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"The promise of cerebral organoids for neonatology"

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Abstract

Purpose of the review

Applying discoveries from basic research to patients in the neonatal intensive care unit (NICU) is challenging given the difficulty of modeling this population in animal models, lack of translational relevance from animal models to humans, and lack of primary human tissue. Human cell derived cerebral organoid models are an appealing way to address some of these gaps. In this review, we will touch on the work that has previously been done to model neonatal conditions in cerebral organoids, some limitations of this approach, and some recent work that has attempted to address these limitations.

Recent Findings

While modeling of neurodevelopmental disorders has been an application of cerebral organoids since their initial description, recent work has dramatically expanded the types of brain regions and disease models available. Additionally, work to increase the complexity of organoid models by including immune and vascular cells, as well as modeling human heterogeneity with mixed donor organoids will provide new opportunities to model complex neonatal pathologies.

Summary

Organoids are an attractive model to study human neurodevelopmental pathologies relevant to work in the neonatal ICU. New technologies will broaden the applicability of these models to neonatal research and their usefulness as a drug screening platform.

Key words: neonatology, cerebral organoid, neurodevelopmental disorder

Introduction

One of the foremost challenges in neonatology today is understanding how to support the developing brain. For basic science researchers interested in clinical applicability, this question poses several challenges. Cutting edge and continually evolving brain imaging techniques have been applied to study brain development in NICU babies and graduates (1–6), but these approaches are not able to uncover molecular mechanisms. While others have attempted to address these questions using rare human tissue samples (7,8), these dissociated cells and tissues cannot recapitulate the complex dynamics of the developing brain *in vivo*. Thus, extensive emphasis has been placed on animal models which do not always recapitulate key aspects of human disease. This gap is evidenced by the large discrepancies between preclinically developed therapeutics and drugs proven to be effective in clinical trials (reviewed in (9)).

An appealing method to address this gap is the use of human derived cerebral organoids. First described by Lancaster and Knoblich in 2013, organoids are self-organizing clusters of neural cells grown in a three dimensional culture environment, which can better replicate functional *in vivo* organs than standard two dimensional cell culture methods (10). Brain organoids can be produced via two main mechanisms. In unguided protocols, stem cells are allowed to spontaneously differentiate into a variety of neural structures with variable regional identities. Alternatively, extrinsic factors can be added to culture media to induce guided differentiation of stem cells into specific regional subtypes, which can improve consistency between organoids. This approach has been used to generate organoids with dorsal (11), ventral (12), thalamic (13), midbrain (14), hindbrain (14–17), and spinal cord (18,19) fates (reviewed elsewhere (20,21)). Thus far, however, most work has focused on cerebral and dorsal forebrain organoids, and this will therefore be the basis for our review.

An important pre-requisite for the use of organoids to model neurodevelopment is that they can recapitulate key elements of human brain development. In mammals, the cortex develops from a pseudostratified neuroepithelium. As neurogenesis begins, the neuroepithelium becomes layered, with the ventricular zone containing the majority of progenitor cell bodies. The first progenitors to be born are the radial glial cells, which will generate the majority of glia and neurons in the cortex. In addition to producing mature cell lineages, radial glial cells also produce intermediate progenitor populations, which express a different set of protein markers, including T-box brain protein 2 (TBR2), reside in the subventricular zone, and undergo symmetric cell division (22,23). In humans, this organization is more sophisticated, with a distinction between the outer and inner regions of the subventricular zone. The outer subventricular zone contains an entirely separate progenitor layer with its own unique population of outer radial glial cells (24,25), which is not modeled well in the majority of animal models of cortical development.

Previous work has shown that organoids derived from human cells can faithfully generate the cell types and organization of the fetal brain and display human specific features of cortical organization that have been lacking in animal models (26). Self-organizing organoids have been found in multiple studies to recapitulate the single cell RNA sequencing (scRNAseq) profile of the developing fetal neocortex (27,28). Other papers have expanded these studies to patterned organoid protocols and similarly shown that these protocols demonstrate cellular diversity that

reflects that observed *in vivo* (29,30). However, important work has identified key limitations of organoid models, showing that they do not always fully represent the diversity of the human brain, with organoids displaying smaller number of cell subtypes and showing abnormal expression of cell stress markers (31).

Thus far, organoids have been used as a model for a wide array of disorders that affect neurodevelopment. Broadly, work falls in two categories – that which uses organoids derived from patients with genetic disorders as a way to dissect the intrinsic effects of these mutations on brain development, and that which uses organoids derived from healthy volunteers to explore the effects of exposure to a broad array of insults, from hypoxia to drugs and toxins. In this review we will highlight the ways that organoids have already shed important light on a number of neurodevelopmental disorders, many related to our work in the neonatal ICU. We will also explore the limitations of organoid models when attempting to model key aspects of neurodevelopment and ways that researchers are attempting to address these limitations.

