BRAIN ORGANOIDS: HISTORY, MORPHOFUNCTIONAL ASPECTS AND APPLICATION PERSPECTIVES

https://doi.org/10.56238/sevened2024.029-002

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ABSTRACT

One of the main goals of neurobiology is to understand the development and dysfunction of the human brain. Many of the tools and techniques that have informed our understanding of human brain development cannot fully capture the unique and dynamic features of human brain development. Recent advances in stem cell technologies that allow the generation of human brain organoids from pluripotent stem cells (PSCs) promise to profoundly change our understanding of human brain development and enable a detailed study of the pathogenesis of hereditary and acquired brain diseases. In this review, we will overview the development of brain organoid technology, its current progress and applications, and future prospects of this technology.

Keywords: Brain organoids. Neurodevelopment. Neurodegenerative diseases. Stem cells. Vasculature.

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INTRODUCTION

There is a growing concern in modern medicine to better understand how the brain forms and functions, as well as the mechanisms that promote and sustain neurological diseases, in order to develop and improve interventions and treatments for human frailties. However, studying neurodevelopment, neural circuits, and associated neurological disorders is highly challenging. The brain is an organ of great complexity and, to this day, remains difficult to access for experimental investigations in humans. Such access is primarily limited to post-mortem tissue samples or surgically removed tissues, as well as the use of non-invasive methods such as neuroimaging, transcranial magnetic stimulation, and electrophysiological monitoring (Komssi & Kähkönen, 2006; Stan et al., 2006; Brammer, 2009; Eyal et al., 2016). All these methods have limitations, necessitating the development of new procedures. Other constraints relate to the existence of differences in brain circuits and anatomical variations stemming from the interplay between individuals' genetic heterogeneity and exposure to the environment, as well as variations in techniques for processing and preserving tissues from the human central nervous system (CNS) (Adams et al., 2019).

Post-mortem analysis of CNS tissue has historically been the most accessible means of studying phenotypes in neuropathological conditions. While the foundations of modern neuroscience rely on centuries of examinations of human post-mortem tissues, enabling the study of specific features of the human brain, such as neuroanatomy (Moon et al., 2010), this approach is insufficient in providing insights into neural functioning and development (Kelava and Lancaster, 2016a; Adams et al., 2019). However, working with in vivo human brain tissues is extremely challenging yet essential for studies on the biological principles of human brain development and pathologies. Consequently, functional neuroimaging and animal models (primarily rodents and primates) have emerged as the most viable alternatives today (Jay et al., 2011; Partridge and Rossmeisl, 2019; Seeman and Madras, 2013). Nonetheless, significant species differences exist, compounded by the heterogeneity of age, sex, and pathology within the species under investigation, posing considerations that must be weighed in the challenge of interpreting what is consistent among scientific findings (Elston et al., 2001; DeFelipe et al., 2002; Roth and Dicke, 2005; Herculano-Houzel, 2009; Mohan et al., 2015; Muotri, 2016; Kelava and Lancaster, 2016b; Adams et al., 2019).

Consequently, researchers have endeavored to develop and optimize in vitro systems for culturing neural cells, aiding in the understanding of CNS development, functioning, and the underlying pathogenesis of neurological diseases. The advancement of

technologies related to the acquisition, manipulation, and cultivation of stem cells (SCs) has emerged as a new alternative for CNS study. Human embryonic stem cells (hESCs) can be induced into neural stem cells and subsequently differentiated into more specialized cells, such as neurons and glial cells. However, due to the ethical concerns surrounding their procurement, hESCs have been minimally adopted in vitro studies. Another option involves considering the use of adult stem cells, commonly known as pluripotent stem cells (PSCs), responsible for the renewal and repair of adult tissue. However, this type of cell is known to be less versatile than hESCs, and unable to mimic CNS development, limiting its use in such research. Cellular reprogramming, however, presents a viable alternative to these challenges. This technique involves the reversal of specific somatic cells from a donor's tissue, through manipulation of gene expression using stem cell transcription factors, to a pluripotent state, resulting in the formation of induced pluripotent stem cells (iPSCs). iPSCs carry the donor's (or patient's) genotype and possess the same differentiation capacity as hESCs, being inducible into the aforementioned cell types. Therefore, iPSCs allow for direct, non-invasive modulation of relevant cellular phenotypes related to the clinical aspects of the diseases of interest, representing a non-invasive, patient-specific, and ethically acceptable modeling system. Additionally, new genome editing techniques, such as the CRISPR/Cas9 system, which aims to specifically alter the genetic information of human cells (Hockemeyer and Jaenisch, 2016), have proven to be significant allies in this regard.

Despite the existence of various in vitro neuronal differentiation protocols, most are based on a two-dimensional culture system (also known as 2D culture or monolayer culture). One of the advantages is the uniform accessibility of SCs to growth and differentiation factors, as well as the uniform differentiation of SCs, aiding in achieving homogeneity in study results. However, among the disadvantages of 2D culture, especially in simulating human brain development, the absence of cell-cell and cell-matrix interactions stands out, which regulate important stages of neurological development (Koo et al., 2019).

Therefore, it became necessary to develop a model system that is more faithful to the human brain's developmental environment. These processes culminated in the generation of 'organoid technology'. Organoids are structures obtained through three-dimensional (3D) cultures, which undergo some level of self-organization and resemble, at least in part, in vivo organs and their specific cell types. The brain organoids currently being developed are still quite distinct from the human brain (which is much more complex) but can mimic, to some extent, in vitro, characteristics present in neurodevelopment and neuropathologies in vivo (Lancaster and Knoblich, 2014a; Qian et al., 2019).

Thus far, a diversity of protocols for organoid generation has been developed and published for different purposes, such as modeling cortical development (Lancaster et al., 2013; Birey et al., 2017), as well as modeling the development of other specific areas and regions of the human brain, for example, the hippocampus (Sakaguchi, H. et al., 2015), midbrain (Monzel et al., 2017), cerebellum (Muguruma et al., 2015), among others. One of the objectives of organoids is precisely to produce models of neurological diseases that affect specific brain regions. Further applications for brain organoids are more thoroughly addressed by Adams (Adams et al., 2019).

Organoid technology has significantly advanced research on diseases related to neurological development, as well as the investigation of neurodegenerative diseases. Despite its potential, however, many technical challenges and limitations remain (DiLullo and Kriegstein, 2017; Pasca, 2018). This technology is still in its early stages. The current protocols are only sufficient to generate organoids that mimic the human fetal brain around the second trimester of development, in terms of cellular and molecular composition (Camp et al., 2015; Pasca et al., 2015; Qian et al., 2016). Further challenges and limitations will be addressed later, including the difficulty of developing a vascularization network alongside the organoid. There is still a need for additional improvements to overcome the limitations of using these organoids, making them more reliable.

This review provides a brief historical overview of the central nervous system through brain organoid models, as well as a general view of some of the neuropathologies that have been studied using these models, highlighting their limitations, as well as hypotheses and strategies being developed to overcome the challenges of in vitro modeling.

DEVELOPMENT OF ORGANOID TECHNOLOGY:

The initial attempts to create in vitro models of neurological diseases were based on the cultivation of neural cells in monolayers (2D culture). Neural cells (neurons and glial cells) were obtained through differentiation from neural progenitor cells (NPCs), which in turn were derived from the differentiation of iPSCs or ESCs (Chambers et al., 2009). Both iPSCs and PSCs have the capability to form large multicellular aggregates, known as embryoid bodies, a natural ability of ESCs. These embryoid bodies undergo development similar to that of an embryo (Pettinato et al., 2015). In 2001, a study used ESCs to generate embryoid bodies that were subsequently directed toward a neural lineage, resulting in NPCs (Zhang et al., 2001). NPCs have the capacity to self-organize into rosettes, a group of polarized neural progenitor cells that can form structures resembling the embryonic

neural tube (critical for the development of the neocortex in human embryos) (Zhang et al., 2001; Shi et al., 2012).

