Chapter 5
Toward Understanding Neurodegeneration Using Brain Organoids

Patompon Wongtrakoongate, Chatbenja Pakiranay, and Narisorn Kitiyanant

Abstract **Introduction**: With the advancement of pluripotent stem cells (PSCs), several brain regions have been modelled through brain organoids that resemble their in vivo counterparts of the human brain in terms of cellular heterogeneity and gene expression. The models can be used for molecular pathogenesis studies of neurodegenerative diseases and can be combined with many recent technologies such as optogenetics, CRISPR/cas9, patch-clamp, or on-a-chip system to create more precise models of brain development and diseases. Moreover, personalised organoids derived from patient-specific induced pluripotent stem cells (iPSCs) can also be used to develop personalised treatment. This chapter introduces the principles of brain organoid formation and the potential uses of brain organoids for modelling neurodegenerative diseases, drug development, and personalised medicine. **Methods**: We performed a literature review in PubMed (https://www.pubmed.ncbi.nlm.nih.gov) using the keywords brain organoids; neural differentiation; neurodegeneration; personalised medicine. **Conclusions**: Personalised brain organoids, which can be derived by several approaches and coupled with genome editing such as CRISPR-Cas9, have proved to be powerful tools for in vitro studies of early human brain development and pathogenesis. Future treatment of incurable neurodegenerative disorders should ideally be tailored to individual patients to obtain optimal efficacy. To this end, using in vitro patient-specific models of neuroectodermal tissues will allow for such customised treatment personalised medicine.

Keywords Brain organoid formation · Human pluripotent stem cells · Neurodegeneration · Personalised medicine

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Abbreviations

2D	2 Dimensions
3D	3 Dimensions
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
APP	Amyloid precursor protein
Aβ	Amyloid β
COVID-19	Coronavirus disease of 2019
CRISPR	Clustered regularly interspaced short palindromic repeats
EGF	Epidermal growth factor
ESCs	Embryonic stem cells
FAD	Familial Alzheimer's disease
FGF-2	Fibroblast growth factor-2
HSV-1	Herpes simplex virus type 1
iPSCs	Induced pluripotent stem cells
LRRK2	Leucine-rich repeat kinase 2
mDAns	Midbrain dopaminergic neurons
PD	Parkinson's disease
PLGA	Poly(lactide-co-glycolide) copolymer
PSCs	Pluripotent stem cells
PSEN1	Presenilin-1
PSEN2	Preselinlin-2
PTEN	Phosphatase and tensin homolog
SFEBq	Serum-free culture of embryoid body-like quick-aggregation
SMAD	Mothers against decapentaplegic
SNO	Sliced neocortical organoid
SOD1	Superoxide dismutase type 1
TNF-α	Tumor necrosis factor-α
WNT	Wingless and Int-1

Introduction

The neocortex of the human brain is populated by a great diversity of neuronal cell types. It is responsible for the higher cognitive functions which cannot be found in any other species. Understanding human brain development under normal and disease conditions is a major goal to discover new therapeutic approaches for the nervous system. The use of animal cells/models for neurobiology research is a longstanding practice. However, animal cells/models do not reflect but only share some features of human diseases. Accessibility to human brain tissue is limited due to ethical concerns associated with their origin. The establishment of in vitro cultures of neuronal and immortalised neuroblastoma cells overcomes this limitation. Although both neuronal

cell culture systems are regularly used in neurobiology research, the conventional 2D culture systems do not recapitulate the brain microenvironment, complexity of neural tissue, and disease phenotypes such as abnormal protein aggregation. Another limitation is that they contain only one cell type and do not have cell-cell interaction between cell types.

In the pre-organoid era, the ex vivo brain tissue slice culture, known as an organotypic culture, has been used in neurobiology research (Fig. 5.1). Many different brain regions can be sliced and cultured for weeks to months. They have been used as a model to study the brain microenvironment, which resembles in vivo conditions of the precision area of the brain [1]. Unlike neuronal cell culture, organotypic culture maintains cytoarchitecture and the microenvironment of the brain. Therefore, organotypic culture is a functional tool for neurobiology research and drug screening.