Modeling of human genetic mutations

Since the introduction of protocols to generate cerebral organoids, this technology has been used to model single gene disorders. An important example has been work on microcephaly, where an array of single gene organoid models (including CDK5RAP2(10), NARS1(32), PTEN(33), WDR62(34)) have identified the way that mutations in disparate pathways can converge to effect neural precursor cell fate. In addition, cerebral organoids have been employed to model a range of other genetic disorders, including Miller Dieker Syndrome (MDS), a form of lissencephaly associated with intellectual disability and intractable epilepsy. One study comparing cerebral organoids generated from patients with MDS and controls used timelapse imaging and scRNAseq approaches to identify disruptions in neural stem cell development and function (26). They found increased apoptosis of SOX2 expressing neuroepithelial stem cells, as well as an alteration of the cleavage plane of ventricular zone progenitor cells, suggesting that the stem cell pool (and thereby production of mature neurons) is diminished both by apoptosis and decreased vertical cleavage events. They also looked specifically at outer radial glial progenitors, a cell type poorly modeled in mice, finding a delay in cell division in MDS organoids. A recent excellent review goes in to further detail about these and other examples of single gene modelling in organoids (35).

Beyond single gene disorders, many of the most common genetic conditions seen in the NICU are from either gene duplications (for example Trisomy 21 (T21)) or deletions (for example 22q11.2). Dorsal forebrain organoids derived from T21 iPSCs display reduced proliferation of the ventricular zone, decreased neuronal distribution in the cortical plate, and are generally smaller overall, consistent with findings of reduced proliferation in both animal models and postmortem human tissue from T21 patients (36). Transcriptomic analysis of organoids identified DSCAM and PAK1 as important genetic pathways that contribute to these phenotypes, and that inhibition of these pathways can rescue defects in neurogenesis (36). Additionally, in a ventral forebrain organoid models of T21, OLIG2 expression was found to drive increased GABAergic interneuron production (37). Implanting neural precursor cells from these organoids in mouse brains resulted in increased GABAergic neuron production and migration into the cortex. This

increased GABAergic neuron proliferation in organoid models is consistent with previously identified changes in excitatory and inhibitory balance in patients with T21.

Organoids derived from patients with 22q11.2 deletion have also been developed to model this important disease ex-vivo (38). While 22q11 deletion was not found to be associated with profound defects in corticogenesis, the presence of 22q11 deletion in iPSC derived human cortical organoids was found to cause abnormal neuronal excitability. These neurons showed changes in spontaneous firing rates related to changes in resting membrane potential and defects in the function of voltage gated calcium channels. These alternations in neuronal activity could be replicated by mutations of a single gene – DGCR8 – and were rescued in 22q11 organoids by replacement of this transcript, narrowing down the genetic locus responsible for altered neuronal activity. Interestingly, these activity defects were also rescued by short exposure to antipsychotics, presenting a possible novel therapeutic avenue to explore for treatment of neuropsychiatric problems associated with 22q11 deletion.

Modeling early developmental exposures

Viral Infection

In additional to using patient cells to model genetic disorders, organoids derived from healthy controls can be used to examine the effect of *in utero* insults on brain development. An important example is the work done on the relationship between Zika virus and microcephaly (17,39–41). These studies showed a causal link between infection with Zika virus and decreases in organoid size, showing a specific reduction in ventricular zone thickness, primarily through infection of neural progenitor cells (17,41). Additional studies have used organoid models to look at the downstream effect of Zika virus infection on progenitors, identifying the TLR3 signaling pathway as a key mediator of the effect of Zika on cortical development (39).

More recent work has employed similar organoid models to study the effect of cytomegalovirus (CMV), a virus commonly seen in the NICU, on the developing brain (42–46). Clinical outcomes of CMV can be highly variable, and include microcephaly, intracranial calcifications, developmental delay, and sensorineural hearing loss (reviewed in (47)). Infection of cerebral organoids by CMV has been shown to result in abnormal calcium signaling and disruption of cortical layer formation (42,46). Organoid approaches allow for dissection of the mechanism by which CMV induces these changes – in one case Sun et al. showed that siRNA knockdown of EGFR and PDGFRalpha in cerebral organoids significantly reduced their susceptibility to CMV infection (46). They also showed that multiple different neutralizing antibodies had the capacity to prevent CMV infection in cerebral organoids (46). Work continues to identify and test the role of downstream signaling pathways in the pathogenesis of CMV infection, which has the potential to identify new and exciting therapeutic avenues for a disease with significant neurodevelopmental impact and no current preventative approaches(43,46).

