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Cerebral Organoids as Models for Neurological Disorders

Esther Karman

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Abstract

Despite the devastating effects of neurological disorders on millions of people each year, for decades, brain research remained stagnant in the face of scientific advancement in other areas. Ethical concerns, debilitating costs, and a lack of suitable models created an unfriendly environment for the study of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (AD), amyotrophic lateral sclerosis (ALS), autism spectrum disorder (ASD), and gliomas. Recent developments in the field of stem cells, including Yamanaka's discovery of the four transcription factors necessary to induce pluripotency, and the subsequent culturing of induced pluripotent stem cell models known as organoids, opened new opportunities for brain-centered studies. Today, cerebral organoids, mini in vitro 3d models of the human brain, are used to determine disease pathogenesis and potential treatments for neurological disorders. This review explores the achievements, challenges, and possibilities of cerebral organoids as disease models.

Introduction

Scientists have long viewed the brain as an unsolved mystery. Its microscopic neurons and intricate pathways make it difficult to observe and understand. A major obstacle in this field of research is the lack of suitable cerebral models. Obtaining functional human brain tissue is a sensitive procedure (Devine et al., 2011; Gabriel, Gopalakrishnan, 2017). Furthermore, the human brain is more complex than many other species', so animal models serve as poor substitutes for live human subjects (Lancaster et al., 2013; Plummer et al., 2019). These challenges limit the availability and effectiveness of treatment for neurodegenerative diseases. Alzheimer's disease (AD) is the leading cause of dementia worldwide, affecting over 40 million people as of 2019 (Park et al., 2021), while Parkinson's disease remains the most widespread neurodegenerative movement disorder (Devine et al., 2011). Other common neural afflictions, such as autism spectrum disorder (ASD) and amyotrophic lateral sclerosis (ALS), also have no cure (Marchetto et al., 2010; Seminary et al., 2018), while glial tumors, responsible for many brain cancers, are among the most fatal human tumors (Linkous et al., 2019; Goranci-Buzhala et al., 2020). Despite these statistics, until recently, brain research was limited and very costly (Plummer et al., 2019). A finding in 2006 changed the status quo with Japanese researcher Shinya Yamanaka's groundbreaking discovery of induced pluripotent stem cells (iPSCs).

Pluripotent stem cells, or cells from which all cells in the body can be derived, are important to science because of their ability to differentiate into many specialized cell lines. Particularly in the field of neuroscience, pluripotent stem cells are intriguing because they have the potential to serve as in vitro models of the ever-elusive brain. Until the 21st century, however, pluripotent stem cells remained out of reach, primarily because they were difficult to obtain and raised ethical questions due to their embryonic origins. With the Nobel prize-winning breakthrough in 2006, in which Yamanaka identified the four transcription factors necessary to generate pluripotent stem cells from adult somatic cells (namely Sox2, Oct4, c-Myc, and Klf4), the entire research industry changed (Takahashi, Yamanaka, 2006). Scientists now use iPSCs to culture mini-models known as organoids, which can then be employed to study disease pathogenesis and drug efficacy. In 2012, a method for generating brain organoids using Yamanaka's four factors was outlined. The process involved inducing pluripotency in human embryonic stem cells (hESCs), with careful introduction of many additional transcription factors to guide the development of these cells into 3d cultures of neuroepithelial tissue (Eiraku, Sasai, 2012). While this method is still in use, further experimentation honed the process to reduce culture time and generate differentiated brain regions, creating what are now known as cerebral organoids. This review aims to determine whether cerebral organoids can serve as effective in vitro models for treatment of neurological disorders.

Methods

Data for this paper was collected using the Touro College Library and PubMed databases. Keywords included, but were not limited to, "cerebral organoids," "brain organoids," "induced pluripotent stem cells," and "stem cell models."

Cerebral Organoids

Cerebral organoids are in vitro 3d models of the brain derived from human iPSCs. The first documented cerebral organoids were produced by Lancaster and Knoblich, researchers who are also credited with coining the term. To produce the organoids, the researchers used embryoid bodies, cells derived from human iPSCs, to generate neuroectoderm, the precursor to the central nervous system (Lancaster et al., 2013). For the purpose of creating cerebral organoids, neuroectoderm can also be generated from hESCs, as was done in earlier studies. However, recent studies have refrained from using hESCs; this is due to ethical concerns rather than ineffectiveness. Both methods have proven equally capable of serving as in vitro brain models (Linkous et al., 2019; Marchetto et al., 2010). In the case of Lancaster and Knoblich, the neuroectoderm was generated from human iPSCs and then cultured in Matrigel, a matrix that serves as a scaffold for tissue development. Most importantly, the Matrigel drops

