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Cerebral Organoids as a Tool to Model Neurological Diseases

T. Madikas, G. Dafnopoulos

Aristotle University of Thessaloniki, Thessaloniki, SKG, Greece

Corresponding Author: Thomas Madikas, MSc; e-mail: tmadikas@auth.gr

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Abstract

Modeling the human brain in order to study its physiology, development and diseases has been very challenging due to its structural and functional complexity and fragility. Brain organoids, derived from human pluripotent stem cells, opened a new approach to create *in vitro* 3D models, that more closely resemble the complexity of the brain, as compared to 2D cell cultures. So far, multiple developmental and neurodegenerative diseases have been generated with organoid technology and used as drug screening platforms not only to accelerate the development of more efficient treatments but also to get one step closer to personalized medicine. This review aims to showcase the current applications of brain organoids and review future perspectives and limitations.

List of Abbreviations

CNS = Central Nervous System, PSCs = Pluripotent Stem Cells, BMP = Bone Morphogenic Protein, Wnt = Wingless/Integrated, TGF = Transforming Growth Factor, FBS = Fetal Bovine Serum, KSR = Knockout Serum Replacement, SHH = Sonic hedgehog protein, MGE = Medial Ganglionic Eminence, OTP = Orthopedia Homeobox Protein, ESCs = Embryonic Stem Cells, SMAD = Suppressor of Mothers Against Decapentaplegic, FGF = Fibroblast Growth Factor, PTF1A = Pancreas Associated Transcription Factor 1a, Math1 or Atoh1 = Atonal homologue 1, SFEBq = serum-free floating culture of embryoid body-like aggregates with quick aggregation, CGE = Caudal Ganglionic Eminence, SS = Subpallium Spheroids, SAG = Smoothened Agonist, CXCR4 = Chemokine Receptor type 4, CO = Cortical Organoids, ALI-Cos = Air-Liquid Interface culture to Cerebral Organoids, MPCs = Mesoderm Progenitor Cells, IBA1 = Ionized calcium-Binding Adapter molecule 1, WDR62 = WD Repeat domain 62, KIF2A = Kinesin Family Member 2A, CEP170 = Centrosomal Protein 170, NARS1 = asparaginyl-tRNA synthetase 1, RGC = Radial Glial Cells, CNV = Copy Number Variation, PTEN = Phosphatase and Tensin homolog, ODC1 = Ornithine Decarboxylase 1, PKB = Protein Kinase B, ASDs = Autism Spectrum Disorders, FOXG1 = Forkhead Box G1, CHD8 = Chromodomain Helicase DNA-binding protein 8, DEGs = Differentially Expressed Genes, DISC1= Disrupted-in-Schizophrenia 1, GSK3 = Glycogen Synthase Kinase 3, RTT = Rett Syndrome, MeCP2 = Methyl-CpG-binding protein 2, ERK = Extracellular signal-Regulated Kinase, MAPK = Mitogen-Activated Protein Kinase, MDS = Miller-Dieker Syndrome, AD = Alzheimer's Disease, APP = Amyloid Precursor Protein, PSEN = Presenilin, APOE = Apolipoprotein E, NFT = NeuroFibrillary Tangles, MMP = Metalloproteinase, PD = Parkinson's Disease , SNCA = Synuclein Alpha, LRRK2 = Leucine Rich Repeat Kinase 2, HD = Huntigton's Disease, GSCs = Cancer Stem Cells, GBOs = Glioblastoma Organoids, TBI = Traumatic Brain Injury, CCI = Controlled Cortical Impact, NSE = Neuron-Specific Enolase.

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Introduction

The human central nervous system (CNS) is one of the most complex biological structures and its complexity is reflected in its pathology. CNS diseases are usually very complex with a wide range of etiologies along with genetic and epigenetic factors. The same disease may present with different phenotypes. A major drawback in the study of human brain diseases is the lack of *in vitro* model, which is able to reproduce as accurately as possible the human brain. Traditionally rodent animals have been widely used in research as a model of several CNS diseases. Discrepancies between the human and rodent brains have questioned the value of animal models in neuroscience research. For example, the cerebral cortex of the mouse is smaller than the human cerebral cortex at least 1000 times. Although, the basic architecture of the brain is conserved along mammals, scientists have discovered differences in the cellular composition regarding the human cortex and the rodent one. Hodge et al[1] performed single nucleus RNA sequencing to identify the cell populations of the middle temporal gyrus within the human cortex of the brain. They showed that in this region there are 75 cell types – 45 inhibitory cells, 24 excitatory cells and 6 non-neuronal cell groups. Most of the cell groups were found also in the mouse cortex (again the results have been acquired by single nucleus RNA sequencing). The differences among the corresponding human and mouse cell groups reside in the levels of gene expression. One example is the major difference between human and mouse brain is the expression of serotonin receptors. This suggests that mouse models cannot be used to study human neuropsychiatric disorders caused by fault serotonin signaling.

The first step towards building a better *in vitro* model of the human brain was the discovery of human induced pluripotent stem cells (hiPSCs) by Yamanaka et al[2] back to 2006, that awarded him with a Nobel prize. Human iPSCs are stem cells similar to embryonic stem cells that can differentiate in all three germ layers (ectoderm, mesoderm, endoderm) and in all their derivatives. In theory any tissue or any cell population can be produced by hiPSCs. These cells were first used to generate *in vitro* 2D cell cultures to study tissues that are hard to access, such as the human brain or the human heart. Then iPSCs were produced from patients in order to study several diseases. But 2D cultures fail to reform a cellular microenvironment, which includes extracellular matrix, cell to cell interaction in a 3D space or even realistic mechanical stress.