On the other hand, neural precursor cells were isolated from various human brain regions, cultured in vitro, and formed free-floating hollow clusters of neural stem/progenitor cells called "neurospheres" [2, 3]. Neurospheres are heterogeneous

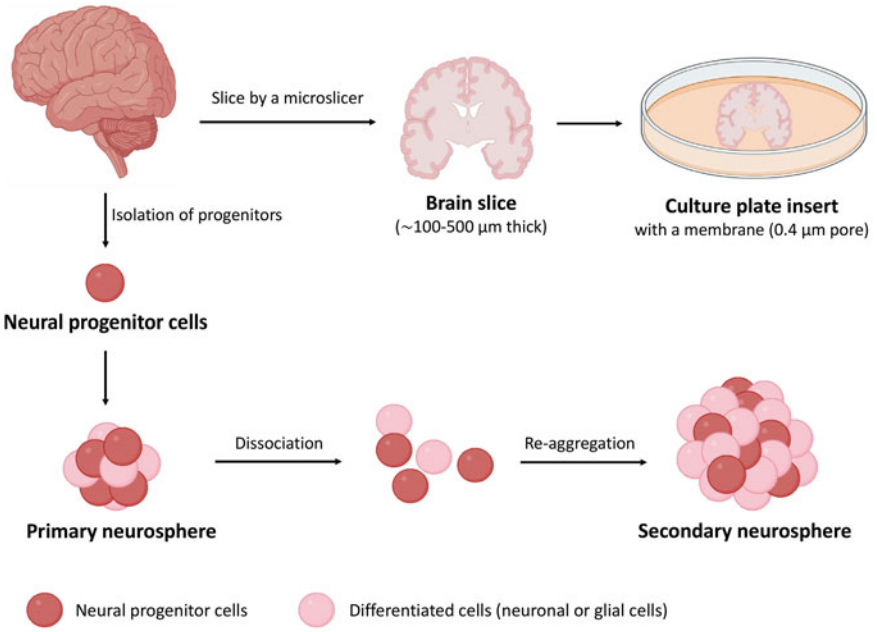

Fig. 5.1 Conventional culture methodologies for brain cells and tissues include ex vivo organotypic culture (top) and neurosphere culture (bottom). For organotypic culture, brain slices at the thickness of 100–500 µm can be prepared from relevant brain tissues using a vibratome and placed in an insert with 0.4-µm semipermeable pores. For neurosphere culture, different brain areas, such as the dentate gyrus and subventricular zone, can be isolated to derive neural progenitor cells. Upon expanding neural progenitor cells, they can further self-renew to give rise to identical progenitors or differentiate to produce neurons and glial cells. Expansion of neurospheres can be performed by dissociation and re-aggregation of the neurospheres (Made in ©BioRender—https://www.biorender.com)

and comprise several hundred astrocytes, neurons, and neural stem/progenitor cells with specific characteristics based on their region of origin [4]. Neurospheres can be propagated by mechanical chopping or dissociation, overcoming the limitation of organotypic culture. Although neurospheres have been known as a tool to study neurogenesis and model for the early development of the human brain, it has lower complexity and less structural organisation than organoids.

The advanced development of human pluripotent stem cells (PSCs), both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have provided a platform of model systems for understanding human biology, physiology, development, and diseases. Treatment with essential growth factors promotes human PSCs differentiation into specific cell lineage. Many groups have developed neural induction protocols to drive hPSCs to become neural cell types in 2D and 3D cultures. A very early method to generate pre-rosette neural stem cells in neurospheres (termed EZ spheres) has been developed by lifting hPSCs colonies and cultured in a neural stem cell medium with a high concentration of EGF and FGF-2 [5]. The EZ spheres can form neural rosettes and further differentiate into several types of neural lineages. Chandrasekaran and colleagues compared the efficiency to generate neural stem/progenitor cells from hPSCs between 2D induction and 3D induction methods. A higher number of neurons with longer neurites were observed in 3D neural induction, suggesting a superior way to generate forebrain cortical neurons from hPSCs [6].

