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Challenges of Organoid Research

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Abstract

Organoids are 3D cell culture systems derived from human pluripotent stem cells that contain tissue resident cell types and reflect features of early tissue organization. Neural organoids are a particularly innovative scientific advance given the lack of accessibility of developing human brain tissue and intractability of neurological diseases. Neural organoids have become an invaluable approach to model features of human brain development that are not well reflected in animal models. Organoids also hold promise for the study of atypical cellular, molecular, and genetic features that underscore neurological diseases. Additionally, organoids may provide a platform for testing therapeutics in human cells and are a potential source for cell replacement approaches to brain injury or disease. Despite the promising features of organoids, their broad utility is tempered by a variety of limitations yet to be overcome, including lack of high-fidelity cell types, limited maturation, atypical physiology, and lack of arealization, features that may limit their reliability for certain applications.

Contents

INTRODUCTION

The discovery and isolation of human pluripotent stem cells (PSCs) have expanded the potential to study human biology as never before. The capacity to evaluate how human cells grow and behave in a dish has the potential to revolutionize our approach to both basic scientific inquiry and the treatment of human disease. PSCs can be derived from the inner cell mass of a human blastocyst or reprogrammed from human somatic cells using a combination of transcription factors until pluripotent; these are referred to as human embryonic stem cells and induced pluripotent stem cells (iPSCs), respectively. PSCs can then be coaxed down a pathway of differentiation utilizing endogenous developmental cues, in either adherent or aggregate cultures, to study cell types of a particular lineage. Recent innovations have advanced the differentiation of stem cells into 3D aggregates. Organoids are self-organizing aggregate cultures derived from human stem cells that generate cell types and organizational properties reflective of a developing organ. Neural organoids can be derived utilizing undirected whole-brain protocols (Camp et al. 2015, Lancaster et al. 2013, Quadrato et al. 2017) where stem cells differentiate spontaneously to resemble a variety of neural structures with variable regional identities. Alternatively, regionally directed protocols utilize small molecules or recombinant proteins that co-opt developmental signaling mechanisms to promote specific regional identity. A variety of brain organoid protocols have been established to generate different regional identities, including those directing dorsal forebrain (Eiraku et al. 2008, Kadoshima et al. 2013, Mariani et al. 2012), ventral forebrain (Bagley et al. 2017, Birey et al. 2017, Xiang et al. 2017), hypothalamus (Qian et al. 2016), hippocampus, thalamus (Xiang et al. 2019), retina (Eiraku et al. 2011), choroid plexus (Pellegrini et al. 2020b), midbrain (Jo et al. 2016), and cerebellum (Muguruma et al. 2015).

Neural organoids offer the potential to study human-specific molecular, cellular, and systems-level processes reflective of the human brain that may give insight into neurological function in health and disease. Currently, many scientific groups are evaluating the strengths and limitations of organoid culture systems by exploring how organoid-derived cells reflect endogenous human brain development from transcriptional, proteomic, morphological, electrophysiological, structural, and functional perspectives. To date, organoid studies have mostly focused on validating and improving organoid models, although attempts to extrapolate features of relevance to complex disease processes have also begun. Increasingly, the future of organoids will include studies of disease mechanisms, personalized medicine, drug screening, regenerative approaches, and cellular transplantation, working toward the treatment of currently intractable neurological diseases. Although organoids are becoming increasingly more complex and manifest a variety of robust features, they remain rudimentary and inherently artificial compared to the intact brain.

FEATURES OF HUMAN BRAIN DEVELOPMENT WELL DEFINED IN ORGANOID MODELS

With the innovation of organoid cultures, it has become possible to probe how human cells develop progressively over time in a self-organizing, neural-like aggregate. It is clear that certain features of the organoid model system are remarkably similar to normal brain development. Organoids can be efficiently differentiated toward an increasingly diverse series of particular regional identities where they self-organize and generate, over time, the broad cell types that are normally resident in the native neural structures (Eiraku et al. 2008, Kadoshima et al. 2013, Lancaster et al. 2013, Mariani et al. 2012, Qian et al. 2016, Quadrato et al. 2017). For example, cortical organoids are first composed of early multipotent progenitor cells: neuroepithelial (NE) and radial glial (RG) cells (Benito-Kwiecinski et al. 2021, Eze et al. 2020, Subramanian et al. 2017). The progenitor cells robustly proliferate and utilize division programs that are remarkably similar to endogenous cortical development at a transcriptional level (Bhaduri et al. 2020b, Pollen et al. 2019, Velasco et al. 2019). Directed cortical protocols, using a variety of different human stem cell lines, reproducibly and consistently produce similar proportions of cortical cell types, including RG cells and their progeny, excitatory neurons, and later, astrocytes (Shah et al. 2020, Velasco et al. 2019). Organoid-derived cells also follow an endogenous differentiation trajectory where NE-like cells give rise to RG and subsequently other progenitor subtypes (Benito-Kwiecinski et al. 2021, Eze et al. 2020, Subramanian et al. 2017), including intermediate progenitor cells (IPCs), before differentiating into astrocytes. The birth order of excitatory neuron subtypes also follows the order in which they arise during development, with cells expressing markers of deep-layer identity emerging before those expressing more superficial-layer genes (Qian et al. 2016). Gliogenesis follows neurogenesis, and astrocytes are generated and mature at a timescale resembling their in vivo counterparts (Andrews et al. 2021, Sloan et al. 2017).