These issues addressed with the utilization of 3D cell cultures. It is now possible to generate 3D cell models from iPSCs (or adult stem cells) that are organ-like and can be produced from healthy donors derived-iPSCs or patient-derived iPSCs. These models are called organoids. The most notable attempt to produce a cerebral-like organoid is that of Lancaster and Knoblich[3] in 2014, when they first generated a 3D culture derived of iPSCs based on the self-organization ability of the stem cells along with the correct environmental cues coming from several growth factors.

From 2014 until know several advancements in the field of organoids have been made that enable scientists to utilize this technology in order to model diseases of the human CNS. In this review we will try to briefly present some aspects of organoid technology and some attempts to produce accurately *in vitro* model of some neurodevelopmental and neurodegenerative diseases.

Organoids Generation - Techniques

Types of brain organoids

Cerebral organoids

Whole-brain organoids or cerebral organoids are not region-specific brain organoids. Their production requires the exclusion of growth factors, which facilitates neural differentiation. The fact that cells can self-organize is manifested by the fact that many structures in the brain organoids can be distinguished simply by allowing the cells to differentiate into a three-dimensional state without external interference. However, due to the stochastic situation of cells, these organoids lack the dorsal-ventral, anterior-posterior and medial position that occurs in an, *in vivo*, brain structure. Biomaterials can support the three-dimensional brain structure of organoids, enabling cells to support an architecture close to that of a normal brain.

Forebrain organoids

The organoids of the forebrain are the ones that have been studied the most. This is because the forebrain produces a number of other brain organoids, such as the thalamus and hypothalamus, the cerebral cortex, the optic cup, the olfactory bulb

and the basal telencephalon. Mariani et al[4] generated forebrain organoids by blocking three signalling pathways, BMPs, Wnt, TGF-β/activin/nodal. More specifically, the brain structures showed signs of forebrain on the 50th day, while on day 70th synapses between neurons could be discerned. Using the same protocol, Pasca et al[5] were able to reproduce forebrain-like structures by inhibiting the same pathways. After 20 weeks of gestation, neurons started to express NEUN, a mature neuronal marker, usually expressed in the human forebrain.

Optic cup organoids

The three-dimensional formation of the eye is crucial for smooth function and vision. This means that structural errors during morphogenesis can result in visual damage that can even lead to blindness. Morphogenesis of optic cup starts with the evagination of optic vesicles from both sides of the brain. Complex cell and tissue rearrangements give a hemispherical structure to the optical vesicles called the optic cup. A fully formed optic cup contains a retinal pigment epithelium, the neural retina, pseudostratified epithelium that consists of neural progenitors, a flat monolayer and the lens. The first report of organoids with optic cup characteristics appears back in 2011 by Eiraku et al[6] . The key point in creating optic cup-like organoids lies in the use of Matrigel, Fetal Bovine Serum (FBS) and Knockout Serum Replacement (KSR) in combination with manipulation of other signalling pathways at different stages of eye morphogenesis [7,8].

Hippocampal/choroid plexus organoids

The telencephalon is one of the most developed regions in the mammalian brain and has been associated with the ability to learn and memorize. The telencephalon consists of the dorsal pallium and the medial, lateral and ventral pallia. It is also incorporating regions of the cerebral nuclei, as the amygdala and septum. The origin of the hippocampus arises from medial pallium which resides in the telencephalon, next to the dorsal pallium. More dorsal to the previous structures are found some midline structures, as the choroid plexus. The neurons forming the hippocampus are mostly pyramidal and granule type. It is known that a gradual decrease along the dorsal-ventral axis, such as wingless-related integration site (WNT), bone morphogenic factor (BMP) and sonic hedgehog (SHH), determines the differentiation of dorsal pallial and ventral subpallial regions of the telencephalon. Lateral to the choroid plexus lies the cortical hem, which secretes WNT and BMP, while the dorsal midline (where the choroid plexus will form) secretes BMP. This system regulates the gradience of morphogenic factors that would guide the differentiation of the different telencephalon regions. Traiffort et al[9] were able to generate a 3D culture of the choroid plexus from human embryonic stem cells (hESCs) using prolonged dorsalization by BMP and WNT activation and the generation of the hippocampus using a transient activation of BMP and WNT. The same team dissociate the 3D cultures to achieve prolonged maturation of the neuronal cells and the establishment of a protocol to generate hippocampal and choroid plexus organoids.

Ventral telencephalon organoids

The cortical cells are sorted in two broad categories, the pyramidal glutamatergic neurons and astrocytes, which proliferate in the ventricular zone of the pallium (dorsal telencephalon) and the GABA-ergic inhibitory interneurons and oligodendrocytes that are generated in the subpallium (ventral telencephalon) and migrate to the cerebral cortex. The ganglionic eminence (GE) is one of the major regions of the subpallium and is further divided in medial lateral and caudal GE. Different types of interneurons are produced in each subregion and since SHH control the development of the subpallium, SHH could be responsible for the differentiation of interneurons[9,10].

When organoid technology was put into laboratory practice, several groups tried to create 3D models of the ventral telencephalon using the SHH signaling. Birey et al[11] were able to generate human forebrain spheroids, and Xiang et al[12] generated medial ganglionic eminence (MGE) and cortex-specific organoids from human pluripotent stem cells.

As mentioned above, oligodendrocytes are also produced in the subpallium, thus ventral telencephalon organoids could be a useful tool in understanding their differentiation path as well as their migration tactic towards the cerebral cortex.

Diencephalon organoids

Diencephalon is the link between the telencephalon and the midbrain, and it is divided into four main structures, the thalamus, the hypothalamus, the epithalamus and the subthalamus. During embryon-

ic development, the diencephalon is differentiated from the anterior vesicle of the neural tube, and in adult humans is located above the brain stem and under the cerebellum. Cerebellum and diencephalon are parts of the forebrain.