Maternal Immune Activation

Increasing evidence from human and animal models suggests that in utero exposure to inflammation (often termed maternal immune activation or MIA) is associated with long term risk of neurodevelopmental deficits in infants, including autism spectrum disorders (ASD) a(48–50). A recent paper from Sarieva et al. establishes a dorsal forebrain organoid model of MIA through expression of a constitutively active form of IL-6, a key mediator of MIA in humans and animals models, for 5 to 10 days starting at day 45 of organoid differentiation (a timepoint corresponding to human mid gestation) (51,52). This model showed an increased number of radial glial cells, but no change in the number of intermediate progenitors or mature neurons (though one specific type of excitatory cortical neuron showed over production in this model). The authors also observed abnormal development of cortical layers, suggesting a migration or maturation defect of differentiated neurons. RNA sequencing in these organoids showed upregulation in major histocompatibility complex class I genes, consistent with human studies that have implicated these proteins in ASD (53). The authors also found that radial glia show the most significant changes in gene expression, with IL6 over activation causing a decrease in expression of genes related to protein translation (which has also been seen in mouse models of MIA (54)). In addition to confirming already-described mechanisms of disruption in MIA, the authors also identified differentially expressed genes not previously found in animal models, including *NR2F1*, which regulates genes involved in synapse organization and cell migration, and may contribute to species-specific effects of MIA.

Hypoxic injury

An essential clinical problem in neonatology is how to assess the impact of hypoxic injury on the developing brain. This question is relevant to preterm infants, who often experience periods of hypoxia during their NICU stay, as well as term babies who experience hypoxic ischemic injury around delivery. A number of studies have used organoid models to make key discoveries about the changes in cortical development induced by transient hypoxia (55–58). Many of these studies have found similar results, suggesting that both outer radial glial cells and intermediate progenitor populations are important mediators of hypoxia's effect on cortical development.

In 2019, Pasca et al. used 10 week old organoids, approximately 19-24 postconceptional weeks, to model hypoxic injury in the preterm cortex (55). After exposing these organoids to low oxygen tension (<1%) for 48 hours, they found reduced density of intermediate progenitor cells in the proliferative region, but no effect on radial glial cells, which persisted even after oxygenation was re-established. Using RNA sequencing approaches, they identified the unfolded protein response pathway as a key mediator of hypoxia's effect on the developing brain. Interventions to increase the unfolded protein response exacerbated hypoxia's effect on intermediate progenitor number, whereas restoring the appropriate protein translation during hypoxia restored the density of these cells. While fewer intermediate progenitors are present, there is no corresponding increase in markers of apoptosis, suggesting that these cells are not dying. The authors hypothesize instead that they are being induced to exit the cell cycle and shunted into early differentiation. A key strength of this work was its validation of these findings in primary human tissue from 20 week samples; the authors generated slice cultures which were then exposed to normal or low oxygen concentration. As they had observed in the organoid

model, the authors noted a decrease in the proportion of intermediate progenitor cells following hypoxia, which was similarly prevented by blunting the unfolded protein response.

Other groups have made similar observations at earlier developmental timepoints. Daviaud et al. 2019 used a human cerebral organoid model with dorsal forebrain specification to examine the effect of 24 hours of hypoxia on 28 day old organoids, which are estimated to approximate 14.5-18.5 post conceptional weeks. They found selective vulnerability of outer radial glial progenitors and other neuroblasts (including intermediate progenitors) to this insult, but relative resilience of neural stem cells in the ventricular zone niche. Interestingly, they noted a change in the cleavage plane of dividing cells, with vertical angles more frequent in hypoxia exposed organoids, which they suggest indicates compensatory division to replace lost neural stem cell populations.

Both of these papers focused on organoids directed to differentiate into dorsal forebrain fates. In a self-directed organoid model, which likely includes a broader diversity of neuronal subtypes, Boisvert et al. 2019 looked at both a model of transient (72 hours) as well as more prolonged (25 days) hypoxia. They found that prolonged hypoxia repressed gene markers of forebrain development, oligodendrocytes, glial cells, and genes important for cortical layering and migration. This effect was mitigated by application of minocycline, a second generation tetracycline antibiotic, suggesting directions for potential future therapeutics.

In utero exposure to toxic substances

There has historically been a large barrier to research and drug testing in pregnant women, out of appropriate concern for negative side effects on the developing fetus. Organoids provide an appealing platform to rapidly assess the effect of potentially toxic substances on the developing brain, which may facilitate drug development for this important population.