In addition to demonstrating robust division and differentiation programs and a neurogenic to gliogenic transition, organoids also reflect features of endogenous tissue organization (**Figure 1**). The cellular composition of organoids includes progenitor, neuronal, and glial cell types that are similar to those observed in the developing brain, and they self-organize into cytoarchitectural structures that also resemble early tissue organization. In cortical organoids, a simplified radial scaffold forms with NE and RG cells forming a stratified, polarized structure surrounding a lumen. Instead of a laminar sheet, however, the RG form ventricular zone–like rosettes, and as IPCs and neurons differentiate, they move away from the progenitor rosettes, creating a subventricular zone–like region. For several months, organoids thus retain a progenitor zone structure that resembles the developing human cortex.

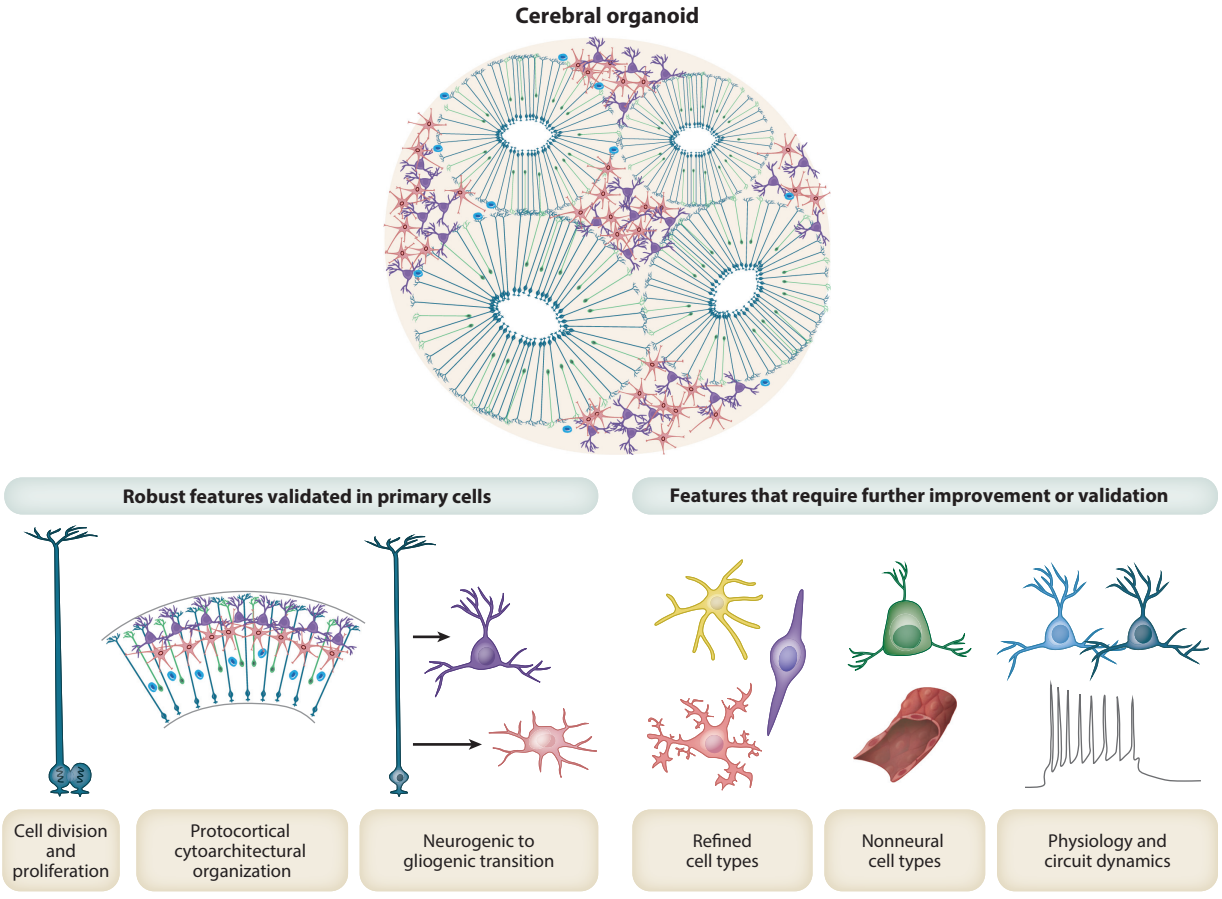

Figure 1

Features of cerebral organoids. Organoids robustly replicate proliferation and division programs and spontaneously organize into a rudimentary radial scaffold. Neural progenitors generate neurons and later differentiate into astroglial cells. However, cerebral organoids still require validation for certain applications and continued refinement to better replicate neurodevelopmental features. Currently, organoids are impaired in gene expression programs that define robust cell type specification. They lack specific nonneural cell types that are vital for brain health and function, including vascular endothelial and mural cells and immune microglia. Organoids also may not replicate complex physiological dynamics that define intracortical and subcortical circuitry. Figure adapted from Bhaduri et al. (2020a).