Self-organization is characterized by the ability to form specific cellular structures without external interference, solely through intrinsic and spontaneous processes. It is a crucial factor in organ formation (Werner et al., 2017). In 2003, a study demonstrated that ESCs produced neural precursors even in the absence of inductive signals, such as growth factors. Subsequently, it was shown that some of these cells acquired neural identity during differentiation. It is important to note that only a fraction of the ESCs were able to acquire neural identity, requiring the treatment of the rest with retinoic acid, which is important in neural differentiation. The study also demonstrated that this restricted differentiation of cells into NPCs did not depend on multicellular aggregation (Ying et al., 2003). Thus, protocols were developed for generating cortical neurons from 2D cultures (Chambers et al., 2009). However, as introduced in this article, the 2D culture of neural cells has limitations in producing the connectivity observed in vivo. Efforts to overcome these limitations led to the development of 3D brain organoids that were capable of representing more advanced in vitro models, more accurately recapitulating the connectivities of the human brain, along with its high complexity.

It is worth noting that the pioneers in creating what is now known as the cerebral organoid were a group of stem cell researchers from the laboratory of Yoshiki Sasai at the RIKEN Center for Developmental Biology in Japan. These researchers laid the groundwork for the research that has been conducted since then (Eiraku et al., 2008; Watanabe et al., 2005). It was in 2011 that the group used human ESCs in a 3D neural culture system to generate cup-shaped self-organizing structures that exhibited characteristics similar to retinal tissue (Eiraku et al., 2011).

Other pioneering works have advanced the field of brain organoids (Lancaster et al., 2013; Qian et al., 2016; Paşca et al., 2015; Lindborg et al., 2016). The work by Qian and colleagues demonstrated that three-dimensionally cultured structures exhibited cortical regions that displayed an organization similar to that of the human cortex in early development, both structurally and in terms of cellular behavior (Qian et al., 2016). Among various studies, new protocols were developed, such as growing organoids using embryoid bodies in Matrigel matrix cultivated in a rotating bioreactor. This matrix was intended to serve as a support to assist in the formation and expansion of tissues, while the bioreactor was used to facilitate gas and nutrient exchanges between the organoids and the medium, resulting in the production of larger and more viable structures. These exhibited growth for longer periods, maintaining viability for up to 100 days (Grebenyuk and Ranga, 2019;

Lancaster et al., 2013; Qian et al., 2016). It is worth noting that new bioreactor models have been developed with the aim of achieving the neural subtypes found in the six cortical layers, adding even more complexity to the model (Qian et al., 2016). All of this has made organoid technologies a model system for the study of the human brain, its development, and related diseases.

Today, the production of organoids can occur in two ways: one is termed non-standardized or whole-brain organoids, and the other, standardized or region-specific brain organoids. Non-standardized organoids, typically cultivated in an extracellular matrix, self-organize into structures from different regions of the brain through the activity of specific endogenous cues from the cell culture itself (Lancaster et al., 2013; Renner et al., 2017). Standardized organoids aim to generate specific regions of the brain by adding specific external growth factors (Paşca et al., 2015; Qian et al., 2016). Indeed, in current protocols, both growth factors and scaffolds composed of extracellular matrix molecules are of great importance (Lancaster et al., 2013; Qian et al., 2016; Quadrato et al., 2017). Some protocols are capable of producing brain spheroids without the presence of scaffolds, solely through extrinsic neural induction. These spheroids undergo both neurogenesis and astrogliogenesis, thereby more faithfully replicating the neural cell diversity, a critical aspect of cortical development (Paşca et al., 2015).

Another aspect that must be considered when studying the development of organoid technology, which is crucial for self-organization, is guided cell migration. This process is essential for the proper assembly of the functional neural circuit, driven by neurotrophic factors and intercellular interactions (Valiente and Marín, 2010). As a result of successful cell migration, we observe successful generation of neurons from NPCs in their respective original locations, as well as their redirection to the expected sites. For example, excitatory neurons migrating to the cortical plate simultaneously with inhibitory neurons, which also migrate to connect with the excitatory neurons in the cerebral cortex to modulate neuronal activity (Kriegstein and Noctor, 2004). Fluorescence labeling confirms the modeling of the migration process in vitro (Bagley et al., 2017).

Approaching a decade of history, cerebral organoids and associated technology have already begun to strongly impact modern medicine. It is expected that this model will become invaluable for a better understanding of the fundamental biology of brain development, function, and disorders.

LIMITS AND ADVANCES

Organoide technology, represented by three-dimensional (3D) culture systems, is the most recent technological development in modeling the central nervous system (CNS), addressing some of the needs not previously met by the analysis of in vivo tissues or the use of 2D cell cultures. Since the creation of this technology, many advances have been made in the development of these models, such as the ability to recapitulate increasingly later milestones of brain development and extend the capacity for the cultivation and maintenance of these organoids. In vivo transplantation is currently a subject of discussion among many researchers. This topic is still subject to ethical and epistemological considerations regarding the potential development of consciousness in organoids (Lavazza and Massimini, 2018; Shepherd, 2018).

Although the benefits of using cerebral organoids represent an advantageous culture system in many respects, with an incredible diversity of neural cells that help us model intercellular interactions during organogenesis as closely as possible, it is important to note that the technology suffers from certain limitations, which are constantly being brought to attention, serving as a starting point for new research aimed at improvement (DiLullo and Kriegstein, 2017).

Regarding reproducibility, organoid development protocols can result in variable batches, presenting differences in the compositions of cells representing certain brain regions, resulting in morphofunctional variabilities (Lancaster and Knoblich, 2014b). Differences have been noted when comparing the same region between two organoids from different batches, and even within the same batch, in terms of cell distribution, density, and composition (Di Lullo and Kriegstein, 2017; Kelava and Lancaster, 2016a). This leads to different regional identities in the organoids, which in turn raises significant concerns about the reproducibility and accuracy of the protocols (Kelava and Lancaster, 2016a).

Indeed, the variability among organoid models can have serious implications when used for disease modeling, drug testing, or studies focused on neurological development, as heterogeneity implies inconsistency in the analyzed phenotypes. This issue can be mitigated by increasing repetitions in quantitative analyses, but this would raise the cost of experiments. On the other hand, qualitative analyses, such as morphology by microscopy, for example, would still be compromised. For this reason, as suggested by Kelava and Lancaster, when investigating phenotypes related to genetic diseases using organoids, the results must be robust enough to be considered (Kelava and Lancaster, 2016b). It is worth noting that improvements in organoid generation protocols are currently being developed to

reduce heterogeneity and optimize reproducibility (Qian et al., 2016; Sloan et al., 2018; Yoon et al., 2019).

A factor that limits the development of organoids in relation to the early stages of organogenesis is the absence or minimal presence of relevant cell subtypes (Pasca et al., 2015; Qian et al., 2016). Various types of non-neural cells make up the human brain, and these cells are not necessarily neural cells derived from the neuroectoderm. Non-neural cells, such as microglia, endothelial cells, hematopoietic cells, as well as meninges, are largely absent in organoids because most current protocols induce the neuroectodermal fate in the embryoid body. Consequently, brain functions or disorders that originate from non-neuronal cells or from interactions between non-neuronal cells and neural cells cannot be adequately modeled in cerebral organoids. A study conducted by Ormel and colleagues, using a lower concentration of heparin (a neuroectoderm stimulant), delaying transfer to matrigel, and not applying brain-derived neurotrophic factor (BDNF) in the cerebral organoid differentiation protocol, obtained mesodermal progenitor cells capable of differentiating into mature microglia. The authors attributed this to the microenvironment of the organoid itself, which presented conditions similar to the CNS that allowed the development of these microglia. Temporal monitoring of organoid development led to the observation that the mesodermal cells present at the beginning originated microglia in subsequent stages. The group highlighted that factors known to drive microglial development in rodents, such as CSF1, IL34, and TGFβ1 (typically used to generate microglia from iPSCs), are the same factors expressed inherently by cerebral organoids. The results showed organoids with functional synapse formation and the detection of microglial ramifications very close to neuronal processes and synaptic structures (Ormel et al., 2018). Other methods of co-culturing differentiated microglia in 2D or introducing these cells into cerebral organoids can also be used in disease modeling or to investigate interactions between microglia and neural cells (Lin et al., 2018).