An organoid refers to 3D suspension culture techniques to generate an organised organ/tissue resembling various in vivo-like cellular architecture features in a dish. Fatehulla and colleagues defined organoid as "an in vitro 3D cellular cluster derived exclusively from primary tissue, embryonic stem cells, or induced pluripotent stem cells, capable of self-renewal and self-organisation, and exhibiting similar organ functionality as the tissue of origin" [7]. Therefore, an organoid is technically different from a neurosphere, which refers to an aggregate of neural derivatives without any cytoarchitecture and morphogenesis found in the brain. By combining PSCs technology and differentiating iPSCs into neuronal cells, an innovational study by Lancaster and colleagues has shown a culture system to generate cerebral cortex-like organoids from human PSCs derived from a patient with microcephaly [8]. Brain organoid or cerebral organoid composed of progenitor, neuronal and glial cells and resemble the human fetal brain [8–10]. Since then, effective protocols for brain organoids cultures have been established. Exogenous cues such as Wingless and Int-1 (WNT) inhibitor and Mothers against decapentaplegic (SMAD) inhibitor were used to mimic the endogenous developing pattern and direct neural induction in a high consistency of brain organoid generation. Although the short-term culture brain organoids reflect the immature state of the brain, neurons in brain organoids begin to mature after 60 days in culture and show spontaneous excitatory post-synaptic currents after 120 days in culture [11, 12].

To date, several brain regions, e.g., thalamus [13], midbrain [9, 14, 15], pituitary gland [16], cerebellum [17, 18], and brainstem [19], have been modelled using brain organoids. As the brain organoids have some main features of the human brain, e.g., cellular distribution and organisation, electrophysiological functions, and neural

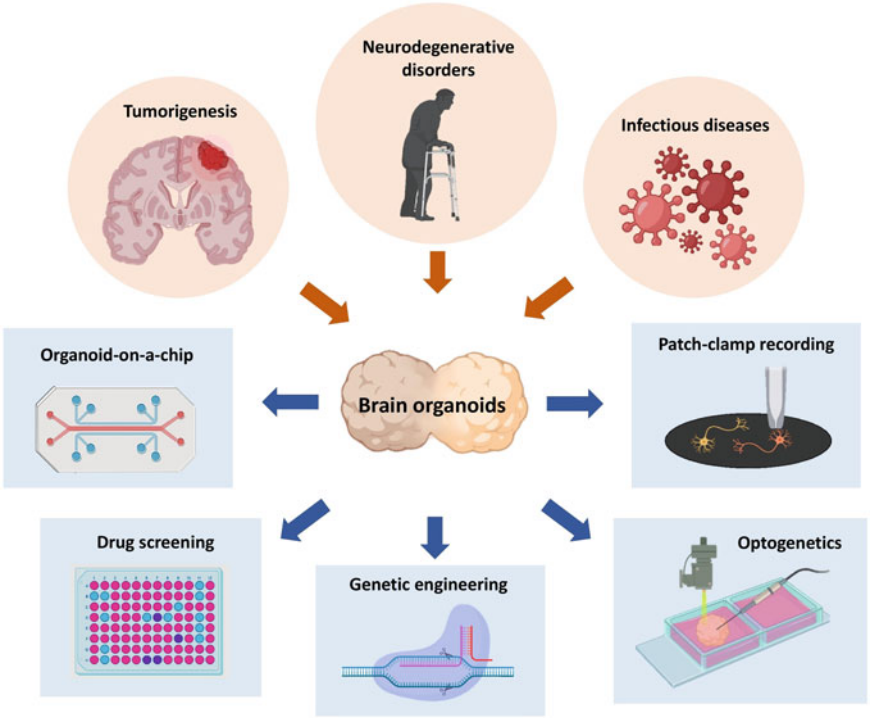

Fig. 5.2 Brain organoids have revolutionised research in neuroscience, regenerative medicine, infectious diseases, and tumorigenesis, as they provide a tool to study brain health and pathogenesis. In addition, brain organoid technology can be coupled with other technological advancements such as electrophysiology using a patch-clamp technique, optogenetics, genetic engineering, drug screening, and organoid-on-a-chip (Made in ©BioRender—https://www.biorender.com)