Qian et al[13] were able to successfully generate iPSCs-derived hypothalamic organoids. They initiated the differentiation protocol by treating the iPSCs with dual SMAD inhibitors to drive the cells fate towards the neuroectodermal fate. After three days, embryonic bodies were formed, which exposed to WNT3A, SHH and Purmorphamine in order to induce hypothalamic differentiation. After eight days the culture expressed markers associated with embryonic hypothalamus development, such as NESTIN, SOX2, NKX2.1, NKX2.2 and FOXA2. After day 40, the culture contained subsets of cell populations that expressed the homeobox protein OTP, which is used for hypothalamic cell specification.

More recently human thalamic organoids were also generated from hESCs by Xiang et al[14] the team who used the same strategy with Qian et al[13] for the neuroectodermal differentiation, using a dual SMAD inhibitor supplemented with insulin. The thalamus is produced caudally in the forebrain and insulin is a caudalization factor. After the initial neural induction, PD0325901 was used to inhibit the MEK-ERK signaling pathway in order to stop further caudalization, which would result in a midbrain differentiation. Along with PD0325901, human BMP7 was used, as previous findings suggested that BMP7 induces thalamic differentiation in rodents.

Midbrain organoids

Mesencephalon, or midbrain, is detected caudal to the forebrain. Communication among the forebrain and the spinal cord, movement control and sensory processing are some of the midbrain functions. Because of its association with motor functions, there is great interest in understanding diseases such as Parkinson's disease, especially the dopaminergic neuron of midbrain[15,16]. Midbrain's development is very closely related from the morphogens WNT1 and SHH and the growth factor FGF8[17,18]. Generation of midbrain organoids achieved from hPSCSs-derived embryoid bodies (EBs) with a combination of WNT1 and SHH activation, FGF8 treatment and dual-SMAD inhibition[19]. Another scientific group reported that midbrain organoids can be produced without FGF8 treatment[20].

Hindbrain organoids

Hindbrain region contains cerebellum, the centre of movement control. Cerebellum can be developed from the hindbrain's rostral part linked to the midbrain. It includes GABAergic neurons like Purkinje cells and glutamatergic neurons like projection neurons and granule cells. GABAergic neurons are produced from the $Ptf1a^+$ cerebellar ventricular zone, while the glutamatergic neurons are generated by the $Math1^+$ rhombic lip[21]. Treatment with Wnt3a and BMP4 intensified the production of $Math1^+$ neural progenitors from mouse ESCs with a further differentiation into granule cells[22]. Same conditions in culture did not contribute to the Purkinje cells production. Eventually, Purkinje cells generated using SFEBq approach and treatment with FGF2, insulin and the SHH inhibitor cyclopamine[23].

Fusion of different types of brain organoids

The formation of the human Central Nervous System (CNS) is a very complex and dynamic process, the mechanisms of which, have not been elucidated yet. During this extremely complex procedure, migratory cell flows are created. This migration results in them settling from one particular area to another. Complex neural networks immediately begin to form, and branches of neurons are projecting in multiple areas. Entanglement increases if we take into account, that at the same time, ancestors of microglia from the mesoderm begin to colonize the fetal brain along with the development of perfusion system.

In recent years a great effort has been made to decode neuronal activity and cell differentiation mechanisms, to better understand the development of the vascular system as well as the production of oligodendrocyte, astrocytes and microglial cells, through the construction of cerebral organoids. Therefore, it is crucial in the coming years for scientists to shed light on the circuits and connections of neural networks through organoid technology.

Interneuron migration

During the development of the human cerebral cortex, a process of assembling brain circuits and establishing neural networks consisting of glutamatergic neurons, in the dorsal forebrain or pallium, and GABAergic interneurons in the subpallium or ventral forebrain precedes[11,24]. Caudal ganglionic

eminence (CGE) and medial ganglionic eminence (MGE) are two areas of the ventral forebrain that contain GABAergic cells. These inhibitory interneurons, begin to migrate from ventral forebrain to dorsal forebrain, after their production. In dorsal forebrain they start to connect with locally born neurons. Imperfection in proliferation of these cells or in their migration could lead to a series of many psychiatric and neurological disorders like, Down syndrome, autism or schizophrenia.

In 2017, Birey et al[11], for the first time manage to model migration of interneurons using brain organoids through an approach using hPSCs. Adding SHH and WNT inhibitory at the early stages of differentiation made their organoids to acquire an astonishing resemblance of the dorsal forebrain (human cortical spheroids, hCS) and the ventral forebrain (human Subpallium Spheroids hSS). After this, scientists proceed into the assemblage of the different parts of the brain organoids which led to interneuron migration along with the creation of connections between cells.

Shortly afterwards, in the same year, two independent scientific groups were also able to use this technology successfully. Specifically, Bagley et al[25] used an inhibitor of WNT pathway, IWP2, and SAG an agonist of SHH receptor for the ventral part and an antagonist of SHH receptor, CycA, for the dorsal part. Using a fusion technique from Lancaster and Knoblich[3] they observed cell migration from ventral to dorsal parts. Administration of AMD3100 an antagonist of CXCR4 disturbed the migration.

Xiang et al[12], produced MGE organoids (hMGEOs) from human embryonic stem cells (hESC) along with cortex-specific organoids (hCOs). Transcriptomic and chromatin analysis disclosed a strong relation between hMGEOs with fetal brain tissues. Fusion between the two organoid systems revealed migration of interneuron progenitors into hCOs. Administration of blebbistatin, inhibitor of myosin II, suspended the migration process.

Neuronal projection

The long-ranged connections play critical roles in different brain function. Corpus callosum connects the neurons of the right and left hemispheres. Internal capsule connects cortex with the thalamus while the forebrain is connected with spinal cord with axons that penetrate midbrain. If these circuits are disturbed the nervous system will not be able to perform its functions properly.

Using thalamic and cortical organoids Xiang et al[14] established a model of axonal connections. When these two specific regions were fused, neuronal projections from both sides of the two organoids began to consolidate. Further analysis disclosed that the maturation of thalamic neurons was affected by cortico-thalamic neuronal projections. Disorders like epilepsy, autism and schizophrenia are linked with thalamic anomalies and this system is a step closer for decoding the mechanisms related with neurological and psychiatric disorders.