AREAS OF ACTIVE STUDY IN NEURAL ORGANOIDS

Cell Type Specification

Although the cell types present in organoids reflect broad expression profiles of human neural cell types, their specification programs are impaired. The level of gene expression and the refined gene networks observed in endogenous development are not perfectly duplicated in organoid-derived cells (Bhaduri et al. 2020b, Velasco et al. 2019). To be clear, many cell type–specific gene expression patterns, as well as modules of gene expression, are reproduced in organoid cells (Bhaduri et al. 2020b, Camp et al. 2015, Pollen et al. 2019, Sloan et al. 2017, Velasco et al. 2019), but others are not. Some type-defining marker genes of specific cell subtypes, such as *PTPRZ1*, which is a marker of outer radial glia cells (oRGs), are nearly absent in oRG-like cells that nonetheless express

HOPX and other oRG markers. Organoid-derived excitatory neuron subtypes also have decreased expression of type-defining marker genes, such as significantly decreased levels of the upper layer marker, *SATB2*, when compared to primary cortical cells. Data suggest that the imperfect gene expression profiles do not resolve over time. The impaired cell type specification can be observed across publicly available single-cell RNA sequencing (scRNAseq) data sets from multiple different organoid protocols, indicating pervasive impairment across neural organoid models (Amiri et al. 2018, Camp et al. 2015, Giandomenico et al. 2019, Marton et al. 2019, Pollen et al. 2019, Velasco et al. 2019). However, the impact of the gene expression differences on the study of human brain development and disease is unclear.

Cell Stress

scRNAseq technology and the availability of transcriptomic data sets have allowed the detection of gene expression patterns in organoids and identification of potential differences from primary tissue. Organoids have increased expression of cellular stress marker genes, indicative of metabolic stress, endoplasmic reticulum stress/unfolded protein response (UPR), and electron transport dysfunction (Amiri et al. 2018, Bhaduri et al. 2020b, Pollen et al. 2019, Xiang et al. 2019). Cellular metabolism is a highly and dynamically regulated process that is not best evaluated through gene expression profiling, and improved approaches to understand metabolomic and proteomic profiling are needed. Importantly, unlike cells in primary samples, which demonstrate different levels, timing, and specificity of expression of metabolic genes (Gordon et al. 2021), organoids ectopically express stress-associated genes chronically in all cell types. As chronic cellular stress is not a feature of normal neural development, it may interfere with developmental programs, including fate specification, maturation, morphology, or connectivity. There are many potential sources of metabolic dysregulation, including hyper- or hypo-oxygenation, lack of requisite nutrients, nonphysiological quantities of sugars, or missing cell types/structures such as vasculature or cerebrospinal fluid (CSF) flow, that could increase stress in organoids.

Hypoxia and Necrosis

Although a general lack of physiological context is inherent to in vitro culture systems, creating a 3D living mass of tissue suspended in culture media poses additional challenges. Hypoxia and lack of perfusion lead to increasingly necrotic tissue within the interior of growing organoids over time. Organoids cultured for many months can grow up to 5 mm in diameter (Paşca et al. 2015); however, the increase in size compounds this problem. Slicing and growing organoids as slice cultures rather than as spheres can increase oxygen permeability and rescue much of the cell death that is otherwise present in the organoid interior (Giandomenico et al. 2019, Qian et al. 2020). An additional benefit of slicing organoids is the opportunity to select slices that have optimal laminar organization. As they grow, the flattened disc–like organoids tend to preserve laminar architecture longer than standard organoids, and they have been observed to extend axon bundles (Giandomenico et al. 2019, Qian et al. 2020). Despite a potentially hypoxic baseline environment, the impact of severe hypoxic injury, such as that associated with prematurity, has been successfully modeled using cortical spheroids (Paşca et al. 2019). Hypoxic organoids demonstrate an increase in UPR and, interestingly, a decrease in neurogenic IPCs.

Vasculature

Although strategies to decrease organoid thickness have generally improved cell health, the lack of vascularization presents a limitation for nutrient delivery, metabolite elimination, endothelial

cell signaling, and the study of blood–brain barrier formation. Specific efforts to include vascular cells within neural organoids have focused on exogenous addition of endothelial-like cells from different tissue sources. Since neural organoids are derived from PSCs guided down a path of ectodermal neural induction while vascular cells are derived from mesoderm, it is technically challenging to differentiate these cell types in parallel in the same organoid. An alternative strategy is to ectopically graft vascular cells, particularly endothelial cells, into neural organoid models. One approach has been to transplant human umbilical vein endothelial cells (Shi et al. 2020), and another approach has been to drive expression of endothelial genes such as *ETV2* in a subset of stem cells (Cakir et al. 2019) to promote induction of vasculature-like channels within the organoids. The presence of endothelial cells and vascular-like conduits in neural organoids decreases cell death and improves neurogenesis.

