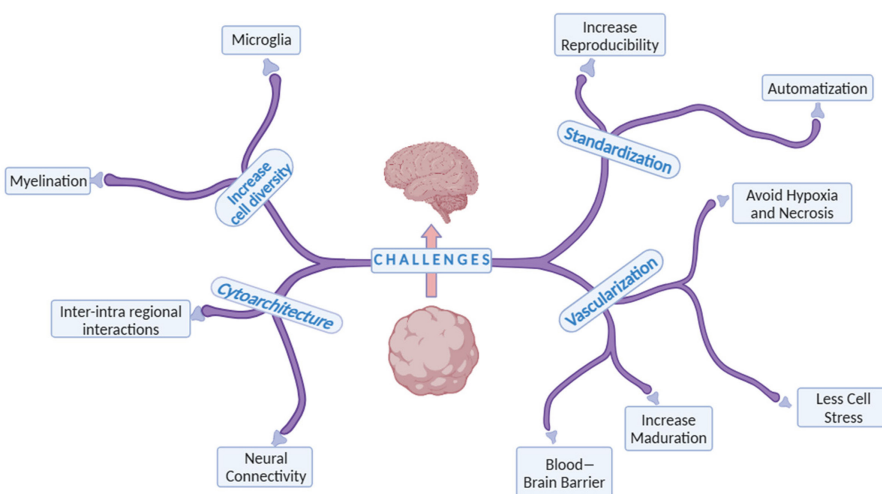


Figure 6 | Challenges in hCO research.

Current challenges include improving standardization, achieving vascularization, increasing cellular diversity, and enhancing cytoarchitecture. Created with BioRender.com. hCOs: Human cerebral organoids.

to generate the niche for neural progenitors, facilitating the proper neural progenitor differentiation, migration, and maturation. Furthermore, efficient oxygen and nutrient delivery and removal of metabolites could improve the regionalization of hCOs, still missing in current protocols (Ye, 2023).

Vascularization is also necessary for the normal development of the human brain as fetal vascularization begins at the third gestation week. The vascular system can be developed by the secretion of some growth factors such as vascular endothelial growth factor and placental growth factor by nearby tissues. A mature vascular system is essential for long-term maintenance of organoids in culture and to better simulate what occurs *in vivo* in the brain (Grebenyuk and Ranga, 2019).

That is why a variety of strategies have been used to vascularize hCOs, for example: (i) Co-culture with vascular cells such as endothelial cells is one of the most common ways to promote vascularization in hCOs (such as human umbilical vein endothelial cells, derived from human umbilical veins). These endothelial cells have the propensity to organize themselves into structures that mimic blood vessels, promoting the formation of a vascular network within the organoids. The generated organoids had reduced levels of hypoxia and cell death and were larger than non-vascularized organoids (Shi et al., 2020; Ye, 2023). (ii) Co-culture of hCOs together with vessel organoids. This strategy can be performed in two ways. One option is to disaggregate the vascular organoid and infiltrate its cells into the hCOs (Ahn et al., 2021). The other option is to directly fuse both types of organoids (Sun et al., 2022). In both studies, brain organoids with structures similar to endothelial tubes and the blood–brain barrier (BBB) are obtained. Interestingly, in Sun et al. (2022), in addition to the increase in neural precursors, they observed the presence of microglia cells. These microglia cells found were not only functional in responding to immune stimuli, but have been shown to engulf synapses (Sun et al., 2022). (iii) Use of biomaterials such as hydrogel scaffolds. Hydrogels, such as Matrigel or alginates, are used to encapsulate the organoids and create a 3D environment that favors the organization of endothelial cells and other vascular cells in a pattern like blood vessels. These biomaterials can be designed to release growth factors or allow vascular cell migration into the organoid. (iv) Reconstructing brain-mimetic microenvironment with decellularized human brain tissue-derived brain extracellular matrix enriched with brain extracellular matrix components. This brain extracellular matrix can recreate brain-mimetic niches necessary to guide neural and glial differentiation for brain organogenesis, which would likely be deficient in the non-neuronal matrix for example Matrigel (Cho et al., 2021). (v) Addition of growth factors to culture medium, such as vascular endothelial growth factor or FGF, which stimulate angiogenesis (formation of new blood vessels), can be used. These growth factors can induce endothelial cells to organize into blood vessels within hCOs without affecting neuronal markers (Ham et al., 2020). (vi) (1) Complex vascular systems such as transplantation