Giandomenico et al[26] adapted an air-liquid interface culture to cerebral organoids (ALI-COs) in order to improve axon outgrowth and neuronal survival. ALI-COs were placed millimetres away from the spinal cord of the mouse embryo, which was also connected with paraspinal muscles and dorsal root ganglia. Neuronal projections from ALI-COs developed and ended in the mouse spinal cord led to the control of the contraction activity of paraspinal muscles.

Vascularization

One of the most important disadvantages of brain models is the lack of vascularization. This problem arises due to the lack of cells coming from mesodermal origin during the cultivation of model organoids. Wörsdörfer et al[27], in an attempt to address this limitation, attempted to integrate mesoderm progenitor cells (MPCs) into neural organoids. This fusion led to the creation of structures that were very close to a classical blood vessel structure. These structures included endothelial cell-to-cell junctions and a basement membrane. The most impressive was the creation of $IBA1^+$ cells which originate from MPCs and behave like microglia.

Developmental diseases

Microcephaly

Microcephaly is defined as the reduction of the circumference of the head above two standard deviations below the average value for age and gender. People with congenital diseases tend to have

smaller brain size. Recent studies have shown that mutations in a population of genes can lead to microcephaly condition. One such mutation is that of the gene CDK5RAP2. Buchman et al[28] found that Cdk5rap2 is highly expressed between the neural progenitor cells. Therefore, the loss of function of this specific gene leads to overexpression of this protein and this results in the depletion of apical progenitor cells through premature neuronal differentiation, as a consequence of rapid exit from the cell cycle. The failure of mouse models to encapsulate the microcephaly symptoms in humans led scientists to turn to the creation of organoid disease modelling. Cerebral organoids that came from a patient with microcephaly with the specific mutation of CDK5RAP2 gene, revealed premature neural differentiation, the exact same phenomenon that shows up in the disease phenotype[29]. This novel study proclaimed for the first time that brain organoids can be used for disease modelling.

Mutations in genes, like WDR62 and KIF2A that encode centrosomal proteins can cause primary microcephaly. A previous research that modelled microcephaly with human cerebral organoids disclosed a WDR62-CEP170-KIF2A pathway and the disruption of it leads to microcephaly[30]. Wang et al[31] also reported that loss of NARS1, a gene that translates an aminoacyl-tRNA synthetase required for support of the Radial Glial Cells (RGC) proliferation in human brain development can cause microcephaly. The cortical brain organoid model revealed a turn-down in RGC proliferation, which further led to smaller organoids, compared with control, a characteristic of the disease. Another study further supports that brain organoids, which came from human, can recapitulate disease modelling caused by a mutation to ASPM gene[32]. One of the most ordinary copy number variation (CNV) that leads to microcephaly is the duplication of the 16p11.2 region. In a recently published research[33], cortical organoids was developed from the 16p11.2 duplication patient fibroblasts. Researchers unveiled a statistically significant decrease in duplication organoids compared to the healthy control and a significant number of pathways disrupted such as, cell motion and mobility, activation of ion channels, synaptic relation processes and Wnt signalling. The phosphatase and tensin homolog (PTEN) protein which is encoded on chromosome 10, regulates human cortical formation. Dhaliwal and colleagues[34] generated human brain organoids with PTEN overexpression. This overexpression resulted in the reduction of neural progenitor proliferation, a precocious neuronal differentiation that led to the development of significantly puny brain organoids.

Macrocephaly

Macrocephaly is a clinical condition in which the patient presents an unusually large head phenotype with a circumference more than 2 standard deviations above the average value for a given gender and age. As beforementioned the duplication of 16p11.2 leads to microcephaly. In the previous study[33], it has been found that not only duplication led to significantly decreased organoids, but when they generated cortical organoids with deletion of 16p11.2 region, they found out that these organoids were significantly larger than the control ones. Ornithine decarboxylase 1 (ODC1) is a gene that its expression linked with cell proliferation of neural progenitor cells. ODC has a critical role for the synthesis of polyamines controlling cell proliferation and transformation. Cerebral organoids from hiPSCs developed to test the hypothesis of correlation between maturity of neurons and ODC1 expression[35]. Results showed that brain medial and lateral regions consisted of high levels of the protein Odc1. This pointed out a significant correlation between gain-of-function variants and neurodevelopment resulting in macrocephaly. PTEN protein along with protein kinase B(PKB/AKT) is a regulator of cortical formation in humans[36], and Dhaliwal et al[34] found out that overexpression of PTEN led to smaller brain organoids. On the other hand, a group of researchers generated brain organoids without the PTEN gene, deleted by CRISPR/Cas9 genome editing, unveiled an increment of PKB activity in human neural progenitors, boosted cell cycle re-entry and delayed neuronal differentiation, something that resulted in an expansion of intermediate progenitor cells. At the level of organoids, their size was significantly bigger and presented surface folding[32]. One more study indicated the importance of PKB through the PI3K-AKT-mTOR pathway. Dysregulation of the specific mechanism led to macrocephaly and autistic-like behaviours[37].

Autism spectrum disorders (ASDs)

Autism spectrum disorders are a group of conditions with a neurodevelopmental and psychiatric background appertaining to a high degree of complexity and heterogeneity in terms of their genetic architecture and structure. The symptoms of ASD, often present in patient's childhood, vermiculate in severity and type. Such symptoms are characterized by language difficulties, inability to communicate with other people, repetitive behaviours, sleep disorders, anxiety and panic attacks, aggressive behaviour and intellectual disability.

The pathophysiology of ASD puzzles scientists and genetic architecture in a large part of ASD incidents is unknown till today. Thousands of genes seem to implicate in ASD according to researchers. Studies showed that a number that varies between 300-1000 genes are potential targets for an abundance of rare mutations associated with ASD. Their complexity and heterogeneity make it hard to model with traditional technologies such as cell culture. In order to comprehend ASD mechanisms scientists begin to turn into stem cell modelling and organoids as a promising experimental technology.