Current vascularization methods often produce endothelial cell networks surrounding the organoid without vascular-like innervation of the organoid center, which is most in need of perfusion. While these conduits excitingly create space for passive media flow through exterior parts of the organoid, active pumping and oxygen/nutrient exchange, akin to blood flow, are absent. Additionally, most attempts to vascularize organoids have focused on endothelial cells without evaluating the contribution of other vascular cell types, such as mural cells that normally surround endothelial cells, or astrocytes that contact endothelial cells and contribute to blood–brain barrier formation. However, a recent study addressed the specific contribution of pericytes, a type of mural cell, by transplanting pericyte-like cells differentiated from neural crest stem cells into organoids (Wang et al. 2021). Physical interactions between pericytes and organoid-derived astrocytes were observed, and basement membranes formed, suggesting a neurovascular-like unit (Wang et al. 2021). In response to viral infection, organoids containing pericytes had a stronger astrocytic inflammatory response than standard organoids. The developing vasculature also normally provides a germinal niche for neural progenitor cells, particularly IPCs (Javaherian & Kriegstein 2009, Stubbs et al. 2009), and the impact of the absence of this niche on neurogenesis in organoids has not been fully evaluated.

Glial Cells

Although glial cells have historically been considered neuronal support cells, they are increasingly appreciated for a range of vital functions that contribute to a healthy nervous system, as well as their role in neurological disease. Gliogenesis usually follows neurogenesis in organoids, as it does in primary developing tissue. While astrocytes are often produced, oligodendrocyte precursor cells (OPCs) and particularly mature myelinating oligodendrocytes (OLGs) are rarely observed. Protocols have been modified to promote oligodendrogenesis within directed cortical spheroids, successfully promoting differentiation of OLGs that have the capacity to interact with neurons and even myelinate axons (Marton et al. 2019). Primary OPCs and OLGs have a distinct temporal trajectory of gene expression, behavior, and function. Organoid-derived OLGs express an altered range of primary OLG genes, similar in degree to what has been observed in other neural populations (Bhaduri et al. 2020b, Pollen et al. 2019, Velasco et al. 2019). However, organoids may nonetheless provide molecular insights into aspects of normal OLG function as well as their dysfunction in demyelinating diseases despite the differences in gene expression.

Microglia, the immune cells of the central nervous system (CNS), serve to identify and destroy invading pathogens and help monitor nervous system health. They engulf unhealthy and unused synapses and are involved in the refinement of circuits. Dysfunction of these microglial processes is related to a variety of disease states. Microglia are nonneural, mesoderm-derived immune cells that migrate into the CNS during gestation. They rarely arise in directed, regionally restricted

organoid differentiation protocols. However, microglia-like cells can spontaneously differentiate in undirected cerebral organoid protocols that contain mesodermal lineage cells (Ormel et al. 2018). Cerebral organoid–derived microglia express marker genes and have similar morphology to cultured primary microglia. Additionally, they can respond to inflammatory signals by secreting cytokines, and they reside in close proximity to synapses with which they may interact. Differentiation of iPSCs to microglia has been achieved in adherent culture (Muffat et al. 2016). iPSC-derived microglia have been transplanted into mouse brains where they demonstrate immune responsiveness and gene expression profiles similar to endogenous microglia (Lin et al. 2018, Svoboda et al. 2019). iPSC-derived microglia can also be transplanted into organoids. Interactions between microglia and neurons, including engulfment of synapses and modeling of inflammation, infection, and disease, could potentially be studied in organoid models.

Cytoarchitecture

Although the early apical-basal organization of NE and RG cells resembles the ventricular and subventricular proliferative zones of the developing brain, they typically form multiple circular rosettes within a single organoid rather than the continuous radially oriented layers found in vivo. As IPCs and, subsequently, neurons differentiate from RG cells, organoid cytoarchitecture loses its resemblance to the increasingly complex developing human brain (Bhaduri et al. 2020b). During neurogenesis, which occurs midgestation, the size of the human cortex expands dramatically. RG subtype composition in the expanded outer subventricular zone, as well as the diversity of excitatory neuron populations in the cortical plate (CP), increases as the developing cortex becomes more complex and intricately organized. In contrast, organoids reach a maximum size of a few millimeters around peak neurogenesis (Paşca et al. 2015) with little additional expansion. While organoid RG cells initially display apical-basal orientation, the lack of a clear basal lamina and the numerous progenitor rosettes within each organoid help to create multiple radial structures with concentric laminar organization. As excitatory neurons differentiate, they migrate away from the multiple VZ-like rosettes, resulting in neurons derived from different rosettes being located adjacent to one another. Ultimately, in most organoid protocols, many different CP-like regions are formed without consistent lamination (Bhaduri et al. 2020b, Lancaster et al. 2013, Mariani et al. 2012, Velasco et al. 2019). Precise localization of specific neuronal and nonneuronal cell types in appropriate proportions is a hallmark of the complexity of the human cortex and is key for establishing both intracortical and subcortical projections that underlie function. The ability of cerebral organoids to produce the major cell types of the developing brain and to replicate many features of cortical organization in vitro is truly remarkable. Nonetheless, the establishment of topographically complex cellular organization and intercellular relationships that are the hallmark of neocortical organization is not well recapitulated in organoids. Although acutely slicing organoids can preserve early laminar features for a longer time in vitro (Giandomenico et al. 2019, Qian et al. 2020), neuronal and glial cell types do not have the same tissue distribution or architectural structure as in vivo. While organoids have immense cellular complexity compared to adherent in vitro culture systems, the relative structural disorganization provides significant challenges for the study of complex neuronal functions such as directed neuronal migration, axonal pathfinding, and circuit formation.