All of this concern stems from the crucial need to understand whether cerebral organoids recreate the neural circuitry observed in the human brain (or at least possess the necessary cellular diversity for such and in proportion close to the ideal), enabling us to advance in the comparison and understanding of the pathophysiological findings in human cerebral organoids.

Another issue related to the absence of mesodermal cells is the resulting lack of vascularization, which poses a significant obstacle in the development and maintenance of organoids. The late-stage developing brain is highly dependent on vascularization for the diffusion of nutrients and oxygenation. Additionally, neural cell groups and vascularization

are intertwined in the brain structure. Given that cerebral organoid models lack an inherent circulatory system with blood vessels, they rely on simple diffusion of the culture medium for the supply of gases and nutrients. When cultivated for extended periods, a significant number of cells within the organoids, particularly in their interior, undergo apoptosis due to oxygen and nutrient deficiency (Lancaster and Knoblich, 2014a). The absence of vascularization also disrupts certain endogenous patterning cues necessary for the differentiation of neural progenitors and the proper development of the organoids. These cues are extremely important for the late-stage development of neural structures. Furthermore, it is noteworthy that some of the neural progenitor cell niches are located near blood vessels.

Confronted with this issue, it becomes imperative to refine existing protocols by simulating the physiological environment, altering the culture conditions, and employing bioengineering to provide vascularization, thereby establishing a nutrient flow system, as well as to promote the emergence and preservation of patterning cues in the organoids (Kelava and Lancaster, 2016a).

Recent vascularization techniques have shown promise in addressing these challenges, demonstrating the generation of blood vessel organoids from human PSCs, containing endothelial cells that have the capacity to self-assemble, forming capillary networks. Following the transplantation of these organoids into mice, the formation of a well-defined vascular system was reported (Cakir et al. 2019, Mansour et al. 2018, Pham et al. 2018, Wimmwe et al. 2019).

It is known that adequate blood supply is essential for the normal functioning of the brain, and a failure in the cerebral vascular network can result in damage and loss of function in brain tissues. The cerebral vascular network is composed of the blood-brain barrier (BBB), which protects the tissue from infections, regulates the passage of nutrients, and removes metabolic waste (Zhao et al., 2015). In addition to the BBB, we can highlight some indispensable functions that glial cells - particularly microglia and astrocytes - perform in the CNS under normal conditions. Microglia serve as the brain's immune monitor and, when activated, release inflammatory cytokines and perform phagocytic functions (Solito & Sastre, 2012). Astrocytes, on the other hand, are involved, among other things, in the secretion of growth factors and the regulation of oxidative stress, as well as synaptic remodeling, energy supply, and homeostasis (Wyss-Coray & Rogers, 2011). Both cell types are involved in the repair, maintenance, and permeability of the blood-brain barrier (BBB) (Abbott et al., 2006). Given the complexity of the biological mechanisms related to the pathophysiology of the many disorders that affect the CNS (many of which are already

reproducible through organoid culture techniques, as will be seen below), it is important to emphasize that future research aiming to model such disorders in vitro should not only focus on the production of cell types such as neurons and glia within the models, but also on the contribution of these cells associated with the cerebral vascular system to understand the onset and progression of pathologies.

There are currently several methods for generating vascularization in organoid models. Pham et al. (2018) generated vascularization in cerebral organoids by incorporating them into Matrigel droplets containing endothelial cells previously derived from iPSCs. Mansour et al. (2018) also achieved vascularization by grafting in vitro-developed cerebral organoids into in vivo murine cortices. This work demonstrated the potential of organoids, as it observed vascular structure and synaptic connections between the organoid and the host CNS, as well as facilitating the study of vascularization and grafting processes in general (Mansour et al., 2018).

As previously mentioned, in addition to the formation of blood vessels, another important component that must be emphasized and developed alongside vascularization is the blood-brain barrier (BBB). Present at all levels of the vascular tree and formed by a continuous monolayer of endothelium surrounded by mural cells, the BBB restricts the endothelial transport of most molecules (especially macromolecules) from the blood, as well as the entry of blood cells (such as leukocytes) and microbial pathogens, thus maintaining the integrity of the brain tissue (Zhao et al., 2015). Some groups did not address the absence or presence of the BBB when generating vascularized organoids, while others have already developed viable BBB models, including the presence of one to three cell types (Cho et al. 2015, Helms et al. 2016, Mansour et al. 2018, Pham et al. 218, Wang et al. 2016). Similar to organoid models, improved performance is expected when providing the model with greater cellular complexity. Thus, the importance of developing in vitro BBB models can be considered. These models can be used for testing new drugs and therapeutic approaches related to the treatment of neurological diseases, providing more specific data regarding the ability of the molecules used to cross the BBB and their subsequent actions, such as glial and neural cytotoxicity. Another important aspect is to provide a better understanding of the interactions between the BBB and the adjacent brain tissue (Nzou et al., 2018). In summary, these models represent a promise for the in vitro modeling of neurodegenerative conditions and injuries, such as amyotrophic lateral sclerosis, Alzheimer's disease, and stroke.

Therefore, organoids have shown promise for investigating neurodevelopment-related diseases, also holding enormous potential for testing personalized medications for

certain brain disorders. In the following sections, the main disorders related to neurological development and neurodegeneration will be briefly presented, discussing the main approaches and challenges related to the study of these disorders. Later on, the focus will be on autism spectrum disorder (ASD), Alzheimer's disease, the production of neuroglia, and the development of the cerebral vascular network.

UTILIZATION OF BRAIN ORGANOIDS IN MODELING NEUROLOGICAL DISORDERS: ADVANCES AND CHALLENGES

For several years, brain organoids have been widely used as a means to investigate disorders affecting the human brain. Due to the resemblance between the developmental stages - especially in the initial phase - that both undergo, organoids have, in most cases, been considered the most suitable models for investigating disorders related to neurological development. Moreover, in modeling neurodegenerative disorders, organoids are excellent for bridging the gap between patients and animal models. Other utilities, such as modeling the progression of brain cancer, coupled with studies for drug development, genomic editing, and epigenomic remodeling, give organoids the status of a promising experimental model for reproducing and standardizing brain disorders ((Bershteyn et al., 2017; Pacitti et al., 2019; Sachs et al., 2018; Sun & Ding, 2017; Yan et al., 2018).

Indeed, since their creation, organoids have been the subject of an exponential number of publications. Pacitti and colleagues (2019), in their review, report the popularity of organoids over the years. As mentioned earlier, while animal models have been extremely important for our current understanding of the pathological mechanisms of many brain disorders, such as the relationship between mutated genes and phenotype, these models have limitations regarding the translation of their findings to humans. One of these limitations is the different cellular composition between organisms. There are cases where disease phenotypes are found in both animal models and organoids, and such cases are important for validating (or not) both models (Bershteyn et al., 2017). Furthermore, organoids can provide knowledge about specific human phenotypes, as well as cellular subpopulations that are not easily observed in other mammals. In addition, organoids have other significant advantages, such as the possibility of using them as models of living tissues and studying cellular functionality or behavioral dynamics more easily and accessibly (compared to in vivo neural tissues). In organoids, functional synapses can be found after 6 months of culture, as well as considerable neuronal maturation, with well-formed dendrites and active neural networks after nine months (Wang, 2018). The possibility of genome editing offers more precise creation of mutations or repairs, further

expanding the modeling of many diseases (Sun & Ding, 2017). As organoids are a relatively new technology, but showing rapid and significant expansion as in vitro disease modeling tools, this section of this review will be dedicated to advances in this field.