Today, cerebral organoid technology is rapidly evolving in terms of research into ASD conditions. In particular, Mariani et al[38] worked on cerebral organoids acquired from families with one member suffered from idiopathic autism. Analyses of transcriptomics revealed differential gene expression and more specifically showed up increased population of GABAergic inhibitory neurons as well as unusual proliferation of neural progenitors in comparison with control groups. Overexpression of GABAergic neurons in appear to be the result of upregulation of the transcription factor FOXG1 in ASD organoids.

The Chromodomain helicase DNA-binding protein 8 (CHD8) is a gene whose mutation has been linked to ASD conditions such as schizophrenia and intellectual disabilities[39,40]. One of its main roles is the negative regulation of WNT signalling, a pathway that plays a critical role in early brain development. In order to investigate the role of CHD8, Wang et al[40] proceeded to a transcriptomic analysis of $CHD8^{+/-}$ cerebral organoids from iPSCs using CRISPR/Cas9 gene editing technology. Analysis revealed that differentially expressed genes (DEGs) were to a large extent similar with the DEGs found in idiopathic ASDs and previous studies[38,40]. A non-coding antisense RNA, DLX6-AS1, and a regulator of GABAergic neurons development, was the most overlapped DEG.

Disrupted-in-Schizophrenia 1 (DISC1) gene's mutations are correlated with a wide range of significant psychiatric disorders like autism, schizophrenia, depression and bipolar disease[41,42]. DISC1 is a scaffold protein and interrelates with various proteins of dopamine system like AKT and GSK-3[43]. A study published in 2017[44] unveiled the mechanism behind the DISC1/Ndel1 complex through human forebrain organoids. The disorder of DISC1/Ndel1 complex formation extended mitosis and affected negatively cell cycle of radial glial cells.

Rett syndrome

Rett syndrome (RTT) is an early-onset neurological condition which mostly affects young females. Symptoms of the syndrome include disturbed brain functions, mental and learning difficulties and repetitive movements of the upper extremities[45,46]. RTT is due to mutations in the X-linked gene methyl-CpG-binding protein 2 (MeCP2) at a rate of a 90% while other mutations associated with the disease are those that occur in CDKL5 and FOXG1 genes with approximately 10% of Rett Syndrome cases[47,48].

MeCP2 is a transcriptional regulator and is highly expressed in brain, spleen and lungs and has lower expression in heart, kidney and liver. In the brain, expression of MeCP2 is mostly detected in neurons but its protein has been found also in oligodendrocytes, astrocytes and microglia[49-51].

An elegant study with organoid models generated by hiPSCs unveiled an untimely development of the deep-cortical layer connected with the formation of specific neurons such as TBR1 and CTIP2 while there was low expression of progenitor neural cells in female dorsal organoids with Rett Syndrome. In addition, when they tried to assembly RTT ventral and dorsal organoids found out problems in interneuron's migration[52]. MicroRNAs (miRNAs) seem to possess a remarkable role in early neurodevelopment while being targets of MeCP2. Mellios et al[53] produced cerebral organoids derived from RTT patients disclosed two MeCP2-targeted miRNAs (miR-199, miR-214) that their upregulation during brain development led to a different modulation of AKT signaling pathway as well as extracellular signal regulated kinase (ERK) and mitogen-activated protein kinase (MAPK).

Miller-Dieker syndrome

Chromosome 17p13.3 is a genomic unstable region linked to rare neurodevelopmental genetic conditions with one of them being the Miller-Dieker syndrome (MDS). The main cause of MDS is the heterozygous deletion of this specific area involving the genes YWHAE, LIS1, PAFAH1B1, OVCA1 and H1C1 and is associated with lissencephaly and smooth cerebral cortex resulting in intellectual disability, congenital anomalies, seizures and abnormal spasticity[54,55].

A group of researchers[56], in 2017, analysed cerebral organoids originated from MDS-iPSCs and control-IPSCs using immunostaining, single-cell RNA sequencing and time-lapse imaging. The analysis revealed a cell migration flaw as well as critical apoptosis of the founder neuroepithelial stem cells. The cell migration error fixed when researchers rectified the chromosomal deletion. Organoids derived from MDS patients were remarkably smaller than the control ones along with asymmetric cell division from radial glia cells of ventricular zone (vRGCs)[57]. Phenotypic changes like the organization of microtubule network in vRGCs led to anomalies regarding the N-cadherin/β-catenin signaling. Moreover, the extrinsic stimulation of Wnt pathway led to the improvement of the phenotype, insinuating that Wnt/β-catenin signalling pathway can be a prospective therapeutic strategy for MDS patients.

Neurodegenerative diseases

Neurodegeneratives disorders are charecterized by degeneration and loss of functional neural cells caused by a variety of reasons as toxic cell environment, protein aggregation, mutations and ageing. Although the mechanisms undelying these diseases have been studied etxenciveley in 2D and animal models, but also cadaveur brain tissue, little is known about its pathophysiology.

Among the available models to study brain disorders, the most suitable for accurate results is the human brain. Post-mortem brain tissue is often hard to find, but also some studies have shown changes in the transcriptome of post-mortem brains[58]. The next most used model to study neurodegenersation is animal models and most often rodent are used to study the human brain due to ease of handle in the lab but also their similarity to the human brain. Although animal model can be used to study the development of a brain disorder, they cannot be used for the development of suitable treatments because of differences in the pathology of the disorder that are species-specific[59]. 2D cell cultures are an alternative solution, but they fail to recapitulate the 3D microenviroment and cell to cell interactions. These reasons made the delopment of new *in vitro* modeling systems mandatory.