Regional Interactions

While directed differentiation protocols produce more consistent cell types than undirected protocols, the more stringent protocols that succeed at specifying uniform regional identity are less useful for exploring interactions between different brain regions. Unguided protocols, while less

consistent and more unpredictable regarding region and cell type composition, have more potential to include multiple regional identities and to display interactions between them. However, a more reliable approach to study interactions of cell types from different brain regions has been the development of assembloids which consist of organoids directed from different lineages that are fused together (Birey et al. 2017). Assembloids have been utilized to model the migration of ventral forebrain–derived inhibitory interneurons (INs) into the dorsal cortex, where they can interact with excitatory neurons (Bagley et al. 2017, Birey et al. 2017, Xiang et al. 2017). Assembloid-derived INs that have migrated from ventral to dorsal cortical spheroids have stereotyped migration behavior, have more complex morphology than in ventral-only cultures, and form functional synapses with excitatory cells. The specificity of migration patterns, the presence of different IN subtypes, and the formation of canonical cortical circuits are features that have not yet been fully explored.

Axonal connections across multiple neural structures are also being studied using assembloids. Connectivity between the cortex and thalamus underlies the processing of sensory information. By fusing organoids that have been directed to either thalamic or cortical identity, interactions between cell types derived from these two structures have been observed (Xiang et al. 2019). Reciprocal axonal projections from conjoined cortical and thalamic organoids can form functional synaptic connections. Assembloids can also be composed of more than two organoids, as in cortical-spinal-motor assembloids that model the connections between cortex, spinal cord, and limb muscles that regulate motor output (Andersen et al. 2020). When the three types of spheroids—cortical, spinal, and muscle—were fused in a row, spontaneous muscle twitches were observed, triggered by corticospinal activity. While assembled organoids can demonstrate cell-cell interactions and functions that appear to mimic endogenous activity, these may not reflect the same cell types and connectivity that are found in vivo. For example, whole-brain cerebral organoids often develop retinal cells, including photoreceptor-like cells arranged in a primitive retinal cup (Lancaster et al. 2013, Quadrato et al. 2017). The organoid-derived photoreceptors are responsive to light, notwithstanding the absence of a lateral geniculate relay, but light activation inhibits spontaneous neural activity, which does not reflect normal in vivo retinocortical function.

Arealization

Gradients of morphogens pattern the developing neural tube into distinct structures along anterior-posterior, dorsal-ventral, and medial-lateral axes. Signaling mechanisms continue to refine these neural structures and diversify cell types within distinct areal regions. Although gradients of key signaling molecules and transcription factors are relatively well understood in mouse cortical development (Cadwell et al. 2019), how the larger and more complex functionally distinct cortical areas are defined in the human brain is an area of intense study. Organoids provide a platform to better understand the relevant signaling mechanisms that are required for cell type specification, tissue organization, and mechanisms of arealization. Approaches to manipulate signaling mechanisms in cerebral organoids and other in vitro cultures through the addition of pathway-activating small molecules have successfully promoted neural induction and differentiation of cortical cell types from undifferentiated neural structures. However, while intrinsic self-organization recapitulates a protocortical structure, parcellation into distinct cortices (prefrontal, visual, somatosensory, etc.) does not occur spontaneously. Some organoids demonstrate areas of graded morphogen and transcription factor expression that suggest anterior to posterior identity (Kadoshima et al. 2013); however, most do not have clear topographical organization and instead demonstrate sporadic localization of areal genes (Bhaduri et al. 2020b, Kelava & Lancaster 2016, Sasai et al. 2012). During normal development, precise levels of morphogens secreted from

specific organizing centers establish areal patterning. One strategy for increasing topographical organization has been to provide a point source of signaling molecules within organoids to progressively pattern the identity of adjacent cells. For example, a PSC line was genetically engineered with an inducible capacity to secrete sonic hedgehog (SHH), a powerful morphogen responsible for ventralization of the neural tube (Cederquist et al. 2019). When activated at a key point in organoid differentiation, this signaling mechanism was able to create a ventral to dorsal forebrain pattern of gene expression within a single organoid (Cederquist et al. 2019). This approach can be utilized to better understand the molecular mechanisms and features of arealization. Conversely, a more refined understanding of the mechanisms of areal patterning in vivo can be applied to improve the areal consistency of organoids and enable studies of more accurately patterned brain structures for disease applications.