What we refer to as neurodevelopmental disorders (NDDs) are all the diseases that compromise some brain functions, such as learning, sociality, and motor coordination, and that originate from the impairment of normal evelopment processes caused by some kind of disturbance. In other words, they are a group of early-onset neurological disorders. Manifestations of diseases resulting from abnormalities in development processes include disorders such as epilepsy, microcephaly, intellectual disability, and language disorders. Also included are disorders such as Autism Spectrum Disorder (ASD), attention deficit/hyperactivity disorder (ADHD), schizophrenia, bipolar disorder, Tourette's syndrome, Rett syndrome, and developmental coordination disorder (Savatt & Myers, 2021). It is said that NDDs affect 4 to 5% of the world's population (Mitchell, 2011), and can be attributed to mutations at more than 1000 loci (Tărlungeanu & Novarino, 2018).

Currently, there are limitations regarding the understanding of the etiology of NDDs. These limitations range from the difficult delineation of the components involved in heredity to the identification of the mechanisms by which specific cellular factors lead to the disorder, including the definition of these factors. The clinical diagnosis of these disorders, often a time-consuming and costly process, is still limited by the heterogeneity in the clinical presentation that patients show (de la Torre-Ubieta et al., 2016). Therefore, understanding the causes of NDDs, such as identifying genetic risk factors as well as environmental factors, and establishing an appropriate preclinical model for the development of new treatments, represent an important step towards the development of personalized therapeutic approaches.

Models of NDDs in animals have certainly been essential for the current understanding of the mechanisms related to the pathology of these disorders. However, there are differences between a human model and an animal model that limit the use of the latter, including biological characteristics related to development, cellular composition, and genetics. Many cognitive and behavioral diseases have polygenic origins and multifactorial environmental influences, making it challenging to study models of evolutionarily different species, such as rodents, with respect to their intellectual and behavioral abilities compared to the human species. Therefore, there is a clear advantage in using in vitro models for these disorders (DiLullo and Kriegstein, 2017). As we saw earlier, the use of hESCs and iPSCs in generating neurons in vitro has allowed researchers to recapitulate and reproduce in the laboratory various neuronal synaptic defects related to NDDs. One advantage of

using stem cell-based models is the fidelity of modeling diseases directly from affected individuals. Thus, the in vitro model, in addition to having the same genetic information as the patient, can reproduce with a high degree of reliability the cellular and molecular phenotypes associated with the disease in question. Another advantage is the generation of stem cell lines, which implies an unlimited source of cells. However, these methods are mostly limited due to the low complexity generated by 2D systems, which results in a lack of high-order connectivity, an immature identity of differentiated neurons in vitro, and high heterogeneity among clones derived from iPSCs (Sun & Ding, 2017). These limitations are being overcome as organoid technology gains ground. Later on, we will see examples of how organoids are contributing as additional tools for studying the underlying mechanisms of NDDs.

Currently, the protocols used for organoid production report the presence of neurons from all six cortical layers in a temporally structured manner. In other words, cortical neurogenesis in the organoid, concerning the emergence of neuron subtypes, appears to respect the timing and sequence of in vivo development (although they are not arranged in the same way as in vivo). The production of radial glial cells and intermediate progenitors is also reported (Lancaster et al., 2013; Qian et al., 2016), as well as the production of human cell subpopulations that are absent in the development of animal models such as rats (Di Lullo & Kriegstein, 2017).

A concern regarding the validation of organoid models is the understanding of the reproducibility of cell types, as well as their cellular diversity in in vitro models. This has led scientists to profile single-cell genomes during neurodevelopment both in vivo and in vitro. The investigation is carried out by observing changes related to cellular diversity and the enrichment of gene expression when comparing organoids at different stages of development (Quadrato et al., 2017). Another concern is the understanding of self-organization, a phenomenon not yet well understood. Seeking to highlight this issue, studies have identified organizing centers of different brain-like structures in organoids (Renner et al., 2017).

There is another major issue, the establishment of reliable neural circuits, especially in the cortical region. Although few, some studies demonstrate the presence of functional synaptic junctions in organoids (Quadrato et al., 2017). Many neurological diseases manifest their phenotype postnatally, such as problems in the formation and refinement of circuits and synaptic pruning. Considering that the maturation of these processes can take years to form the neural networks observed in vivo, the ability of organoids to faithfully

portray these complex characteristics related to human brain development and maturation is questionable.

As seen earlier in this review, organoid technology as a model better represents the early stages of neurological development. Thus, its use is more advantageous for modeling early-onset neurological diseases during fetal or embryonic stages. In fact, in vitro modeling of neurodevelopmental disorders is perhaps currently the most impactful approach in the use of organoids. This approach allows the study of the onset and progression of the disease during neurodevelopment, enabling a greater understanding of the underlying pathological mechanisms. Organoids constitute a versatile model, as their use allows the modeling of diseases through both genetic factors and those mediated by the environment. New techniques, such as the development of functional networks, promise to broaden studies to understand intracellular mechanisms and cell-cell interactions in more detail (Trujillo et al., 2018ª). Below are some examples of disorders that are more amenable to in vitro modeling.

MICROCEPHALY

Microcephaly is a condition characterized by a reduced head size and is accompanied by intellectual disability and seizures. This disease was the first neurodevelopmental disorder to be modeled using brain organoids. Lancaster and colleagues (2013) generated microencephalic organoids derived from iPSCs from a patient with a mutation in a gene related to the coding of Cyclin-Dependent Kinase 5 Regulatory-Associated Protein 2 (CDK5RAP2), known as a genetic risk factor for microcephaly. A difference in size was demonstrated between the microencephalic organoids and those in the control group. As expected, the smaller organoids were from the microencephalic patient, showing premature neural differentiation and reduced proliferation in their neural progenitor cells (NPCs). This study and its results were of paramount importance for organoid models, as they indicated them as useful tools for modeling brain disorders, presenting them as a means to understand the underlying mechanisms of the phenotype observed in patients (Lancaster et al., 2013).

ZIKA VIRUS INFECTION

Some neurological diseases, including microcephaly, can be promoted by environmental factors that compromise the normal development of the fetal brain. A highly studied example is viral infection during pregnancy. In 2016, the Zika virus was epidemiologically linked to congenital microcephaly in children of mothers infected during

pregnancy (Heymann et al., 2016). Due to the lack of experimental evidence confirming the causality hypothesis in humans, brain organoids and 2D culture of neural progenitor cells were key elements in understanding the mechanisms and pathways by which the virus induced damage to the fetal brain. When exposing iPSC-derived organoids to the Zika virus, it was discovered that the virus has tropism for NPCs, and the infection resulted in reduced organoid growth and decreased NPC numbers (Cugola et al., 2016; Garcez et al., 2016; Qian et al., 2016). Cell signaling pathways during infection were also discovered using organoids through transcriptome analysis (Cugola et al., 2016; Watanabe et al., 2017). However, considering the limitation of the models in depicting the complexity of a human brain (cellular composition, tissue architecture, etc.), the data were not sufficient for a complete understanding of the infectious process. The use of primary tissue, in addition to presenting tropism for NPCs, identified infection and vulnerability in astrocytes and microglia (Retallack et al., 2016), contrary to research conducted in organoids, which showed occasional infection in these cell types (likely due to an underrepresentation of astrocytes and microglia in the organoids). Retallack and colleagues also used primary tissues to demonstrate the vulnerability of astrocytes and radial glial cells to infection via the AXL receptor (a tyrosine-protein kinase receptor abundant in these cell types).

These examples, in addition to highlighting the utility of organoids for investigating the etiology of neurodevelopmental disorders, also emphasize the need for constant improvement in human brain organoid production protocols to ensure better accuracy in results. As we will present later, new protocols aiming to create glial cells within organoids can resolve this impasse.