were placed in a spinning bioreactor for better absorption of nutrients, a novel technique that formed larger, more consistent, and more stable models than those that were kept stationary. At 8-10 days, the neural cells differentiated into brain cells with specialized functions; at 15-20 days, a fluid-filled cavity formed inside the neuroepithelium; within a month, the neuroectoderm differentiated into clearly defined brain regions, containing a cerebral cortex, choroid plexus, and meninges, among other identifying brain regions (Lancaster et al., 2013). As in earlier models, the cerebral organoids contained both neurons and glial cells, as well as the axons, myelin, and synapses that characterize the human brain and enable cross-communication between cells (Linkous et al., 2019). Once prepared, cerebral organoids are useful for studying the pathogenesis and treatment of neurological diseases. In addition to these critical functions, which will be discussed shortly, cerebral organoids offer the pivotal opportunity to observe brain development in real-time, a role that cannot be filled by fully-developed animal or human brain models. Previously, brain development was mapped out by in utero imaging such as ultrasound, a method that, although successful, has its limitations. With the advent of cerebral organoids, scientists can use reverse transcription polymerase chain reaction (RT-PCR) and staining techniques to track neural development. They observed the differentiation of early neural tissue into three regions, with the forebrain expanding faster than the hindbrain, according to forebrain and hindbrain markers. However, care must be taken when using cerebral organoids to model brain development; researchers found that the early hippocampus and ventral forebrain were not structured identically to those formed in vivo (Lancaster et al., 2013).

Disease Modeling

Cerebral organoids can be induced to exhibit disease pathogenesis by culturing them from iPSCs derived from affected patients. The first recorded incidence of cerebral organoid disease modeling was concurrent with the first instance of cerebral organoid culture. The researchers used the organoids to model microcephaly, a neurological disorder caused by mutations in the CDK5RAP2 gene, resulting in a drastic reduction in brain size. They converted a patient's skin fibroblasts into iPSCs using Yamanaka's four transcription factors, then used the iPSCs to culture cerebral organoids. The organoids displayed markers of microcephaly such as smaller embryoid bodies, indicating an accurate disease model (Lancaster et al., 2013). Another way to generate cerebral organoid disease models is to introduce patient-derived stem cells into a fully-formed cerebral organoid culture. This method was used to create cerebral organoid glioma (GLICO) models. Glioma stem cells, the parent cells for tumor formation, were obtained from cancer patients; they were then co-cultured with individual, healthy cerebral organoids. The cells proliferated in the organoids, imitating the process of tumor formation in vivo (Linkous et al., 2019). This latter method has the added benefit of enabling observation of disease pathology.

In some cases, disease pathogenesis can be induced in healthy stem cells. This can be especially useful where affected patients are not accessible as subjects, or to provide isogenic stem cell models, which allow diseased organoids to be compared to healthy controls. This was the case in a study of AD, which obtained stem cells expressing the parental apolipoprotein E3 (APOE3) gene. CRISPR/Cas9 technology was used to induce the cells to express apolipoprotein E4 (APOE4), a gene commonly associated with increased risk for AD. Using the two cell lines to derive cerebral organoids, the researchers were able to compare the effects of both gene variants on neural development, an added benefit over using a single diseased cell line (Lin et al., 2018). A similar technique was employed to derive stem cell models with markers of Parkinson's disease (PD). Healthy embryonic stem cell lines, induced to express the SNCA-A53T mutation associated with PD, were used to generate dopaminergic neurons as disease models. Some cells were deliberately not induced and were set aside as isogenic controls. For the same reason, patient-derived iPSCs carrying the SNCA-A53T mutation were used to generate dopaminergic neurons; the mutation was corrected in some cells to provide isogenic controls (Ryan et al., 2013). It must be noted, however, that only dopaminergic neurons, and not cerebral organoids, were generated in this case. Thus, although they expressed the ^{a}-synuclein and Lewy body pathology characteristic of PD, it is unclear whether these stem cells serve as effective models for the disease. In addition to providing healthy and diseased isogenic models, cerebral organoids confer the added advantage of allowing scientists to choose which genes or characteristics to express. One study on autism spectrum disorder (ASD) examined 53 iPSC lines from 25 affected patients, each carrying different genetic variants of the disease. The stem cells were differentiated into glutamatergic neurons, which were used to compare neural activity in the various mutations (Deneault et al., 2019). However, because the iPSCs were not cultured into GABAergic neurons, which have an inhibitory effect on the brain, or into the many other cells that make up cerebral organoids, the results may have been skewed. Nevertheless, the approach could

be used to compare the effect of different mutations of a disease. With regard to characteristics, an in-depth study of AD generated 1300 organoids with different key markers of the disease. Their iPSCs included Pittsburg compound B (PiB) positive and negative cell lines, a second strong indicator of AD, in addition to iPSCs derived from APOE3 and APOE4 cell lines (Park et al., 2021). They were then able to generate organoids with any combination of these markers, among others. This diversity enables scientists to model a disease under a variety of circumstances, creating the potential for drug tailoring based on which hallmarks of AD a patient possesses.