Brain organoids are a propitious system to model neurodegenerative diseases, as they could recapitulate precicely the cellular interaction that happens *in vivo*, the cellular microenviroment and cellular composition of the tissue of interest. In addition they could be used as a tool for personalised medicine. Patient-derived brain organoids could be used as a drug screening platform, predicting a patient's outcome to a specific theurapeutic approach. This technology relies heavily on the innate ability of cells to self-organize given the correct extracellular cues, thus they could also help the scientific communtity shed some light in the development of the human brain but also the development of some neurodegenerative diseases.

Modeling Alzeihemer's disease

A new case of Alzheimer's disease (AD) occurs every seven seconds worldwide, making it the most common cause of dementia[60]. AD is classified by the age of onset, to early onsert AD and late onset AD. Early onset AD occurs in roughly 1%-6% of all cases and manifests betwwen the ages of 30 to 60. Late onset AD begins when the patient is older than 60[61].

AD is a multifactorial disease, with enviromental, genetic, and developmental problems, which play a part. The major risk factor is age, with complimentary factors being head trauma, AD in the family, gender (females have a higher risk of developing AD), assosiated deppression, diabetes, vascular issues and hyperlipidemia[62,63].

There are three key genes responsible for causive early onset AD. These genes encode, the amyloid precursor protein (APP) on chromosome 21q21, presenilin 1 (PSEN1) on chromosome 14q24 and presenilin 2 (PSEN2) on chromosome 1q42. There are several other genes that contribute to the pathophysiology of the desease. Mutations in the gene that endoces the apolipoprotein E (APOE) that resides in chromosome 19q13, have been assosiated

with late onset AD. The full molecular profile of AD is still in research, but these four genes constitue half of the total genetic risk[61] .

The above genetic factors contibute to create the histological profile of AD. Mutations in the APP gene cause increase despotition in the brain of the pathogenic form of amyloid-β (Aβ) petpides. PSEN1 and PSNE2 mutations also play a role in the high concetration of aβ peptides, as they, under normal conditions, provide instructions for making a protein complex called γ-secretase. The major role of this complex is to cut proteins to petpides, thus the disfunction of the complex contributes to Aβ-peptide production[61].

Another histopathologic characteristic of AD is neurofibrillary tangles (NFT). The number of NFTs is linked to the degree of the dementia, suggesting that NFTs are more correlated with neuronal loss than Aβ peptides. The main compartments of NFTs are higly phosphorelated forms of the microtubule-assosiated protein tau. The accumulation occurs early in neurons, before the formation of NFTs[64].

Several attempts have been made to model AD, using animal models and 2D cell cultures. Animal models of AD that overexpress AD-related mutations are able to develop amyloid related pathology but NFTs are absent as well as the neuronal loss that accompanies NFTs. 2D neural cell cultures have been generated using AD patient-derived iPSCs. Although, the cells carried the mutations that the patients had, the Aβ peptides that were produced by the mutant cells could not recapitulate the pathophysiology of AD, as they were removed from the culture when the media was changed[65-67].

The first attemp to create o 3D cell model of AD was made by Choi et al[68]. Their approach deviates from the classical organoid protocols, although they were able to generate a 3D model that recapitulate the pathology of AD, incorporating both NFTs and Aβ-petide desposition. They started by differentiating the cells in 2D before embedding the cells in matrigel for the creation of the 3D model. The cells were overexpressed APP and PSEN1 mutations, and the research group were able to demonstrate the extracellular increase of Aβ in compariton with the 2D culture. NFTs were also present in this model. Finally they demonstated that treatment with inhibitors of β- and γ-secretase could decreade the huperphosporylated tau protein and toxic levels of Aβ-peptides.

Raja et al[69] used patient-derived iPSCs to generate brain organoids that harboured the mutations of AD patients in APP and PSEN1 genes. The organoids were cultured for one month and they consisted of different subpopulations of cells that recapitulate different brain regions. Raja et al[69] describe the aβ-peptide presence in the organoid model as well as the presence of NFTs with poshorylated tau protein. Moreover, they were able to show that treatment with β- and γ-secretase inhibitors causes a significal reduction of tau and amyloid pathology in AD-patient derived organoids, proving that these models could be used as a drug screening platforms in preclical studies.

Yan et al[70] also generated cerebral organoids, derived from iPSCs from familiar AD patients carring PSEN1 mutations. They also managed to recapitulate Aβ-peptides accumulation, tau pathology and also cellular apoptosis. They were also able to show that glutamatergic neurons decreased after 17 days of organoid culture but increased after 28 days of culture, which is consistent with the hyperactivation of neurons that follows a cycle that orchestrated by Aβ-amyloids and is observed in animal models. They also showed that lactate dehydrogenase was higly active in AD cerebral organoids, which leads to neuronal loss through toxicity, elevated gene expression of inflamatory factors such as interleukin-6 and tumor necrosis factor. This leads to apoptosis of astrocytes and neurons. They also observed downregulation of metalloproteinase-2 and 3 (MMP2, MMP3). These extracellular matrix proteins are fundamental for correct neuronal activity as they take part in neurogenesis, remodeling of the basement membrane, axon regeneration, synaptic remodeling and Aβ-amyloid degradation. Arber et al[71] attempted to model familiar AD using iPSCs that carried PSEN1 mutations showed accelerated aging in neural cells and increased neurodegeneration. They also showed a decreased production of new neurons that aggreed with the premature aged neuronal profile.

The above models focused on efforts to model familiar AD. Research teams such as Lin et al[72] and Zhao et al[73] have tried to model sporadic AD. The first group used the CRISPR/Cas9 technology to promote APOE3 mutations in human pluripotent stem cells. They observed Aβ-peptides accumulation and tau pathology, which became apparent after longer time of organoid culture than the familiar AD organoids. Zhao et al[73] generated sporadic AD

cerebral organoids with APOE mutations from iPSCs derived from AD patients. They also showed increased levels of both tau and Aβ.