circuits, they have become a promising tool to explore the mechanisms of nervous system diseases (Fig. 5.2). Brain organoids have been used to model neurodegenerative diseases such as Alzheimer's disease [20, 21] and Parkinson's disease [9, 14, 22], brain tumorigenesis, Zika virus infection to the brain [12, 23] and neurological COVID-19 [24].

Technical Principles of Brain Organoid Formation

As mentioned above, a hallmark of brain organoids that makes them different from neurospheres is forming cytoarchitectures and tissue morphogenesis of the former [25]. This property allows brain organoids to recapitulate region-specific brain architectures. Following a paradigm of directed differentiation, an original approach toward organoid culture was developed by Yoshiki Sasai to derive cortical

layers from human PSCs using a three-dimensional system termed the serum-free culture of embryoid body-like quick-aggregation (SFEBq) [26]. Since then, various approaches have been devised to generate brain organoids from PSCs. Fundamentally, we will summarise four technical principles employed for the derivation of brain organoids, including (1) factor-primed, (2) self-patterned, (3) fusion, and (4) co-culture approaches (Fig. 5.3).

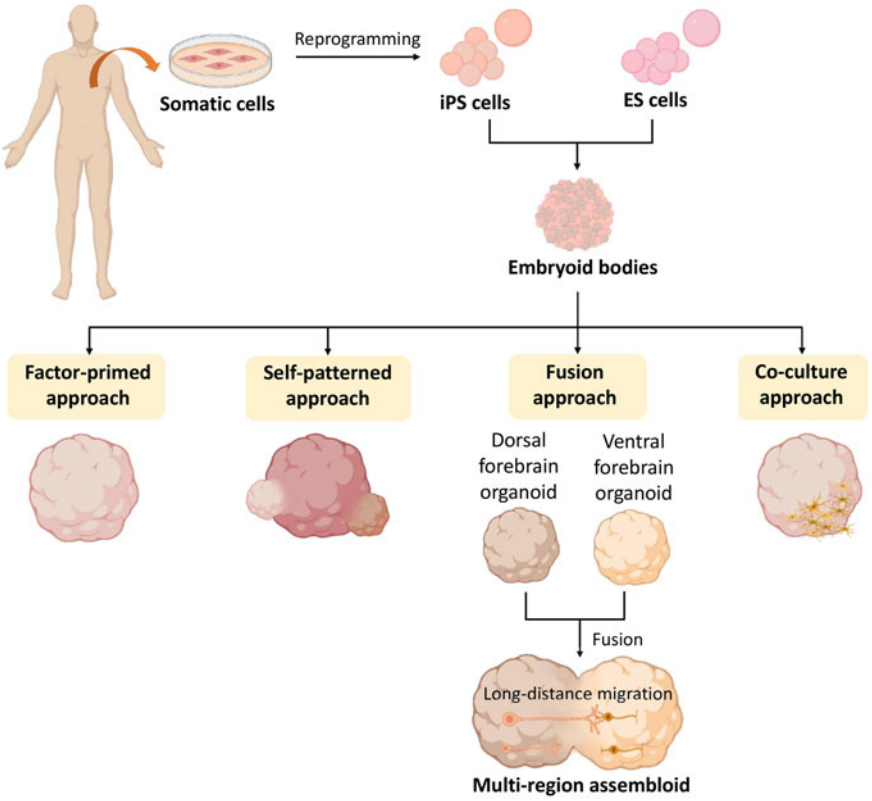

Fig. 5.3 Brain organoids can be derived from human iPSCs and ESCs through the aggregation of EBs using four different methods. The factor-primed approach offers a consistent and reproducible method. The self-patterned approach can deliver diverse cell heterogeneity and extensive morphogenesis. The fusion approach is suitable for the derivation of at least two distinct interconnected yet defined compartments, for example, dorsal and ventral forebrain regions. The co-culture approach gives rise to a brain organoid harbouring not practical cells derived from neural differentiation such as microglia or brain tumour cells (Made in ©BioRender—https://www.biorender.com)