or microfluidic systems: Transplanting hCOs into the brains of immunodeficient mice: They showed progressive neurogenesis, gliogenesis, synaptogenesis, and improved maturation in the transplanted hCOs. However, this strategy is difficult to scale up and can cause differences in hCO development (Mansour et al., 2018; Revah et al., 2022). (2) Microfluidic systems (also known as Organs-on-a-Chip) can be integrated with organoids to mimic a functional vascular system. These devices allow for precise control of medium flow and nutrient delivery, facilitating vascularization and organoid growth (Cho et al., 2021). (vii) Genetic strategies. In some cases, organoids or cells that compose them have been genetically modified to express growth factors that promote angiogenesis. For example, hESCs engineered to ectopically express human ETS variant 2 in hCOs contributed to forming a vascular network. Interestingly, this vascularization resulted in increased functional maturation of organoids. These hCOs formed vasculature that resembled the early prenatal brain and acquired several BBB characteristics such as tight junctions and nutrient transporters (Cakir et al., 2019).

Microglia in the human cerebral organoids

A cell type that is often missing in hCOs is microglia. This is the main immune cell in the brain, representing more than 5% of total brain cells. Microglia origin is not ectodermal, but mesodermal, generated from erythro-myeloid progenitors in the developing embryonic yolk sac, which migrate to the brain during development, where they differentiate into microglia (Utz et al., 2020). The main function of microglia is immune surveillance, acting as resident phagocytes that monitor the environment. In addition, microglia also plays critical roles in neuronal development, synaptic formation, plasticity, and neuronal network maturation (Zhang et al., 2022). During postnatal brain development, microglia actively engulfs synaptic material and eliminates weak synapses to shape neuronal circuits through synaptic pruning (Paolicelli et al., 2011). Microglia dysfunction is also implicated in the pathology of several neurological disorders (Zhang et al., 2022).

Although previous studies have demonstrated that microglia can spontaneously emerge in unguided hCOs (Ormel et al., 2018), it is necessary to improve the representation of this cell type in hCOs to have a more accurate model of the brain. In this sense, some authors have used the overexpression of the transcription factor PU.1 in hESCs to increase the proportion and maturity of microglia in hCOs, although not all the PU.1 overexpressing cells inside the organoids were differentiated into microglia cells, which indicates that more factors are necessary to obtain a microglia identity (Cakir et al., 2022).

Different approaches have been developed for generating hCOs containing microglia cells: (i) Co-culture of exogenous microglia with hCOs. For example, the co-culture of hCOs containing neurons, astrocytes, and oligodendrocytes with microglia derived from human iPSCs. This microglia was capable of integrating, mature, and being affected by an injury similar to *in vivo* cells (Abud et al., 2017). Another interesting study showed evidence of how microglia and

hCOs affect each other (Popova et al., 2021). The authors found that exogenous microglia implanted into hCOs induced transcriptional changes and reduced cell stress. Microglia also increased the synchronization and frequency of oscillatory bursts in the hCOs that facilitated the maturation of neural networks, indicating the microglial contribution to neural development. Other studies applied exogenous microglia into hCOs to investigate how microglia acted in brain pathology and under different infectious agents, such as ZIKA, Dengue or HIV-1 virus (Muffat et al., 2018). Taken together, these studies show that microglia can incorporate into hCOs and somewhat remodel hCOs function and immunity. However, there is considerable variability in the level of maturity and responsiveness to immune stimuli of microglia among the different studies, which may lead to inconsistent and highly heterogeneous results following the addition of microglia to hCOs. (ii) Co-culture of myeloid precursor cells with hCOs. Since microglia are cells of mesodermal origin, the addition of myeloid precursor cells into hCOs could, in theory, generate microglia under the organoid environment. This strategy can mimic the development process of microglial progenitors migrating into the developing brain, and has been used successfully to generate midbrain organoids, increasing neuronal maturation and functionality (Sabate-Soler et al., 2022). However, still, the amount of microglia obtained is uncontrolled and low as compared to the control human brain.