MACROCEPHALY

Like microcephaly, the macroencephalic phenotype is also the result of some mutations. In this case, silencing of the PTEN gene is the main factor (Butler et al., 2005). The use of PTEN knockout hESCs for the production of brain organoids resulted in organoids with larger volume and surface area accompanied by increased neuroepithelial cells, increased cell proliferation, and delayed neuronal differentiation (Li et al., 2017).

CONGENITAL LISSENCEPHALY OR MILLER-DIEKER SYNDROME

Miller-Dieker Syndrome (MDS) is a congenital form of lissencephaly, a neurological development disorder characterized by the absence of normal brain convolutions, resulting in intellectual disability and seizures (Blazejewski et al., 2018). Studies conducted with the help of brain organoids identified underlying mechanisms of the syndrome phenotype.

Iefremova and colleagues (2017) developed organoids from iPSCs of patients with MDS. The organoids showed reduced size and slower expansion rate compared to controls, as well as other structural modifications. Another study also modeled the syndrome through patient cells and observed a deregulation of neuronal migration and the mitotic axis of glial and neuroepithelial cells (Bershteyn et al., 2017). These data suggest that organoids may recapitulate important cellular and molecular mechanisms in the formation of the disease.

SANDHOFF DISEASE

Sandhoff disease is a neurodevelopmental disorder characterized by lysosomal accumulation of GM2 ganglioside and is related to a defect in the hexosaminidase enzyme due to a mutation in the HEXB gene (Sandhoff et al., 1971). In addition to developmental delay, patients with this disease present macrocephaly and seizures (Allende et al., 2018). Allende et al. (2018) produced brain organoids from cells of na affected patient and from isogenic iPSCs with a HEXB mutation generated by CRISPR/Cas9. The organoids derived from the patient's cells exhibited na increase in organoid size parallel to increased cell proliferation compared to the control.

RETT SYNDROME

Rett Syndrome, a neurodevelopmental disorder, is most commonly caused by mutations occurring on the X chromosome, in the MECP2 gene that encodes the methyl-CpG-binding protein 2 (a protein that specifically binds to methylated DNA sequences, with its main function being transcriptional repression). Clinically, symptoms vary by sex; females experience motor and language impairments, while males suffer from severe congenital encephalopathy and typically have an early death (Ip et al., 2018). Brain organoids from patients with the syndrome were instrumental in identifying the role of over-regulated microRNAs (miR-199 and miR-214) in important signaling pathways for neurogenesis and neural differentiation. The organoids from patients with Rett syndrome exhibited an increased ventricular area with a decrease in ventricular wall thickness, as well as an increase in the number of neural progenitors due to exacerbated proliferation, leading to the increased cell density typically observed in patients with the syndrome (Mellios et al., 2018).

TIMOTHY SYNDROME

Another example concerns Timothy syndrome (TS). It is a neurodevelopmental disorder characterized mainly by the presence of abnormal inhibitory neurons. The

syndrome is caused by a mutation in the CACNA1C gene, which encodes proteins that compose calcium channels, especially the L-type, related to the migration of interneurons, regulating the frequency and termination of migration. Birey et al. (2017) were responsible for the first study based on a fused organoid system to investigate interactions between different brain regions (a subject discussed later). In this syndrome, there is a deficit in GABAergic interregional cell migration from the ventral to the dorsal prosencephalon. To reproduce this migration, organoids from different regions were generated from patient iPSCs and subsequently fused. Fluorescent marker tests revealed that inhibitory neurons had impaired tangential migration. When a ventral prosencephalon organoid was fused with a dorsal prosencephalon organoid, both obtained from TS patient cells, the number of hops required for migration increased, as the hop amplitude was significantly reduced compared to control organoids.

SCHIZOPHRENIA

2D models have been important for studying underlying mechanisms of schizophrenia (Brennand et al., 2011). These findings are being complemented with studies that used brain organoid models. For example, one study observed that in organoids derived from patients with a mutation in the DSC1 gene, there was a delay in mitosis. The gene in question is associated with schizophrenia and has one of its functions as regulating mitotic events (Ye et al., 2017).

AUTISM

Autism spectrum disorder (ASD) is a complex neurobiological developmental disorder commonly observed early in an individual's life. It is characterized mainly by neuropsychological and behavioral deficits such as cognitive impairment related to social communication difficulties and the presence of repetitive or stereotyped behaviors. For a better understanding of the main characteristics found in an autistic patient, see Mukherjee (2017). The most likely accepted hypothesis for the causality of autism is the interaction or conjunction of multiple factors, such as genetic, epigenetic, and environmental factors (Fett-Conte et al., 2016). It is worth noting that one of the possible genetic origins of ASD occurs through mutations in genes on the X chromosome, such as PTCHD1, responsible for approximately 1% of ASD cases (Noor et al., 2010). To some extent, these factors lead to an imbalance of neurotransmitters, as well as an abnormality related to neuronal connectivity and synaptogenesis, which, in turn, can lead to the dysfunction of neuronal pathways. These abnormal connections of functional brain regions may reflect

morphological abnormalities typically found in autistic children (Misic et al., 2014; Just et al., 2012), resulting in communication and learning impairments (Verly et al., 2013) (Schipul et al., 2012). Abnormalities regarding the size of the corpus callosum (He et al., 2010) are also described, as well as cortical thinning in the frontal, parietal, and occipital lobes (Zielinski et al., 2014) and a reduction in neural connectivity between these lobes (Tyszka et al., 2014). Irregular synaptic pruning mediated by microglia in autistic individuals is related to weak synaptic transmission and decreased functional brain connectivity, which in turn implies repetitive behavior and deficits in social interaction (Zhan et al., 2014). Beyond these factors, disturbances in the gut-brain communication axis (mainly promoted by the intestinal microbiota) (Sharon et al., 2016) may contribute to various aspects of the autistic brain.

Studies based on RNA sequencing indicate that a large portion of the cells found in organoids have a gene expression pattern corresponding to that of a human fetal brain (Ilieva et al., 2017). This gives a very promising character to research using organoid technology. A study conducted by Mariani et al. (2015) used brain organoids produced with iPSCs derived from patients with ASD, which, when compared with a control group, showed: less presence of neurites and synapses; differences related to cytoskeletal regulation; and deficiencies in potassium ion channel function. These organoids also showed an accelerated cell cycle and increased production of inhibitory GABAergic interneurons, characteristics that can be found in autistic patients. An analysis of the transcriptome showed an overexpression of the FOXG1 gene, which was positively correlated with excessive formation of inhibitory neurons. This result was validated by experiments that promoted the knockdown of the FOXG1 gene, which reduced GABA production to the level considered normal (Mariani et al., 2015).

Aberrations in the development in areas that concentrate a larger number of neural stem cells (NSCs) have a greater influence on the overall development process. This is the case for the subventricular zone (SVZ). Genes that regulate proliferation, migration, and cell differentiation in this area in question are found to be dysregulated in young autistic patients. Research also suggests that autistic patients have different DNA methylation profiles in genes related to these characteristics. Ilieva et al. (2017) observed an accumulation of methylation in the developing brain of autistic patients, suggesting abnormal epigenetic regulation (Ilieva et al., 2017). Studies of this type using organoids as a model can easily provide answers about epigenetic regulation since organoids recapitulate most of the epigenomic characteristics of fetal brain development.