Factor-Primed Approach

Brain organoids can be derived by defined factors. To this end, a factor-primed approach, by which defined extrinsic and trophic factors are added into the culture medium, can be adopted to prime human PSCs to differentiate along neuroectodermal lineages. Scaffolds can also be included in the system to instruct cytoarchitectures and morphogenesis. A well-established protocol of the factor-primed approach is serum-free culture of embryoid bodies (SFEBq), which has been utilised to generate forebrain [27–29], midbrain [9], cerebral cortex [30], cerebellum [31, 32], hippocampus [33], neocortex [34] and pituitary [16]. Moreover, this approach has led to a recapitulation of rostral-caudal organogenesis [35]. A key advantage of using the factor-primed approach is relatively more consistent in cellular heterogeneity and a higher degree of differentiation than the self-patterned approach (see below). However, less advanced-stage morphogenesis is a drawback of this approach as opposed to the other methods.

To avoid limited morphogenesis, step-wise protocols for priming PSCs and their progenies with guiding factors have been established, in which a transient induction by extrinsic and trophic factors is employed to derive radial organisation of the cerebral cortex midbrain organoids and hypothalamic organoids [12, 36]. This temporal manipulation of cell signalling allows brain organoids to be further self-instructed upon removing or diluting the signals. Moreover, the biomaterial poly(lactide-co-glycolide) copolymer (PLGA) can be successfully applied for priming cell attachment and hence facilitating morphogenesis of the organoids around the scaffolds [36]. One study has compared PLGA with carbon fibres for the generation of midbrain organoids and found an increase in expression levels of genes specific to dopaminergic neurons from carbon fibre-primed cultures, structurally more stable than PLGA and does not alter the pH of culture environments [37]. In addition, micropatterned arrays made from the organosilicon polydimethylsiloxane have been shown to improve the derivation of forebrain organoids with homogeneous and singular neural rosettes [38].

Self-Patterned Approach

In contrast to the aforementioned factor-primed approaches, self-patterned approaches are organoid derivation techniques utilising the ability of spontaneous differentiation and subsequently spontaneous morphogenesis of PSC aggregates [39, 40]. Hans Clevers pioneered this protocol for the development of intestinal organoids [41]. Later on, cerebral organoids were successfully derived from matrigel-embedded embryoid bodies. A key success of this method came from using a spinning bioreactor to enhance absorption of nutrients and trophic factors and allow the aggregates to develop self-patterned morphogenesis in a free-floating format [8]. Importantly,

this technique led to a generation of various cell lineages belonging to the forebrain, midbrain and hindbrain in single organoids, indicating a potential of the self-patterned approach to model diseases of the human brain, which might require a crosstalk mechanism among different brain regions.

Nonetheless, in contrast to the signal-primed approach, two drawbacks of this method are a massive cell death inside the organoids and an inconsistency of cellular heterogeneity in the organoids. To increase nutrient absorption and oxygen diffusion and reduce cell death, a multi-well spinning bioreactor system has been engineered for simultaneous expansion of brain organoids, improving the growth of brain organoids and increasing the efficiency of organoid derivation [12]. Furthermore, to overcome a limited expansion of self-patterned brain organoids, genetic deletion of phosphatase and tensin homolog (*PTEN*) led to enhanced cell proliferation of ventricular and outer neural progenitors, in agreement with expansion and folding of human cortical organoids [42]. Moreover, Ming and colleagues have recently developed the self-patterned approach by combining the sliced neocortical organoid (SNO) technique to increase the diffusion of nutrients and trophic factors into SNO, leading to higher cell viability and more expansion of the organoids [43].