Circuit Formation and Activity Dynamics

In vitro systems are inherently nonphysiological, as cell culture media and incubation conditions differ from the endogenous neural niche. Nonetheless, many major cell types present in developing cortex can be produced, and synaptic connectivity and electrophysiological activity can be observed. In vitro–derived human neurons can extend axons, express markers indicative of synapse formation (Andersen et al. 2020, Giandomenico et al. 2019, Xiang et al. 2019), and form synaptic contacts on dendritic spines (Quadrato et al. 2017). Electrophysiological approaches, including whole-cell patch clamp recording (Watanabe et al. 2017), multielectrode array (MEA) recordings, and calcium imaging (Quadrato et al. 2017, Trujillo et al. 2019), have been applied to assess individual synapses or to gain understanding of overall circuit dynamics. Although ensembles of organoid-derived cortical neurons appear to oscillate in ways that have been compared to infant electroencephalograms (EEGs) (Trujillo et al. 2019), the manner in which spontaneous activity arises in organoids may not reflect the way groups of neurons function in the developing human brain. Neural cell types, including a proper complement of inhibitory INs, as well as regional structures key for appropriate connectivity and circuit dynamics such as the thalamus, are absent from organoid model systems suggested to have EEG-like activity. Moreover, organoid physiological dynamics often reflect hyperexcitability, mimicking the functional dynamics of cells prone to seizures rather than the refined, carefully controlled function indicative of regulated neuronal activity. Human clinical data sets acquired through extracranial or invasive electrocorticography approaches or from surgically resected samples are difficult to compare to MEA recordings obtained from in vitro organoids. It is currently unclear how reflective organoid-derived neuronal activity may be of intrinsic human brain activity, but the structural and cellular composition differences suggest minimal direct homology.

Maturation

Neural organoids are derived from pluripotent cells directed down a differentiation trajectory, so it is not surprising that they represent early developmental stages as documented by gene expression and morphological and physiological features (Kadoshima et al. 2013, Pollen et al. 2019, Quadrato et al. 2017, Sloan et al. 2017, Velasco et al. 2019). Neural organoids follow a human developmental timescale, which is more prolonged compared to other mammalian species, including nonhuman primates (Benito-Kwiecinski et al. 2021, Camp et al. 2015, Pollen et al. 2019). While cerebral organoids are able to differentiate into a diversity of neural and nonneural cell types, the capacity of these cells to fully mature is less clear. For example, organoid-derived radial glia lack the maturation network signatures indicative of temporal progression in primary radial glia (Bhaduri et al. 2020b). Astrocytes collected from cortical spheroids across one year of differentiation, however,

do demonstrate gene expression that reflects a transition from fetal to early postnatal development (Sloan et al. 2017). Maturation of synapses and circuits past fetal-like stages toward more mature, adult-like circuits has yet to be demonstrated, but this may be dependent on longer culture times. Gene signatures relevant to aging-related neurodegenerative pathology, including Alzheimer's and Parkinson's diseases, such as *APOE*, *APP*, and *PSEN2* and *PRKN1*, *UCHL1*, *SNCA*, *PARK7*, *PINK1*, and *LRRK2*, respectively, have been detected in organoids cultured for relatively long periods (>250 days) (Gordon et al. 2021). Neurodegenerative disease–relevant gene expression suggests that organoids may be useful for the study of age-related disease mechanisms and therapeutic approaches. However, the capacity to reveal disease-relevant functional defects in specific cell types, given potentially imprecise specification and maturation state, is still unknown.

MODELING NEUROLOGICAL DISEASES USING ORGANOIDS

Studying human cells in a complex, organ-like structure that can be easily manipulated to explore molecular and cellular mechanisms of human tissue development and disease has enormous utility. But while organoids can help evaluate human-specific features missing in animal models, they are a reductionist model that reproduces certain features of brain development very well, and they do not reflect the entirety of human brain composition, organization, or function. The previously highlighted limitations regarding cell type diversity, specification fidelity, physiological function, and maturation (**Figure 1**) are all relevant challenges when using organoids to study human brain health and disease.

Stem Cell Line Variability and Use of Patient iPSC Lines

iPSCs have revolutionized the promise of personalized medicine for disease modeling, drug discovery, and, notably, autologous cell transplantation. However, the variability of stem cell line behavior in culture is a continuing challenge. Maintenance of pluripotency, spontaneous differentiation, and lack of appropriate differentiation capacity can all present challenges. The method of reprogramming, whether viral or episomal, may have different effects on the genome and epigenome outside of the intended genetic differences inherent to patient lines (Allison & Lowry 2017). Different iPSC line clones, isolated from the same biological individual and reprogrammed with the same method, can also display different phenotypes in vitro (Kyttälä et al. 2016). Due to the various sources of technical variability, multiple iPSC line replicates derived from several different individuals are now the standard for rigorous organoid studies aimed at defining disease phenotypes, particularly when studying polygenic or idiopathic disease models. For studies of monogenic disorders, genetic background differences can be circumvented by gene editing that allows comparison of isogenic mutant and control cell lines. Even so, disease phenotypes may vary across different genetic backgrounds.