With the aim of investigating the interaction between neurons and astrocytes and neuronal connectivity in individuals with autism, Russo et al. (2018) employed iPSC models

derived from non-syndromic ASD patients cultivated together with astrocytes in a 2D culture model (neuronal population grown on top of the astrocyte population). The results were intriguing as the ASD-derived cell culture exhibited disease characteristics, such as a decrease in glutamatergic neurotransmitter release, as well as alterations in the expression of genes related to synaptic formation. Consequently, these factors altered the spontaneous firing rate. Co-culturing healthy neurons with ASD-derived astrocytes revealed the glial cells' interference in neuronal development (synaptogenesis and neuronal morphology), resulting in neurons exhibiting ASD-related cellular characteristics. Conversely, when the co-culture combined healthy astrocytes with neurons derived from ASD, the "normal" phenotypes related to synaptogenesis and neuronal morphology were restored. IL-6 secretion by glial cells was identified as a possible cause of the phenotypes, confirmed by the cytokine's levels being blocked. This influence had been previously suggested in other research, and this study confirms the relationship (Russo et al., 2018). This work brings promising results to autism research using iPSC technology. Brain organoids can be used as models to further expand these findings and aid in the development of future therapeutic strategies, as 3D models allow for the recreation of a more complex cellular environment (Dezonne et al., 2017).

ORGANOIDS AS MODELS OF ALZHEIMER'S AND OTHER NEURODEGENERATIVE DISORDERS

Neurodegenerative diseases (NDs) are responsible for the progressive loss of cognitive and/or motor function in patients, with these symptoms often associated with the progressive and irreversible death of neurons leading to the loss of brain functions. Precursor mutations and common risk alleles associated with the development risk overlap in different neurodegenerative disorders. Additionally, some syndromes may have overlapping clinical manifestations. For example, common cognitive deficits in Alzheimer's disease (AD) are also present in vascular dementia and Lewy body dementia (LBD). Another example is motor system impairment, common to Parkinson's disease (PD), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), and spinocerebellar ataxias (SCAs). Moreover, aging is a common risk factor for some of these diseases. Hence, there is a direct relationship between increased life expectancy and the increased prevalence of these diseases that develop later in life (Prince et al., 2013). Despite the variety of clinical manifestations, neurodegenerative diseases share similar mechanisms. One characteristic is the regional aggregation of cytosolic or nuclear proteins, such as beta-amyloid plaques (Aβ) in AD, polyglutamine protein aggregates in HD (and

other similar diseases linked to CAG nucleotide repeat - glutamine codon), and alpha-synuclein aggregates in synucleinopathies such as PD (Taylor et al., 2002).

As discussed earlier regarding NDDs, complex genotype-phenotype relationships are also found in NDs. Multiple genes give rise to similar clinical entities in different diseases. When identified, these genes have helped elucidate the pathways of diseases such as AD and PD, suggesting new therapeutic approaches (Hardy & Orr, 2006). On the other hand, a neurodegenerative process evoked by a mutation can cause a spectrum of clinical signs (DeJesus-Hernandez et al., 2012; Renton et al., 2011; Schöls et al., 2015; Zimprich et al., 2004). Moreover, disorders with overlapping pathologies tend to share genetic risk loci (Zimprich et al., 2004; Scholz et al., 2009). An example is the shared genes between LBD and AD, among which is the apolipoprotein E (APOE) gene, considered the primary risk gene for AD (Huang & Mahley, 2014; Guerreiro et al., 2018). Carrying an APOEε4 polymorphic allele increases the patient's risk (3 to 4 times) of developing late-onset AD; possessing two alleles further raises this risk (9 to 15 times). Additionally, APOEε4 is associated with earlier onset of AD. Studies indicate that APOE4 is directly related to factors that impair normal brain function, such as beta-amyloid accumulation, and neurodegenerative processes mediated by tau and alpha-synuclein. Furthermore, this gene is linked to neuroinflammation (due to its significant role in regulating innate immune response), synaptic degeneration, glucose metabolism dysfunction, and cerebrovascular dysfunction. For a more in-depth study of the implications of the APOE gene in AD and other neurodegenerative diseases, see (Yamazaki et al., 2019).

Some tauopathies also share genetic risk (Höglinger et al., 2011). Tauopathies are a group of clinically heterogeneous NDs whose main pathological characteristic is the formation of aggregates of tau protein forming neurofibrillary tangles within the cell. Also known as "microtubule-associated tau protein," this protein is related to microtubule stability. Among the best-known tauopathies are AD, progressive supranuclear palsy, and corticobasal syndrome (Orr et al., 2017). Even in cases where clinically different syndromes are promoted by variants of the same gene, there can still be an overlap of genetic risks. As previously mentioned, many neurological diseases may share common mechanisms. However, generalization is not possible because there are still unique aspects of genetic risk that promote different mechanisms for some NDs.

Despite the extensive history, we still do not have complete clarification on the pathogenesis of AD, but known markers can aid in the understanding of its pathogenesis (Forestier et al., 2015; Liu et al., 2015). Macroscopically, it is possible to observe atrophy of the hippocampus and cerebral cortex, which in AD is related to increasing age (DeTure &

Dickson, 2019). Microscopically, the formation of amyloid plaques and neurofibrillary tangles can be observed. Both deposits lead to extensive neuronal loss, while they are essential markers for AD (Forestier et al., 2015; Liu et al., 2015; Stancu et al., 2014; Perl, 2010; DeTure & Dickson, 2019).

Specifically, AD is characterized by the deposition of beta-amyloid peptides (Aβ) in the extracellular environment of neurons and the formation of neurofibrillary tangles (NFTs) resulting from intracellular accumulation of hyperphosphorylated tau protein. The amyloid cascade hypothesis, formulated in 1992, postulates that these characteristics constitute the main pathological event linked to the clinical picture of the disease (Hardy & Higgins, 1992). The proteolytic cleavage of the beta-amyloid precursor protein (APP) by the action of two enzymes, beta-secretase 1 and gamma-secretase, is the event responsible for Aβ production (O'Brien & Wong, 2011). The accumulation of Aβ in the brain can lead, among other impairments, to the hyperphosphorylation of microtubule-associated tau protein and, consequently, the formation of neurofibrillary tangles (Niedowicz et al., 2011).

The cerebral organoid model is also promising in the field of ND modeling, being considered by many as an alternative to animal models. It is known that rodent models are not capable of reproducing the entirety of the pathophysiological processes of diseases such as PD and AD found in humans. We can take, for example, some points mentioned by Dawson et al. (2018), namely: inherent differences related to methods of generating animal models such as the artificial overexpression of proteins, which, when circumvented, generate models that demonstrate mild disease phenotypes; the reduced "lifespan" of rodents, which may ultimately contribute to the incomplete development of pathological neurodegeneration phenotypes; differences in the development and function of rodent and human brains, leading to errors when comparing or interpreting results of models and humans; the genetic differences between both (Dawson et al., 2018). However, respecting the limitations of organoids (mainly those related to in vitro neuronal immaturity), they have been pointed out as tools to investigate the early stages of diseases and their most common processes. For example, Raja et al. (2016) generated organoids from cells of AD patients, and the models presented the two biomarkers of the disease (Aβ deposition and tau protein hyperphosphorylation). These results were encouraging because 2D culture models were unable to mimic the extracellular environment and its necessary complexity to observe these biomarkers (Wang, 2018). In addition, a significant reduction of these biomarkers in organoids was demonstrated after treatment with β and γ secretase inhibitors (Raja et al., 2016).

Another group of researchers succeeded in developing organoids that showed progressive accumulation of Aβ, forming structures similar to plaques, preceding the appearance of phosphorylated Tau and neurofibrillary tangles (Gonzalez et al., 2018). Recently, aiming to confirm the hypothesis that anterior brain organoids formed by iPSCs from AD patients can accurately recapitulate the extracellular microenvironment present during neural degeneration, Yan et al. (2018b) generated prosencephalic cortical organoids with iPSCs with a mutation in the PSEN1 gene (responsible for the expression of presenilin-1, which plays an important role in Aβ generation). In the organoids, high levels of Aβ concentration, inflammatory phenotypes related to AD (elevated gene expression of IL-6 and TNF-α), increased expression of matrix remodeling protein (resulting in synaptic dysfunction and loss of neurons during pathology) were found. Treatment and responses to DAPT (a γ-secretase inhibitor), heparin, and heparinase were also evaluated. The results of drug treatment were encouraging, as they showed that treatment with DAPT inhibited endogenous Aβ aggregation, leading to a decrease in cytotoxicity, while heparin and heparinase III were able to reduce Aβ concentrations, probably by hindering the binding of Aβ peptides to neurons (Yan et al., 2018).