Fusion Approach

For factor-primed approach and self-patterned approaches, key drawbacks are limited heterogeneity and uncontrolled cellular diversity, respectively. The latter also usually comes with the irreproducibility of tissue morphogenesis. Specifically, an uncontrolled size of brain regions and tissue organisation are hurdles of this approach. Hence, a novel method has been devised to improve brain organoids' quality in heterogeneity and reproducibility. This is a fusion approach whereby different regions of brain organoids can be fused from individual region-specific brain organoids. Organoids of different brain regions can be fused to generate an expanded architecture, so-called "assembloids". This approach connects multiple brain regions in vitro for long-range and multi-synaptic interconnection. The fusion approach has been used to study the migration of human GABAergic interneurons and to integrate cortical circuits between neurons from the ventral to the dorsal forebrain [44]. This circuit integration comes from interneurons and glutamatergic neurons, which can be found in a microphysiological niche. The authors also utilised the model to study Timothy syndrome and observed a defective neuronal migration. The migration of GABAergic interneurons from ventral to dorsal forebrain is CXCR4-dependent [45]. A similar study has modelled the development of human medial ganglionic eminence of the ventral brain, which hosts neurogenesis of cortical interneurons. Two different region-specific organoids, medial ganglionic eminence organoids and cortical organoids, were fused to observe the migration and integration of interneurons produced by the former [46]. In addition, a method aiming at the generation of thalamus-cortex assembloids by fusing thalamus-like brain organoids to cortical organoids has also been established. Remarkably, the reciprocal thalamocortical

projections between the thalamus and cortex were observed in the fused assembloids [13]. Using the fusion approach, a human multi-synaptic circuit has been recently demonstrated by generating the cerebral cortex or the hindbrain/spinal cord assembled with human skeletal muscle spheroids to generate 3D cortico-motor assembloids [47]. A novel method has been invented for which the midbrain-to-forebrain mesocortical pathway was modelled. This method utilises a hexagonal acoustofluidic device to generate dynamic acoustic fields that can move and fuse one organoid with another in a contact- and label-free manner [48]. Nonetheless, even though the fusion approach offers a path to engineer and expand multi-regional brain organoids with high reproducibility, not all aspects of brain physiology can be implemented, for example, brain-microglia interaction and blood-brain barrier.

Co-culture Approach

A common key limitation in factor-primed, self-patterned, and fusion approaches is that not all cell types present in brain tissues can be obtained from these methods, especially cells belonging to other germ layers such as microglia and endothelial cells. Thus, co-culture protocols have been developed to obtain a complete niche or systems by adding particular cell types into or onto brain organoids.

In order to incorporate microglia into brain organoids, microglia were differentiated from human iPSCs and were tested for their interaction with brain cortical organoids lacking microglia. Upon addition of microglia, by day 3, the cells had migrated into the organoids. The formation of activated microglial clusters was observed when the injury was applied to the organoids [49]. A similar study investigated the role of microglial co-culture in Alzheimer's pathology using brain organoids with Aβ aggregation. The authors found that integrating microglial co-culture can attenuate the accumulation of Aβ plaques [50]. Microglia migrated faster into dorsal organoids than ventral organoids in a comparative study between dorsal and ventral organoids. Immune response upon microglial incorporation was also altered. Specifically, microglia-incorporated dorsal organoids possess higher anti-inflammatory cytokine secretion than ventral organoids, whereas microglia-incorporated ventral organoids express higher TNF-α upon treatment of Aβ42 oligomers [51]. Further, to closely mimic brain microenvironments, Gage and colleagues have successfully transplanted cerebral organoids into adult mouse brains and have established that the engraftment can lead to extensive neuronal differentiation and maturation, gliogenesis, axonal outgrowth, integration of microglia, and vascularisation of endothelial cells [52].