Treatment of Disease

It is important to determine whether cerebral organoids can serve as models to test potential treatments for disease. To determine the efficacy of ionizing radiation in treating glioma, scientists compared the effects of radiation on their 3d GLICO disease models to the effects of radiation on simpler, 2d glioma models. They observed that radiation had reduced ability to inhibit tumor growth when compared with the drastic apoptotic effects of radiation on 2d glioma models. The poor response observed in organoids concurs with the real-life observation that radiation is usually ineffective in treating glioma patients (Linkous et al., 2019). Thus, the outcomes indicate that 2d glioma models are unreliable because they give artificially inflated results, whereas cerebral organoids serve as better models because of their greater accuracy. Although this doesn't irrefutably prove that cerebral organoids are ideal models for treatment of disease, it does indicate that they perform better than the 2d models formerly used.

When it comes to testing treatments for disease, cerebral organoids can also be used to rule out ineffective treatments, saving years of wasted effort. In their study of microcephaly, scientists used RNA interference (RNAi), a process that uses RNA to selectively suppress gene expression, to inhibit destructive CDK5RAP2 activity in microcephalic organoids. Accordingly, they observed that the number of neurons increased in vitro, confirming that CDK5RAP2 is the gene behind microcephaly. This conclusion suggests that selective RNAi of CDK5RAP2 might be a possible treatment option for patients with microcephaly. The researchers also made another important conclusion; when they introduced healthy CDK5RAP2 into the diseased culture, larger neural tissue was observed. The latter method, however, proved toxic to cells, eliminating it as an effective treatment option (Lancaster et al., 2013). Likewise, in a study of Rett Syndrome, a neurodevelopmental disorder classified as an ASD, iPSC-derived neurons were used to test treatments for the disease. The neurons, while not complete organoids, possessed MeCP2 mutations, a major disease marker, as well as the decrease in synapses and soma size present in Rett Syndrome. Treatment of the cells with IFG-1, an insulin-like growth-factor often studied in relation to Rett Syndrome, had a positive effect on synapses but stimulated excitatory neurons to abnormal levels. While this does not preclude IFG-1 as a potential candidate for treatment of the disease, it does indicate that care must be taken when the therapy is used (Marchetto et al., 2010). Thus, although stem cells models, and not cerebral organoids, were used in this case, the study highlights the capacity of organoids to identify or eliminate treatment options.

Drug Testing

Cerebral organoids provide the opportunity for accurate, personalized medicine through the use of brain models derived from iPSCs of an affected patient. One study on anticancer therapy used glioma tissues derived from Johns Hopkins surgical patients to test the efficacy of temozolomide (TMZ) and doxorubicin in treating glioblastoma. The success rates, at approximately 30% reduction and 80% dose-dependent reduction of cultured tumor cells, respectively, indicate that patient-derived iPSCs can serve as effective models for drug testing (Plummer et al., 2019). Whether the indicated dosages would be ideal for said patients is unknown, as the study did not examine the effect of these drugs on the patients. This could potentially be an area for future study. Another study compared the effects of TMZ and bis-chloroethylnitrosourea (BCNU), another anticancer therapy, on both GLICO and 2d models. They found that while the drugs exhibited drastic dose-dependent decrease of tumor cells in 2d models, with 80% and 90% success rates, respectively, the drugs were only moderately effective in reducing tumor cells in GLICO models, with respective 24-43% and 5% decreases (Linkous et al., 2019). The limited effectiveness observed in GLICO models compares to the weak response to these drugs evident in live subjects. Once again, this confirms the theory that cerebral organoids are superior to 2d cultures as glioma models, because their behavior more closely resembles that observed in actual glioma patients. Additionally, the similar outcomes obtained by both studies in regard to the efficacy of TMZ in treating gliomas (a 30% vs 24-43% reduction in tumor cells) indicates the reliability of cerebral organoids as models for drug testing.