Examples of the use of organoids combined with genome editing by CRISPR/Cas9 can also be mentioned. Organoids with mutations in the APOE gene generated by genetic editing showed an increase in biomarkers for AD. Subsequently, the pathology was attenuated by further editing, converting APOE4 into APOE3 (Lin et al., 2018). As cerebral organoids can be exposed to drugs, there is hope that these models will be a promising platform for the discovery of drugs for the treatment of neurodegenerative diseases.

Despite the above, it is not yet clear how effective organoids can be for modeling neurodegenerative diseases. As we will see later, new techniques and improvements in the models promise to elevate cerebral organoids to a level of protagonist for modeling even late-onset diseases such as dementias, PD, and HD (Wang, 2018). The use of cells derived from PD patients is promising, as studies using specific mesencephalic type organoids derived from iPSCs to investigate the pathophysiology and genetic basis of the disease (Kim et al., 2019; Smits et al., 2019).

MODELING OF PRENATAL AND PERINATAL EXPOSURE TO DRUGS

Another applicability of cerebral organoids that may or may not be associated with the investigation of NDs is the prenatal exposure to drugs or substances, whether legal or illegal, to understand how and to what extent these substances can impact neurogenesis. Studies have exposed organoids to different types of substances (such as cocaine, ethanol,

nicotine, for example) to analyze the consequences of this interaction (Lee et al., 2016; Zhu et al., 2017; Wang et al., 2018). Another approach for organoids besides exposure to drugs of abuse is the investigation of neurotoxic effects of various substances such as valproic acid or other environmental chemicals as promoters of neural teratogenic effects (Schwartz et al., 2015; Belair et al., 2018). Regarding cocaine, Lee and colleagues (2016) demonstrated the inhibition of neocortical NPCs proliferation, premature neuronal differentiation, and consequently, disruption of neural tissue development after exposing neocortical organoids to the substance. The suggestion is that these effects are mediated by the production of reactive oxygen species, which could be a future therapeutic target.

MODELING OF BRAIN CANCER

The nature of the carcinogenic processes makes them difficult to cure, and na effective treatment could be based on a model system that incorporates the patient's genetic characteristics and reflects the complex 3D environment of tumor tissues. Cerebral organoids could represent this system in the investigation of the progressive nature of cancer as well as its resistances, serving as a good model for drug testing. Organoids derived from patients can bring more personalized approaches. Among several examples, glioblastoma (the most common and aggressive type of malignant brain tumor that affects humans) has been the most studied. This type of model, called brain tumor organoids or simply tumor organoids, was produced from patient tumor cells (obtained directly from neural tissue with this type of tumor) and grafted into previously prepared brain organoids from human embryonic stem cells (hESCs). The 3D model obtained is considered superior to the 2D environment (more commonly used) as it better mimics the microenvironment and progression of cancer, as well as presenting resistance to chemotherapeutic treatments similar to the live tumor in patients (Linkous et al., 2019). Chemotherapeutic effects are another focus of study, and the effects of "anticancer" drugs can be tested on organoids (Plummer et al., 2019). Another method of generating tumor organoids is through the use of CRISPR/Cas9 (Bian et al., 2018).

FUSION OF ORGANOIDS: APPROACHES TO MODELING COMPLEX FEATURES

Taking into account the barriers to be overcome by organoid technology, mainly its limitations in reproducing some complex characteristics of the human brain such as neuronal migration and synaptic connectivity, the organoid fusion technique brings new horizons and approaches to the model. As we have seen, self-organization occurs intrinsically in some protocols for generating cerebral organoids, but in many cases, this

self-organization does not lead to a great cerebral complexity, mainly due to interrupted neuronal migration and inter-regional connection deficiency. Models that mimic specific regions of the brain have greater reproducibility (Birey et al., 2017). However, separately created regional models do not provide the opportunity to recapitulate processes such as connectivity between regions and those related to cell migration (Lodato & Arlotta, 2015). These deficits lead to a gap in the study of cortical circuits. The fusion of organoids from pre-specified brain regions was presented as a solution (Birey et al., 2017). A study, previously presented in this review, conducted by Birey and colleagues (2017) is a good example of organoid fusion to identify deficits in interneuronal migration (GABAergic, in this case) in NDs (Timothy syndrome, in this case). The technique used is based on co-culture that promotes subsequent fusion of organoids from distinct brain structures, in this case, between the anterior (excitatory) and ventral (inhibitory) brain. The fusion occurs simply: when incorporated into matrigel, the organoids are placed as close as possible, and the fusion process takes place in approximately one week (Bagley et al., 2017). Thus, this technique represents a viable method for modeling phenotypic defects of disorders, such as migratory routes and cortical circuit formations.

NEUROVASCULAR MODELS

The process of vascularization of cerebral organoids is the next step in research aiming to reproduce (or part of it) the human brain in vitro. As we have seen, since the models do not have blood vessels, their growth and longevity are quite limited. Until 2018, it was not clear whether the co-culture of organoids with endothelial cells could lead to the formation of vessels, and even if the presence of these vessels would have any implication in the development (self-assembly process) of the model (Pham et al., 2018). To answer this question, a protocol for the vascularization of cerebral organoids derived from iPSCs with endothelial cells from the same patient was developed. Vascularization was verified, showing to be viable, as it did not interfere with the normal development of the in vitro organoid (self-assembly and cytoarchitecture). The authors then transplanted the vascularized organoids generated in vitro into rodents, as well as non-vascularized control organoids. As a result, the vascularized organoids had a longer survival compared to the controls. In this sense, the authors presented the viability of vascularization, especially temporally (Pham et al., 2018).

We have seen in this review that in vitro models of the BBB are indispensable tools for studying the development and drug transport to the CNS. The production of BBB organoids developed to date, largely through co-culture in low-adhesion environments of

organoids and endothelial cells, shows that generated pericytes and astrocytes mimic the main properties of the barrier, such as the presence of tight and adherent junctions, P-glycoprotein (P-gp), and active molecule transport (Bergmann et al., 2018; Oliveira et al., 2019). Bergmann and colleagues (2018) succeeded in creating BBB organoids as a reliable model for in vitro drug screening.

Nzou et al. (2018) generated an organoid model equipped with a neurovascular unit, which better mimics that found in humans, containing neurovascular cells, such as human brain microvascular endothelial cells, human pericytes, and neural cells, such as human astrocytes, human microglia, human oligodendrocytes, and human neurons, in a ratio of 30%, 15%, 15%, 5%, 15%, and 20%, respectively. In this model, Nzou and colleagues first produced a cerebral organoid containing human astrocytes, human microglia, human oligodendrocytes, and human neurons. Human brain microvascular endothelial cells and human pericytes were then added to coat the neuro-glial organoid, thus generating an organoid model with endothelial cells surrounding the brain parenchymal cells. Once formed, the resulting organoid was evaluated for BBB permeability properties, such as the expression of tight and adherent junction proteins and transporter proteins. Additionally, assays were conducted to investigate BBB permeability to IgG in untreated organoids and in others pretreated with histamine (a known agent for transiently opening the BBB). As a result, the analysis showed that the barrier organoids were more selective to antibodies compared to the non-barrier organoids, while histamine-treated barrier organoids showed increased permeability compared to untreated barrier organoids. Another finding was related to protection against neurotoxic components such as mercury, where the barrier organoids exhibited less cellular depletion compared to the non-barrier organoids.