Bioengineering approaches

Advances in hCOs maturation and functionality involve joint developments from different research fields, such as stem cell research, bioengineering, and biomaterials to overcome the current challenges and accelerate hCOs clinical applications in biomedicine (Figure 5B).

As highlighted earlier in this review, hCO cultures lack the maturity of the human brain due to limitations in the culture environment, including the absence of critical cellular components that interact with neurons, such as vasculature and microglia. In this section, we present cutting-edge technologies in cerebral organoids engineering designed to address these limitations, which could pave the way for optimizing the use of hCOs.

3D bioprinting of hCOs involves the automated deposition of biocompatible materials, or bio-inks, to fabricate intricate constructions. Recently, this technology has been applied to create structures featuring microchannels vascularized with human umbilical vein endothelial cells, leaving spaces where hCOs could be infiltrated (Cadena et al., 2024). Likewise, 3D printing has been used to create artificial meshed vessels in hCOs that mimic brain microvasculature and improve nutrient diffusion and oxygen supply (Xu et al., 2024). Although various bio-inks are available (including hydrogels, Matrigel, alginate, and modified gelatin such as GelMA), a significant challenge lies in developing bio-ink formulations that support hCOs survival, differentiation, and maturation. These formulations must also effectively model extracellular components and maintain appropriate mechanical properties for printing (Mierke, 2024).

Brain-on-a-Chip (BoC) technology develops devices by combining the principles of tissue engineering with advanced microfluidic systems. Some BoC models include distinct compartments with microfluidic conditions, providing structural and functional scaffolding. These devices can also integrate microelectrodes and biosensors (electrical, electrochemical, or those based on changes in optical properties such as absorption, scattering, or luminescence) to record electrophysiological activity and monitor metabolic parameters (Yoon et al., 2022).

In particular, Brain-Organoids-on-a-Chip could facilitate the homogeneous distribution of nutrients, oxygen, and growth factors to promote greater maturity, control the expansion with chip size, and integrate vascularization and microglia in the hCOs (Garreta et al., 2021; Amirifar et al., 2022). The advantages of Brain-Organoids-on-a-Chip models may include the following aspects: (i) Reduced variability and improved reproducibility in hCO generation. The use of chemically defined synthetic hydrogels allows for precise control over biochemical parameters (Hofer and Lutolf, 2021). (ii) Improved cellular viability. The implementation of a constant flow of nutrients and oxygen helps reduce apoptosis, promoting higher cell survival rates. This is essential for maintaining hCOs cultures over extended maturation periods, which is particularly beneficial for long-term studies on brain tumors and neurodegenerative diseases, where the persistence and maturation of hCOs are crucial (Kang et al., 2023). (iii) Enhanced cellular diversity. BoC models allow for the inclusion of a variety of non-ectodermal cell types, such as microglia, pericytes, and endothelial cells, within hCOs. This increased cellular diversity is critical for recreating key physiological characteristics of the brain, such as inflammation, the BBB, and neural connectivity.

The inclusion in the hCOs of vasculature (Amartumur et al., 2024) and immune cells (Ao et al., 2022) will improve the accuracy of the hCOs models in simulating pathological conditions facilitate the study of disease mechanisms and therapeutic responses (Amartumur et al., 2024). BoC models would be possible to study for example the responses to relevant chemical stimuli in brain development or neurodegenerative diseases such as AD (Kang et al., 2023, 2024), the evaluation of drug efficacy and toxicity (Kang et al., 2021), the study of the CNS and neurodegenerative diseases (Park et al., 2018), pathogen infections (Tran et al., 2021), and microgliosis induced in GBM (Alves et al., 2022), among others.