Viral Infection

Organoids have been very effective for detecting neural tropism to viral infection, including Zika and SARS-CoV-2 viruses. Multiple studies have demonstrated clear vulnerability of cortical progenitor cells to the Zika virus, resulting in increased cell death and decreased cortical organoid size, mirroring the clinical microcephaly phenotype (Nowakowski et al. 2016, Qian et al. 2016, Watanabe et al. 2017). Stem cell–derived neural cells have also been used as platforms for drug screening to identify therapeutic agents that block Zika virus infectivity or replication (Retallack et al. 2016, Xu et al. 2016, Zhou et al. 2017). More recently, the vulnerability of the brain to the

SARS-CoV-2 virus has been an area of intense study due to the neurological consequences associated with coronavirus disease 2019 (COVID-19). Organoid studies have suggested viral tropism for particular cell types, including choroid plexus CSF-producing cells, cortical astrocytes, and dopaminergic neurons (Andrews et al. 2021, Chen et al. 2021, Jacob et al. 2020, McMahon et al. 2021, Pellegrini et al. 2020a). The range of neurological symptoms associated with COVID-19 supports the experimental evidence that some neural cell types may be vulnerable to infection. However, these in vitro studies must be validated with clinical evidence from patient-derived tissue samples. Multiple studies have now established human organoids as a platform to better understand the vulnerability of specific neural cell types to viral infection and screen for potential therapeutic agents.

Neurodevelopmental Disorders

As brain organoids are reflective of gestational and early postnatal stages of development, they are currently most relevant for the study of neurodevelopmental disorders. Disorders with a known genetic cause and potential manifestation in early development are well suited to study using organoids. Features of brain development, including neurogenesis, differentiation trajectories, and early organization, are sufficiently robust in neural organoids that their disruption may be readily assessed. Disorders that impact proliferation and result in global changes in cortical size, such as microcephaly and megalencephaly, have been successfully studied using organoid models (Bershteyn et al. 2017, Esk et al. 2020, Lancaster et al. 2013, Li et al. 2017). Mutations in genes associated with these disorders change progenitor cell number, cell type proportions, and cell death dynamics, all of which can be evaluated using organoids.

Many neurodevelopmental disorders, including cortical malformations, are associated with features that are more challenging to model, including generalized neurodevelopmental delay, epilepsy, ADHD, and autism. Some features of autism have been successfully modeled in organoids, including changes in the ratio of inhibitory GABAergic neurons as a consequence of increased *FOXG1* expression (Mariani et al. 2015). Moreover, gene regulatory networks associated with autism, identified through postmortem patient analyses, can be identified in organoids (Amiri et al. 2018, Gordon et al. 2021). However, neurodevelopmental disorders are often comorbid, have complex and heterogenous genetic causes, and are characterized by behavioral manifestations, complicating their study using organoid models. For example, disruptions to mTOR signaling are associated with a range of neurodevelopmental disorders often accompanied by epilepsy, including focal cortical dysplasia, tuberous sclerosis, fragile X syndrome, and autism (D'Gama et al. 2017, Mirzaa et al. 2016, Sharma et al. 2010, Winden et al. 2018). These related disorders termed mTOR-opathies can result in disorganized laminar structure and altered cellular morphology, migration, and connectivity as well as disrupted ion channel abundance and function affecting physiological activity (Andrews et al. 2020, Li et al. 2017). The relevant organizational and physiological features may not be easily modeled in organoids, and given the variability in stem cell lines and differentiation batches, it would be prudent to validate findings in in vivo animal models whenever possible. However, the presence of progenitor and neuronal subtypes in human organoids that are not well represented in animals underscores the unique potential of organoid models for studies of human disease.

Many neurological diseases and, in particular, neuropsychiatric disorders are associated with or identified by behavioral changes, a disease phenotype that simply cannot be modeled with in vitro cultures. An additional challenge concerns the timing of onset; while autism may be diagnosed within the first few years of life, schizophrenia often does not present until early adulthood,

and major depressive disorders often appear decades after birth. Years of sensory experience that establish and refine circuits across brain structures are impossible to replicate in organoids. However, dysregulated processes early in brain development may have consequences that manifest later in life. Causative developmental events that may take place in utero are prime for modeling in organoids and could shed light on the early etiology of neuropsychiatric disease.