The co-culture approach can benefit from studying the normal physiology and neurological diseases and elucidating tumorigenesis. In one study, cerebral organoids were formed to model gliomagenesis [53]. The cancer cells can infiltrate into and proliferate in the organoids after co-culturing with patient-derived glioma stem cells. Moreover, gap junction mediated-interconnecting microtubes can be observed using two-photon microscopy, facilitating the tumour invasion. In addition, co-culture

approaches can offer a means to serially expand brain tumours into subsequent organoids [63] and understand tumour heterogeneity [54].

With all these four techniques for derivation brain organoids, including factor-primed, self-patterned, fusion, and co-culture approaches, fruitful information has been made regarding fundamental neuroscience, developmental biology, tumorigenesis and drug discovery. Hypotheses for specific research purposes will guide which technique should be employed for the generation of brain organoids. Future approaches may combine several of these techniques to better recapitulate the brain's anatomy and physiology.

Brain Organoid and Neurodegeneration

Neurodegenerative diseases, including Alzheimer's diseases (AD), Parkinson's diseases (PD), Amyotrophic lateral sclerosis (ALS), and Huntington's disease, are prevalent in the elderly worldwide. Previously, studies with human brain tissue, cell cultures, and animal models have been used to study the mechanisms of diseases. Human cerebral organoids and several 3D culture systems exhibit key neuropathological features of the diseases and can be used as disease models.

Alzheimer's Disease

Alzheimer's disease is the most common age-related, irreversible, and progressive disease that slowly destroys the brain. Individuals with early AD develop brain grey matter volume loss in many brain regions such as the hippocampus and the basal forebrain. The disease is clinically characterised by cognitive decline, severe memory impairment, and severe enough life-altering. AD. is characterised by the presence of extracellular amyloid beta-protein deposition, so-called amyloid plaque, and intracellular neurofibrillary tangles. Familial AD. (FAD) is caused by variants in the amyloid precursor protein (APP), presenilin-1 (PSEN1), or presenilin-2 (PSEN2). Sequential cleavage of APP by β and γ-secretase results in a production of Aβ peptide, which aggregates into insoluble amyloid plaques. The deposition of amyloid-beta and hyperphosphorylation of tau could be observed in a 3D culture system of human neural stem cells with amyloid precursor protein (APP) and presenilin1 (PSEN1) mutation [20]. Moreover, brain organoids derived from multiple FAD patients induced pluripotent stem cells to develop continuous amyloid deposition and tau hyperphosphorylation in an age-dependent manner [21].

Recently, Cairns and colleagues described a new model of AD. Using HSV-1 infection to a 3D brain model. This model can develop amyloid plaque-like formations, gliosis, neuroinflammation, and decreased functionality [55].

Parkinson's Disease

Parkinson's disease (PD) is the second most common neurogenerative disease after AD. PD is characterised by resting tremor, bradykinesia, rigidity, and postural balance instability. The major cause of clinical symptoms is the degeneration of midbrain dopaminergic neurons. To model PD in brain organoids, midbrain-specific organoids were developed [9, 14]. The midbrain-specific organoids contained functional tyrosine hydroxylase-positive midbrain dopamine neurons (mDAns) after 2 months in culture. These mDAns express midbrain markers, such as FOXA2 or dopamine transporter, and show cytoplasmic neuromelanin accumulation. Patient-specific iPS cells from PD patients could be used to model PD with midbrain-specific organoids. The early reports of PD modelling in midbrain-specific organoids focused on the effects of the LRRK2-G2019S variants. CRISPR-Cas9 has been used to introduce the mutation in control human pluripotent stem cell lines [56] or create isogenic mutation corrected lines from patient-specific cells [22]. Kim and colleagues observed no difference in size between LRRK2-G2019S midbrain-specific organoids compared to control. However, less neurite length of mDAns and lower expression of dopaminergic neuron marker were noted [56]. On the other hand, a smaller number of mDAns and lower complexity of their neurites were observed in the midbrain-specific organoid derived from LRRK2-G2019S mutated patient iPS [22]. Midbrain organoids may also be used to study sporadic forms of PD by exposing the organoids to exogenous stressors, such as MPTP.