Cakir and colleagues (2019) developed a fully in vitro vascularized cortical organoid model, one of the most recent works on the subject. The team produced cortical organoids from induced hESCs expressing an ectopically variant of human ETS transcription factor (ETV2). The expression of this transcription factor played an important role in reprogramming human fibroblasts into endothelial cells. The authors further demonstrated that overexpression of ETV2 induced VEGF-independent differentiation. Following this gene expression, some markers of vasculogenesis were observed, such as genes related to cell adhesion. Thus, vascularization of the organoid was achieved, and the presence of vascular structures led to improved functional maturation and survival (reducing apoptosis levels) of the organoid cells. The model also exhibited BBB-like features, including increased expression of tight junctions, transporters, such as the glucose transporter, and the presence of pericytes.

Despite these advances, vascularized organoid models still do not possess a fully functional vascular network in terms of oxygen and nutrient supply (Oliveira et al., 2019). As mentioned earlier, the in vivo grafting of human brain organoids into animals, especially in mice, has been developed as na alternative to achieve vascularized organoids for in vitro experiments. This approach (performed between 30 and 50 days after organoid creation) leads to the progressive vascularization of the model through the invasion of the host vasculature, providing blood flow; cell viability is higher compared to in vitro organoids, as well as showing greater maturation, progressive differentiation of neuronal and glial cells (including registered microglial interactions), and axonal growth, suggesting functional graft-host integration (as recorded by optogenetics) (Mansour et al., 2018).

FUTURE PERSPECTIVES

Based on all that has been discussed above regarding the evolution of organoid technology, it is remarkable how rapidly it has evolved in recent years, providing us with a wide variety of in vitro 3D models of the human brain for various applications, showing that the technology is a great ally in medicine. There is still much to be done when aiming for in vitro human neurodevelopmental modeling. Brain organoids still lack some developmental clues and standardization that would allow for an in vivo-like organization not present in vitro, such as the lack of supporting tissue (Lancaster et al., 2013; Lancaster et al., 2014b; Kelava & Lancaster, 2016a).

Variations within the same batch ("batch effect") are one of the first challenges to be overcome for the reproducibility of organoid experiments. Since the cells typically used for organoid generation (iPSCs) have a certain degree of variability among themselves, a possible solution would be the use of selected lineages and standardized iPSC generation methods in organoid generation protocols, which would benefit the entire scientific community in future research (Kelava and Lancaster, 2016a). Still within this theme, bioengineering techniques, such as the development of scaffolds and extracellular matrices, may be useful in prolonging the viability and development of organoids, providing tools that introduce complexity to the models, influence tissue architecture, and maintain organoid self-organization (Yin et al., 2016).

Another important point is the enhancement of protocols aiming at amplifying the cellular diversity in organoids, crucial for studying the complex interactions that occur in the brain, such as neuron-glia interactions. As seen in this review, the presence of glial cells within the organoids is essential, as they are constituents of the nervous system and play important roles: astrocytes, oligodendrocytes, and microglia act in synaptogenesis, circuit

maturation, myelination, and homeostasis, besides being involved in stages of neurological diseases. Difficulties still exist in the spontaneous generation of these glial cells, but the research conducted by Ormel et al. (2018), where microglia were generated within organoids, symbolizes the window of opportunity for advancement in this area. An alternative to modifying organoid generation protocols is the addition of previously differentiated glial cells from iPSCs to the model (Muffat et al., 2016).

However, we must remember that there are pros and cons regarding the degree of structural complexity of brain organoids, as the high degree of cell diversity, which on one hand can reproduce with some fidelity the complex networks of intercellular communication, can also add analytical difficulties when the research objective is to test hypotheses related to the contribution of specific cell types to specific intercellular processes. Supplementing results observed from a 3D model, combined with results obtained through 2D culture (which has a more homogeneous structure and environment) of specific cells related to specific processes will facilitate the initial understanding of the mechanisms associated with neurological disorders, allowing for the comparison of data regarding cellular interaction and intrinsic molecular mechanisms.

When combined with other approaches in cellular and molecular biology, such as whole-genome analysis using single-cell sequencing, organoid generation techniques can open doors for us to investigate the widespread cellular diversity in various stages of CNS development, including its later stages or even during aging, as well as to investigate the etiology of neurological diseases from their molecular mechanisms (Camp et al., 2015; Quadrato et al., 2017). The genetics related to the etiology of neurological diseases is quite heterogeneous, for this reason, techniques that define more efficiently the different impacts of genetic variants on neurodevelopment are indispensable. Current genetic editing technology is an ally since it has the ability to modify a variety of genes with some safety, allowing for gene silencing and induction of others, mainly in iPSCs (Ilieva et al., 2017). New genetic engineering techniques, such as the CRISPR/Cas9 system, have provided us with genome manipulation and expanded horizons in in vitro research (Waddington et al., 2016). These techniques have enabled mutations to be induced or corrected in wild-type or patient-derived cells (Trujillo & Muotri, 2018b; Adams et al., 2019), and organoids, in turn, have shown adaptability to these techniques (Yin et al., 2016). The process of human brain development can also be influenced by epigenetic mechanisms: a perspective not addressed in this review. In this sense, organoids can be used as a model for evaluating the epigenomic remodeling that occurs during in vivo neurodevelopment (Luo et al., 2016).

As we have seen in the previous sections, in addition to the supply of oxygen and other nutrients, vascularization is intimately related to brain maturation due to its role in the cellular differentiation of NPCs. Modifications in the methods of organoid production and culture using bioengineering approaches are essential tools in this regard. The examples mentioned above demonstrate that the combination of endothelial cells in the organoid culture to promote in vivo vascularization has been a significant advancement in protocols for generating brain organoids. Research on vascular phenotypes in neurodevelopmental and neurodegenerative disorders also represents an indispensable area for future studies, as cerebral vasculature is involved in multiple pathogenic processes that compromise cognition during these pathologies. Furthermore, by analyzing the techniques of organoid implantation in animal tissue, it is possible to investigate vascularization processes for tissue repair understanding, improvement of transplant techniques, understanding of carcinogenic mechanisms, among others. Future review research should focus on these topics and integrate the available data in the literature for better discussion.

Due to the fact that brain organoids maintain the main characteristics of a developing brain with genetic information identical to that of patients (Sachs et al., 2018; Yan et al., 2018), two other fields of great interest are personalized medicine and pharmacology. These fields can benefit from the production of personalized organoids, i.e., models derived from patients, which aim to faithfully reproduce an individual's cellular and molecular mechanisms associated with physiological processes, pathogenesis, and therapeutic responses. Such an approach is essential for investigating future prognostic methods as well as personalized treatments.

In general, future bioengineering approaches are still needed to favor more advanced methods for generating brain organoids. Due to the enormous potential of this technology, biobanks consisting of a collection of model organoids representing different CNS-related pathologies would greatly facilitate research and, consequently, a better understanding of brain disorders, as well as serving as a basis for therapeutic approaches, as mentioned earlier. We can draw a comparison with biobanks of tumor organoid types, which, with their established collections, demonstrate the benefits of implementing organoid biobanks; among these benefits, the development of therapeutic tests for precision therapies stands out. If we compare the difficulty of obtaining neural tissue as opposed to tumor tissue, brain organoid biobanks would prove to be a valuable resource (Sachs et al., 2018; Yan et al., 2018).

The recognized capacity for self-organization, differentiation, and generation of brain regions and structures with a certain degree of complexity makes organoids ideal in vitro

models and, to some extent, necessary for the study of CNS development. They have shown great promise for the field of modeling neurodevelopmental disorders. Once technical and ethical hurdles are overcome, future organoids will serve as reliable models because they possess a microenvironment and cellular diversity closer to what is observed in vivo, strongly impacting disease modeling and drug screening tests. In this review, we have provided a brief history of the technology and why it is revolutionizing the way we study the human brain. It is important to remember that it is still not a perfect model, as we still face some limitations and undoubtedly others will emerge in the future.

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