Neurodegenerative Disorders

Because organoids, from both directed and undirected protocols, are derived from ectoderm and differentiated by promoting neural induction, the diversity of cell types is particularly lacking in nonneural cell types such as endothelial and inflammatory cells normally found in the intact human brain. Nonneuronal cells participate in blood–brain barrier formation, synaptic regulation, and response to inflammation (Abbott et al. 2006, Chung et al. 2015, Haruwaka et al. 2019, Paolicelli et al. 2011). Dysregulation of glial and other support cell types is associated with the onset and progression of neurodegenerative disease (Bachiller et al. 2018, Kwon & Koh 2020, McGeer & McGeer 1998). The absence of immune microglia and vascular cells, as well as limited astrocyte diversity and maturity in organoids, makes the study of these relevant interactions challenging. Additionally, many neurodegenerative diseases, including Parkinson's disease, Huntington's disease (HD), and amyotrophic lateral sclerosis, preferentially impact specific subtypes of neurons (Bano et al. 2011, Cowan & Raymond 2006, Dauer & Przedborski 2003, Piao et al. 2021, Rojas et al. 2020, Rowland & Shneider 2001, Sulzer & Surmeier 2013). Thus, when using organoids to dissect cell type vulnerability in neurodegenerative disease, it may be important that stem cell–derived neurons are truly faithful copies of their native counterparts. The refinement of cell subtype identity, as measured by gene expression, appears to be reduced in organoids compared to primary human cells (Bhaduri et al. 2020b). Whether this will limit the capacity to analyze cell type targeting in disease organoids is unclear.

3D culture systems may reveal neurodegenerative disease phenotypes not apparent in conventional 2D cell culture. For example, human amyloid-β precursor protein (APP) and/or presenilin, harboring familial Alzheimer's disease (AD) mutations, was overexpressed in an immortalized human neural progenitor cell line and then cultured in a 3D Matrigel matrix. Neurons differentiated for 6 weeks contained filamentous tau and high levels of phospho-tau aggregates, and notably, Aβ and β-amyloid plaques accumulated in the extracellular spaces (Choi et al. 2014), features that had not been observed in 2D cell culture. More recently, cerebral organoids from AD patients carrying *APOE* ε4/ε4 were found to have increased apoptosis and decreased expression of synaptic markers after 12 weeks in culture (Zhao et al. 2020). AD patient–derived cerebral organoids at 8 weeks of culture were also observed to have increased levels of Aβ and phosphorylated tau compared to non-AD organoids. These results are intriguing and may lead to increased understanding of disease phenotypes, but they remain surprising as organoids reflect an immature stage of development.

Most neurodegenerative diseases are age-related and manifest in later life, while neural organoids reflect fetal or neonatal stages of development. This fact would appear to limit the utility of organoids to model this important set of nervous system diseases. However, evidence has emerged suggesting that some neurodegenerative diseases that are typically diagnosed in the fourth or fifth decades of life, such as HD, may manifest at fetal stages. A prenatal onset of HD was suggested by findings in HD patient–derived cortical organoids where defects in rosette formation and cytoarchitecture were observed (Conforti et al. 2018). These observations are consistent with a recent report that detected changes in cortical progenitor cells in postmortem HD fetuses (Barnat et al. 2020). The possibility that neurodegenerative diseases may alter early brain development would improve the utility of organoids for studying these diseases.

Organoids provide a unique human cell–based platform for drug discovery. Patient-derived disease-bearing organoids can be differentiated into large batches, including technical and biological replicates, and assayed in multiwell plates to efficiently evaluate potential therapeutics (Shah et al. 2020). However, it will be important to identify relevant disease phenotypes, a not insignificant challenge given the limitations of a reduced in vitro model system. In addition to drug screening, organoids could provide donor material for cell transplantation to replace cells or tissue impacted by disease. Robust directed differentiation of a single relevant cell type may be most effectively accomplished in 2D culture, which has been the predominant strategy to replace dopaminergic neurons lost in Parkinson's disease (Kim et al. 2021, Piao et al. 2021). However, the diversity of cell types that comprise organoids, potentially including multiple interacting cell types, may prove to be more optimal for cellular replacement therapies in particular conditions, for example, in the case of stroke or brain injury, though such a prospect remains theoretical.

CONCLUSIONS

Stem cell–based organoids provide a platform to explore human brain development, with the potential to model features of normal and pathological development. The opportunity to explore human-specific features is especially compelling, as the human brain is dramatically different in terms of structural complexity, cell number, and diversity of neural cell types compared to commonly used animal models. PSC-derived neural cultures and, in particular, complex neural organoids provide an excellent platform to begin to unravel features of human brain development and explore relevance to human disease. However, the limitations of organoid models must be considered when assessing suitability for the study of functional processes and disease phenotypes. In particular, the study of highly specified cell types, functional circuit formation and activity, and interactions between CNS-resident neuronal and nonneuronal cell types may be limited in organoids. These limitations are compounded when cellular composition, organization, and interactions require evaluation simultaneously, as in complex neurological diseases. Differences in genetic background and inherent variability across stem cell lines expose vulnerabilities for misinterpretation of phenotypes. Validation may require replication across multiple cell lines. The method of culturing human stem cells must be consistent and reproducible, preferably with the use of isogenic controls or multiple biological and technical replicates. As is true for any in vitro model, it is important that findings are not overinterpreted as being a ground truth for human biology, particularly in the absence of validation in primary tissue or in an in vivo model. No model system is perfect, and organoids are no different; the potential for new discoveries is nearly limitless, but they should be utilized with a clear understanding of limitations.

DISCLOSURE STATEMENT

A.R.K. is a cofounder, consultant, and member of the Board of Neurona Therapeutics.

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