Modelling Parkinson's disease with brain organoids

Parkinson's disease (PD) is one of the most common movement disorder caused by accumulation of Lewy bodies. These structures consists of insoluble aggregates of α-synuclein. This accumulation leads to degeneration of dopaminergic neurons located in the substantia nigra and then it expands to cortical areas[74].

The molecular backround of the pathology of PD incorporates a number of genes, including the SNCA gene that translates to a-synuclein protein, but the most common mutation is in the LRRK2 gene that translates to Leucine Rich Repeat Kinase 2. This mutation is widely used as a basis to generate PD-organoids, especialy midbrain organoids which are rich of dopaminergic neurons.

Kim et al[20] produced midbrain organoids that carried LRRK2 mutations and were able to observe increased levels of α-synuclein. They also studied the transcriptomic profile of PD midbrain organoids and find a strong correlation with post mortem brain tissues from PD patients with the same molecular profile. Smits et al[75] used the same PD midbrain organoid model to show a reduction in neuronal branching and lower dopaminergic neurons complexity accompanied with elevated levels of FOXA2.

Modelling Huntington's disease with brain organoids

Huntignton's Disease (HD) is an autosomal dominant neurodegenerative disease caused by alterations in the Huntington's gene (HTT). The alterations are caused by a CAG triplet repeat expansion which leads to neurodegeneration in the cortex and stratium. Total loss of HTT protein is lethal at an embryonic stage. HD patients suffer from motor impairments (dystonia, chorea), cognitive decline (impaired judgement, forgetfullness, learning difficulties), phyciatric disorders (depression and isomnia) as well as irritablity and aggression.

The mutations in the HTT gene causes degeneration of neurons in the stratium and cortex through mechanisms that invole altered gene expression, disfunctional mitochondria and metabolic processes, toxicity caused by the mutant protein and low ATP levels[76].

It has been found that the length of CAG repeat has a correlation with the severity of the symptoms but also with the onset of the disease. Less than 36 repeats leads to a normal phenotype, 36 to 39 repeats cause mild symptoms, 40 to 60 repeats causes late onset HD and more than 60 repeats cause juvenile onset HD[77].

2D cell culture models have been successful in recapitulating deficit in neuronal rosettes, neuronal specification, cytoarchitectuires and cellurar migration. Conforti et al[78] constructed HD organoids and showed that the expansion of CAG repeat leads to abnormalities in early telencephalic induction and a false differentiation path of progenitor cells. This leads to false neuronal specification and abnormal cellular organization. There were able to correlate the length of the CAG repeat expansion with the severity of symptoms as well as with the onset of the disease, which were in line with the clinical findings.

A more recent study generated cerebral organoids using iPSCs derived from HD patients and observed a disregulation in the cell cycle accompanied with decreased symmetric division of progenitors cells that leads to abnormal neuroepithelial structures along with premature neurogenesis.

Other Diseases

Cancer organoids

In the last decade scientists have been able to generate cancer organoids. These organoids can be generated either from cancer patients' samples (cancer stem cells) or genetically modified healthy organoids and are a promising platform for translational and pre-clinical research. Cancer organoids have been generated for various solid tumors. Cancer organoids are found to sustain the molecular and histological characteristics of the tumor they are generated to simulate. They also express the same level of intertumoral heterogeneity, which is a mandatory property of a valid drug screening platform but also as a tool in personalised medicine correlated with individual treatment response[79].

One of the most aggressive and common brain cancers is glioma. Glioma occurs in the brain and spinal cord and are originated from glial cells. Gliomas are categorised by which type of glial cells the tumor comes from. There are three types of gliomas: astrocytomas, ependymomas and oligodendrogliomas. The initial cell population that initiates the generation of gliomas are called glioma cancer stem cells (GSC). These cells are responsible for tumor growth, invasiveness but also for cancer recurrence after glioma resection. GSC have similar characteristics with neural stem cells which is evident by the expression of neural stem cell markers and their proliferation capabilities[80].

The first attempt to create an *in vitro* model of gliomas was 2D cultures in adherent conditions. This platform was used to investigate the molecular profile of glioma tumors as GSCs maintain their molecular background of their parental tumor in early passages[81]. The downside in this approach is the fact that 2D glioma cultures favour specific cell types which results in loss of tumor heterogeneity. The loss of some cancer cells subtypes makes 2D glioma cultures an inadequate drug screening platform. Another problem with these cultures is the fact that they do not allow to study the invasive capabilities of the tumor because glioma requires a 3D brain tissue scaffold as a host.

To address the above issues, scientist took advantage of brain organoid technology in order to construct a more reliable glioma *in vitro* model. The starting cell population for the generation of glioblastoma organoids (GBOs) were GSCs. GBOs were able to maintain the molecular background and heterogeneity of the parental tumor in a 3D brain-like environment, thus they could be used as a drug screening platform for anti-cancer treatments and as a model to study tumor invasiveness to brain tissues[82].

Ballabio et al[83] used human cerebral organoids in order to study the molecular mechanisms that leads to the formation of medulloblastoma. They transfected the organoids with viruses that targeted specific genes that they have found to be expressed in higher levels in medulloblastoma (Otx2 and c-Myc) cells than healthy brain cells in order to initiate cancer in healthy human cerebral organoids. They also study the anti-tumor effect of Tazemetostat, a drug that antagonize tumors initiated by Otx2 and c-Myc mutations.

Linkous et al[84] co-cultured human brain organoids derived from hESCs with patient-derived GSCs. They showed that GSCs exhibit the same behaviour regarding invasion and tumor formation in in-vivo and in-vitro conditions.