Although the amount of research available on drug testing using cerebral organoids is limited due to the relative novelty of the process, multiple drug screenings performed using alternate stem cell models indicate the

strong potential of cerebral organoids for drug testing. For example, a drug screening of motor neurons cultured from patient-derived iPSCs identified ropinirole, a drug used to treat PD, as a candidate for treatment of sporadic amyotrophic lateral sclerosis (ALS), a disorder involving loss of movement. To evaluate drug efficacy, a new screening system designed to screen for multiple phenotypic changes was introduced. Disease models were treated either with DMSO, a solvent, designating them as controls, or with a drug dissolved in DMSO. They were then evaluated for multiple markers of ALS, including changes in neurite length, number of formed stress granules, and leakage of FUS protein aggregates and LDH. Subsequent increases and/or decreases in these markers were used to indicate the success of a particular drug (Fujimori et al., 2018). Although this technique was only tried using motor neurons, and not cerebral organoids, the system could be applied to other diseases.

Similarly, a comprehensive screening of over 1,000 drugs on iPSC-derived AD cortical neurons indicated that a drug cocktail of bromocriptine, cromolyn, and topiramate could lower the count of toxic β-amyloid plaques. Three different solvents, used as positive and negative controls, helped evaluate the efficacy of the drugs and narrow down the drug pool to 27 possibilities. A widely accepted fingerprinting method was then utilized to determine the best combination, taking factors such as efficacy and toxicity into account (Kondo et al., 2017). It is unknown whether this cocktail demonstrated improvement in actual trials, but it's possible that the methodology used in this study could be applied to screen cerebral organoids.

Challenges of Cerebral Organoids

One challenge to using brain organoids for drug testing is finding a technique for visualizing markers of disease in the organoids. To overcome this challenge, researchers developed a new screening platform called microTMA, a spheroid tissue microarray that enables the viewing of multiple organoid cross-sections on a single slide, for a comprehensive 3d image. Using this platform, the developers successfully tracked the effect of anticancer drugs on glioblastoma brain sphere models. This screening system performs similarly to the older Polaris/Inform system, a screening technique that is beyond the scope of this paper, but reduces the image acquisition time by more than 95% compared to the original screening technique (Plummer et al., 2019). For disease-specific screening, a more customized approach may be necessary. For instance, when developing in vitro models of AD, one study used two forms of positron emission tomography (PET), PiB-PET and tau-PET, to screen cerebral organoids for β-amyloid plaques and phosphorylated tau protein, two pathological hallmarks of AD. They consistently applied these screening techniques throughout the study to monitor drug efficacy (Park et al., 2021).

Critics of cerebral organoid research point out that organoids vary in size from sample to sample, which may lead to inconsistent results. However, this argument can be made irrelevant by using identical samples. For instance, researchers of AD put their organoids through intensive quality control to determine that only organoids uniform in shape and size were used (Plummer et al., 2019). Nevertheless, it should be noted that this method requires the discarding of many organoids that fail to meet physical requirements. Another solution, employed on stem cell models of ALS, is to use a bulk culture system in which multiple models are derived from many stem cell lines. Rather than disposing of heterogenous models, a clustering system was developed to separate the models based on different disease pathologies. The models were then used to study differing forms of ALS, suggesting that there is an advantage to diversity (Fujimori et al., 2018). However, there are times when uniformity is needed. A novel protocol, adapted from the original method proposed by Lancaster et al., gives researchers more control over organoid development, avoiding the problem of heterogeneity altogether. In this process, neurons are differentiated directly from iPSCs, avoiding the formation of embryoid bodies that spontaneously form unwanted germ cell layers. This method, successfully used to model microcephaly, results in brain organoids with fewer variations and defects (Gabriel, Gopalakrishnan, 2017). Perhaps further research using this protocol could contribute to uniformity of cerebral organoids.

Areas for Future Research

There remains the question of whether drug absorption in brain spheres is comparable to drug absorption in vivo. In actuality, the human brain is isolated from circulation by a blood-brain barrier (BBB) that selectively allows only 5% of drugs across its borders in significant enough dosages to have a pronounced effect (Ribecco-Lutkiewicz et al., 2018). To avoid the pitfall of testing drugs that are not permeable to the BBB, researchers of AD tested only FDA-approved drugs known or suspected to have BBB permeability (Park et al., 2021). While a solution, this is a severe limitation of the organoids because it confines the study to available treatments. Towards that end, several attempts have been made to generate an in vitro model of the BBB using iPSCs to generate a 2d monolayer of epithelial cells. These models have been shown to effectively evaluate drugs for BBB-permeability (Ribecco-Lutkiewicz

et al., 2018). However, to this author's knowledge, no study to date has cultured an in vitro model of brain organoids in conjunction with an epithelial BBB. The introduction of a BBB might provide more therapeutic options.