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a devastating neurogenerative disorder caused by the loss of motor neurons. The most common cause of familial ALS is superoxide dismutase type-1 (SOD1) mutations, resulting in increasing aggregated and soluble misfolded forms of SOD1, leading to the death of motor neurons [57]. Seminary and colleagues generated motor neuron cultures from human iPSC lines carrying mutations in SOD1. Accumulation of insoluble SOD1 can be observed in ALS iPSC-derived motor neurons. However, the heat shock response or stress granule formation in response to protein accumulation cannot be observed [58]. To date, there is no publication using organoids to model ALS. This might be because the motor neurons can be divided into upper motor neurons and lower motor neurons. The upper motor neurons are in the motor cortex, and the lower motor neurons are in the ventral horn of the spinal cord. Therefore, brain organoids cannot mimic the lower motor neurons physiology and environment. Kawada and colleagues developed a protocol to generate a motor nerve organoid from human pluripotent stem cells using a microdevice equipped with a narrow channel to provide a microenvironment for axonal growth. The generated motor nerve organoid mimics the development and dysfunction of a human motor nerve [59]. Later, a protocol to generate a 3D

spinal cord organoid from human induced pluripotent was established [60]. Different spinal cell types were observed with this protocol in the spinal cord organoids and patterned along the rostro-caudal axis, mimicking the ventral spinal cord. Fusing the motor cortex brain organoid to the motor nerve organoid or spinal organoid could be a possible model for further ALS study.

Other Applications

Besides, brain organoids and assembloids could serve as an innovative tool to model pathology and study disease mechanisms from a healthy individual and patient nervous system. Brain organoids and assembloids can be combined with many recent technologies such as optogenetics to use light to control neurons, CRISPR/cas9 for genome editing, patch-clamp for electrophysiology study, and on-a-chip system to control continuous perfused cultures to create more precise models of brain development and diseases.

Brain Organoid for Drug Development and Personalised Medicine

For clinical translation, brain organoids can be used to model patient-specific molecular and cellular pathogenesis, thus guiding the most effective treatment for individual patients, a process called personalised medicine. Personalised organoids can be derived from a specific patient. Briefly, the cells would be obtained from the patient, reprogrammed into iPS cells, and grown brain organoids on a large scale. Personalised brain organoids can be used to test the effectiveness of a compound library (new drug development) to find the ones most appropriate for the patient. Recently, Park and colleagues used 1300 cerebral organoids, including CRISPR/Cas9-edited isogenic lines, from 11 AD patients to assess blood-brain barrier-permeable FDA-approved drugs and purposed a strategy for precision medicine by integrating those cerebral organoids and mathematical modelling. Their results demonstrated the possibilities of drug repositioning and simplified the drug approval process in preparation for precision medicine [61]. In addition, since autism spectrum disorder is a polygenic disease, it is difficult to precisely develop a curable treatment for the patients. To overcome this multi-genetic barrier, cerebral organoids made from the patients via iPS reprogramming have been proposed for personalised drug discovery [62]. However, the production scale of the cerebral organoids is a challenge for the high-throughput drug screening. Specifically, most of the established protocols have been developed using 96-well plates. Therefore, the automation system is required to produce cerebral organoids on a large scale, which will eventually accelerate the development of novel personalised therapeutic strategies for brain disorders.

Conclusion

Brain organoid technology is a powerful tool for researchers to study early human brain development and diseases. Four approaches can be considered for generating brain organoids: (1) factor-primed; (2) self-patterned; (3) fusion; (4) co-culture approaches. Pathogenesis of Alzheimer's, Parkinson's, and ALS diseases, among others, have been successfully modelled using brain organoids. When coupled with genome editing tools such as CRISPR/Cas9, patient-specific brain organoids are key for personalised and precision medicine.

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