More laborious work on the subject was made by Goranci-Buzhala et al[85]. They focused on the invasion capabilities of GSCs taking into consideration organoids age, the number of GSCs and whether GSCs was embedded as single cells or spheres. They were able to show different invasion behaviours between primary GSCs and GSCs from recurrent patients. Their findings also suggest that GSCs invasion capabilities have a strong correlation with organoid age, suggesting that GSCs need a microenvironment of mature neural cells in order to proliferate. This finding comes with agreement with previous studies indicating that mature neural cells provide essential mitogenic factors that support glioma cell proliferation. To evaluate this notion, Goranci-Buzhala et al[85] added the synaptic protein Neuroligim-3 (NLGN-3) and they noticed that NLGN-3 could promote GSCs invasion capability in immature brain organoids.

Glioma tumoroids

An alternative to GBOs are glioma tumoroids. These 3D cultures are created from samples taken from cancer patients and cultured under conditions which maintain the stem cell niches. Glioma tumoroids are highly invasive to neighbour tissues, they maintain the histological properties of the parental tumor as well as the molecular background and cellular heterogeneity[82].

Traumatic Brain Injury (TBI)

Traumatic brain injury (TBI) definition is the damage to the brain due to extrinsic mechanical forces, like rapid acceleration or deceleration, collision and penetration of objects[86]. TBI is classified according to the severity, anatomical characteristics of the injury and the mechanism (causal forces) of its induction. TBI occurs when an external force causes an injury to the brain. TBI is particularly common in the world, affecting 69 million people globally as determined by World Health Organization (WHO)[87]. TBI is a vital cause of disability and mortality globally in all age groups. Causes involve falls, car accidents, sports injuries and violence.

Along with the damage caused at primary injury, it can also cause secondary damage, a variety of events occurring minutes and days after the incident. These procedures, which include changes in the blood flow to the brain and the pressure inside the skull, contribute significantly and aggravate the damage from the initial injury[88].

Mechanism-based classification divides traumatic brain injury into closed and penetrating injury. The first one means that the brain is not exposed. Open injury or penetrating injury takes place when an object pierces through the skull and causes the dura mater, the outer protective membrane that surrounds the brain, to rupture. A large percentage of patients who end up due to some kind of craniocerebral injury do not die on the spot, but days or even weeks after the incident. Instead of improving after hospitalization, a large percentage of TBI patients, are getting worse. This escalation of the symptoms is caused by secondary damage, during which, a complex of cellular and biochemical processes begins minutes to days after the injury. These secondary procedures can significantly intensify the damage as a result of the injury and are responsible for the largest percentage of deaths from traumatic brain injury that occur in hospitals. Secondary damage includes rupture of the blood-brain barrier, release of inflammatory agents, excessive release of neurotransmitters, entry of calcium and sodium into neurons, and mitochondrial malfunction. Injured axons in the white matter of the brain can separate from the cell body as a result of secondary damage, resulting in their death. Other factors of secondary damage are changes in the blood flow to the brain while not only neurons, dendrites and their synapses are damaged but also astrocytes and oligodendrocytes, which are responsible for the production of myelin[89-92].

In order to comprehend the complexity of biological cascade events in TBI scientists developed rodent brain models. However, human and rodent brain appears a lot of differences like gene expression profiles, complexity or distribution of brain areas[93]. Cerebral organoids (COs) generated from hiPSCs showed remarkable resemblance to the human brain in cell composition, organization of various anatomical region, gene expression and epigenetic signature. In a recent study[94], a group of researchers generated a human organoid model of TBI in order to construct a reliable *in vitro* model. To achieve that, they adapted Controlled Cortical Impact (CCI) to their COs. CCI is an established model for TBI in rodents. It allows the control of parameters like velocity of the contact, time of contact and depth of the injury in order to regulate the severity of the damage taken[95]. Nerve cells from the COs that impacted from CCI exhibit a damage response, one of the main characteristics of the primary brain injury. In order to map the metabolic changes that happens to neurons after TBI, scientists evaluated neuron-specific enolase (NSE) levels. NSE is a glycolytic enzyme, a biomarker of late neural maturation which can correlate positively with TBI's severity[96,97]. Indeed, their results pointed out an accumulation of NSE and an escalation of apoptotic cells in COs. All the results were compared with mice brain models and the scientists concluded that COs could become a reliable alternative for the study of the TBI. Moreover, vascularization needs to be facilitated in the transplanted area. In a recent study, the development and transplantation of COs led to the successful vascularization of the system and promoted brain repair[98].

Stroke

Stroke is a neurological disease that affects millions of people worldwide. WHO refers to this specific disease as the next epidemic of the 21st century. Therefore, developing strategies to prevent strokes is a major issue for the scientific community, as research shows that a large percentage of strokes, approaching 85%, can be avoided. One of these techniques developed for understanding strokes is the development of brain organoids to model the disease. Brain organoids intended for stroke modeling are usually kept in hypoxic conditions, lack glucose and oxygen while N_2/CO_2 medium replaces the normal O_2/CO_2 medium. The cellular platforms used for stroke studies are primary cells, brain slices, cell lines, iPSCs and ESCs. Boisvert et al[99] developed brain organoids developed cerebral organs in order to study the effects of hypoxia at the cellular level and attempted to mitigate damage using minocycline, while another study found a reduction in intermediate progenitor cells and an increased number of proteins that were not properly folded[100]. Oxygen deprivation in brain organelles derived from induced nerve stem cells reduced their size and injured them, while the attempt to re-oxygenate did not reverse the negative effects of hypoxia on both the organic and cellular levels[101]

Conclusions

Modeling the human brain to study its physiology, development and diseases has been challenging due to its structural and functional complexity and fragility. Brain organoids derived from human pluripotent stem cells opened a new approach to create in vitro 3D models that better resemble the complexity of the brain, as compared to 2D cell cultures. So far, multiple developmental and neurodegenerative diseases have been generated with organoid technology and used as drug screening platforms to accelerate the generation of more efficient and personalized treatments.

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Both authors contributed equally to this manuscript.

ORCID ID:
Thomas Madikas: 0000-0002-8753-0551
Georgios Dafnopoulos: 0000-0001-8606-4934

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