Brain organoids with a BBB may provide a more complete model for drug testing, but still fail to accurately model drug penetration due to their lack of a circulatory system. Some propose that this factor is negligible due to previous studies indicating high drug penetration in organoids (Plummer et al., 2019). However, it is possible that drug screening platforms using current in vitro models are misleading. The developers of cerebral organoids note that cell death occurs in the core of the brain tissues after several months in vitro, most likely because of their lack of a blood supply, which restricts their oxygen and nutrient intake. Also of note, these missing factors probably explain why the organoids stop growing once they reach a certain size (Lancaster et al., 2013). Perhaps further research could lead to the development of a cerebral organoid containing a capillary system, which would better simulate the real-life internal environment.

Moreover, some cerebral organoids developed to model AD did not contain microglial cells due to the unique circumstance regarding their origins (Park et al., 2021). This is a major downside because microglial cells function in an immune capacity in the central nervous system. In addition, microglia express certain genes that are associated with an increased risk for late-onset AD, making them an important component in any in vitro AD model (Abud et al., 2017). In a study that did incorporate microglia-like cells into their cerebral organoids, the cells expressing APOE4 experienced reduced β-amyloid uptake and impaired response time when cultured in organoids, indicating that microglia may contribute to the β-amyloid buildup that is so detrimental to AD (Lin et al., 2018). Thus, introducing microglial cells into cerebral organoids might generate different responses to drug testing. Although actual microglia are difficult to obtain, owing to their complex lineage which traces back to yolk sac erythromyeloid progenitors that migrate into the neural tube during development, iPSC-derived microglial cells make an acceptable substitute. In fact, microglia-like cells generated from iPSCs are shown to mimic the activity of human fetal and adult microglia (Abud et al., 2017).

There is one area where cerebral organoid research is critically lacking. Although PD is the second most common neurodegenerative disease, stem cell research on the topic is severely lacking (Zambon et al., 2019). Several studies have generated midbrain dopaminergic neurons with markers of PD, but these are missing the complete cerebral environment necessary for accurate drug testing (Ryan et al., 2013; Laperle et al., 2020; Devine et al., 2011; Fernandez-Santiago et al., 2015; Zambon et al., 2019). Others have developed full cerebral organoids, but with a focus on external factors, such as response to viral infection, rather than treatment options (Schultz et al., 2021). Despite the capacity of cerebral organoids for identifying potential drug candidates, little research has been done in this realm with regard to PD. In fact, to this author's knowledge, no published study to date has produced cerebral organoid models of PD with the express intention of performing a drug screening. It is likely that future cerebral organoid PD models, with an emphasis on treatment, could lead to a breakthrough in this disease.

Conclusion

Since their entrance into the scientific industry in 2013, cerebral organoids have changed researchers' approach to the study of neurological disorders. Derived from the iPSCs of affected patients, or from the stem cells of healthy subjects, cerebral organoids exhibit optimal growth when cultured in Matrigel placed in a spinning bioreactor. Perhaps avoiding the step of embryoid bodies could further enhance the process by reducing inconsistencies. Once formed, cerebral organoids can serve as in vitro models, enabling the observation of brain development and/or disease progression. If healthy organoids were produced, disease pathology can be introduced via affected stem cells, offering the opportunity to study isogenic disease and control groups. In addition to RT-PCR and staining, advanced screening techniques, such as the MicroTMA platform or other disease-specific imaging technology, may be necessary for visualizing disease pathology in the organoids. Overall, cerebral organoids perform better than their earlier, 2d counterparts, indicating success in that their results compare to phenomena seen in human subjects. Thus, they appear to serve as effective models for the investigation of potential treatments, and can likely be used to rule out ineffective therapies and explore new treatment options. Furthermore, cerebral organoids show satisfactory progress in the area of drug screening. Although more studies are necessary to make a definitive conclusion, similar trials carried out on alternate stem cell models suggest that cerebral organoids could be used to identify drug candidates. Cerebral organoids could be improved by the introduction of a blood-brain barrier and a capillary system, which would portray drug permeability and penetration more accurately. Moreover, the addition of microglia-like cells, which were omitted in some organoids, seems to be a critical aspect of some disease models, especially of AD. Further research centered on treatment, particularly in regard

to PD, could possibly lead to breakthroughs in this field. Consequently, while there is a long way to go, the signs indicate that cerebral organoids can successfully serve as in vitro models for the analysis and treatment of neurological disorders, potentially opening new doors in medicine.

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