More complex *in vitro* models have increasingly shown themselves capable of reproducing aspects of human physiology, overcoming the limitations of conventional animal models. This limitation is particularly evident when investigating the interaction between the brain and other organs, as neural networks cannot be accurately reproduced in 2D systems (Moradian et al., 2024).

In contrast, more advanced systems, such as Multi-Organs-on-a-Chip models, could more accurately replicate the pathophysiological complexity of complex organs, such as the brain, and recreate microenvironments that resemble *in vivo* conditions. These tools are promising for observing biological mechanisms and their

relationship to disease. In addition, it is essential to advance the integration of multiple organs into interconnected systems, such as the gut–skin–brain axis, which, although widely described in the literature, is still little explored experimentally. This approach has the potential to provide deeper insights into systemic interactions and their impact on specific conditions, such as skin diseases, neurodegenerative disorders, and the role of microbiota in these processes. Understanding these connections is crucial to elucidate the mechanisms underlying these diseases and to develop more effective and personalized therapeutic strategies (Zhang et al., 2024b).

The development of a microfluidic system capable of modeling interactions between three organs (intestine, liver, and brain) by integrating the immune system and purified microbial metabolites into the cultures, has revealed that microbiome-associated short-chain fatty acids increase the expression of genes related to the maturation of neurons, astrocytes, and microglia, evidencing positive effects against PD progression (Trapezar et al., 2021).

Kim et al. (2021) developed a microfluidic chip with intestinal epithelial cells and brain cells, which were cultured to simulate the intestinal barrier and the BBB, respectively, to simulate a response of the gut–brain axis, to observe whether dietary components and microbiota can influence behavior, emotions, and cognitive abilities in the brain. The results demonstrated that there was an interaction between the intestinal epithelium and the BBB. In addition, it was observed that there was a transport of exosomes across the intestinal barrier towards the BBB, thus demonstrating that this *in vitro* model can be used to understand the interactions that occur in the gut–brain axis (Kim et al., 2021).

Furthermore, assays for *in vitro* modeling of the human CNS have expanded our understanding of neural development, neurodegeneration, and drug toxicity (Vieira de Sa et al., 2021). Recent advancements also emphasize the integration of vascular networks within these models to simulate better the dynamic interactions between the brain and other tissues.

Conclusions

The discovery of the possibility of generating 3D cultures of hCOs in the last decade has undoubtedly been a major revolution in biomedical research at all levels. It is making it possible to advance in the knowledge of the development and evolution of the human brain, to delve into the molecular pathology of numerous diseases affecting the CNS, during neurodevelopment, neurodegenerative and tumor diseases, and to study the effects of toxic and infectious agents. However, several shortcomings need to be addressed in the current hCOs models. Some of the main weaknesses of hCOs are the scarce presence of immune cells such as microglia and the lack of vascular systems that lead to increased cellular stress that hinders cell type specification and maturation of brain organoids. In addition, the lack of complex neural circuitry also limits the maturation of hCOs to study adult brain and late-onset brain disorders.

The introduction of microglia cells into the hCOs may be a promising model to illustrate the role that microglia play in brain function and disease etiologies. These organoids are not only valuable for studying microglia, but also for building hCOs that better mimic the real human brain. With these models, we have the opportunity to advance our understanding of how the human brain develops and functions.

Moreover, the vascularization of hCOs is an area of special relevance today, due to its impact on the development, maturation, and long-term survival of these cultures. Although there is still much to be done, advances in tissue engineering, new biomaterials, and molecular and stem cell biology are seeking promising strategies. These advances will not only improve the organoid model for studying the brain and brain development, but could also have applications in regenerative medicine, the study of the pathophysiology of neurological diseases, and the testing of new drugs and compounds for the future treatment of these diseases.

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