Work focused on known toxic substances has characterized the neurodevelopmental effects of these exposures (59,60). Adams et al. showed that ethanol changes post-translational histone modification and chromatin accessibility which ultimately result in impaired calcium signaling, synaptic development, and astrocyte function (59). Looking at organoids exposed to nicotine, Wang et al. showed premature neuronal differentiation and disruption of cortical regionalization as well as abnormal neuronal migration (60). Lee et al. exposed organoids to cocaine, and saw similar reduction of progenitor proliferation and premature differentiation. They further found this effect was mediated through induction of reactive oxygen species via CYP3A5 (61).

In a recent paper Anton-Bolanos et al. explored the effect of ethanol as well as the anti-seizure medicine valproic acid (VPA) (62). This unique study developed a “chimeroid” model, or an organoid model created with contributions from multiple different donors cell lines. Combining cells from multiple donors allowed this group to create organoids that address interindividual variation in susceptibility to neurotoxic triggers. Exposing these chimeroids to ethanol and VPA caused significant changes in cell type and composition. In response to VPA there was an increase in immature interneurons and choroid plexus cells and a decrease in intermediate progenitors. In contrast, ethanol caused a decrease in callosal projection neurons.

The chimeroid model allowed the authors to go further to examine donor specific responses to these toxic substances. Different donors differed in the extent of gene expression changes found in response to VPA. For example, one particular donor line was found to be preferentially sensitive to ethanol, but resilient to VPA, and the degree of gene expression response to VPA varied amongst donors. This application of chimeroids to study donor specific responses to medications or toxins is an appealing approach to determine how a drug might act across a wide population of individuals and truly activate on precision medicine.

Limitations and possibilities for future applications

While organoids have already been shown to be a powerful tool for dissecting mechanisms of health and disease in the developing brain, there remain key limitations to the applicability of this technology, particularly for neonatal pathologies.

Vascularization

A major limitation of most organoid protocols is the lack of intrinsic vasculature. This absence limits organoid size, since the core will eventually become necrotic, but also limits studies of the biology of the blood brain barrier. A number of different approaches have been taken to address this limitation, including transplanting organoids into the mouse brain where they can then be vascularized, organoid fusion protocols, mixed co-culture protocols, and microfluidics(7,63–69). Our own group has used a transplantation model, whereby endothelial and mural cell populations purified from human brain tissue are implanted into 7-9 week old cortical organoids. We have found that these cells integrate into organoids, and display progression towards more differentiated vascular fates(7,70). These novel approaches set the stage for further application of organoid models in neonatology. One of the most common neurologic sequelae of prematurity is intraventricular hemorrhage (IVH), where immature fragile blood vessels in the germinal matrix rupture and bleed in close proximity to the developing subventricular zone. This feared complication of prematurity results in intellectual disability, cerebral palsy, seizures, and/or hydrocephalus in 50-75% of survivors (71,72). As of today, there have been no papers published using an organoid approach to model IVH. This absence is likely multifactorial and due to both a lack of vascularized organoid approaches and the early focus of the field on cortical development rather than the subcortical structures that are particularly at risk for IVH (though there are some groups addressing this gap(13,73)). The advent of novel vascularized organoids and expansion of the regions modeled by organoid approaches may make an organoid model of IVH possible in the future, allowing us to study the pathophysiology of this important condition.

Neuro-immune interactions

Similarly, resident immune cells such as macrophages are increasingly being recognized as key drivers of neurodevelopmental disorders, but are not usually created in the standard generation of cerebral organoids. As with vascularization, approaches thus far have focused on co-culture as well as xenografting developing human microglia into the mouse. In one approach, Schafer et al. describe a xenotransplantation procedure whereby both human brain organoids as well as human pluripotent stem cell derived microglia are both transplanted into the mouse brain (termed

immunocompetent human brain organoids or iHBOs) (74). These immune cells were found to be capable of surveilling the transplanted organoid and responding to environment induced injury. The authors further use this model to explore the immune response in a patient derived model of autism (ASD) with macrocephaly. They found that transplanted iHBOs from ASD subjects had a microglial morphology more consistent with activation. Interestingly, when microglia lineages from controls were introduced to the organoids from ASD patients, the authors found that they too became reactive, suggesting that something about the developing brain itself may be driving immune activation in these patients. In the future, an important question to address is whether there is non-self recognition in organoids by immune cells. Towards this goal, a recent study created human intestinal "immuno-organoids", with endogenously derived tissue-resident memory cells (75) The multiple potential sources of microglia in the brain add complexity to this task for cerebral organoids (76).

Connectivity and Circuit Function

Understanding the development and function of neuronal circuitry in the human brain is a key challenge of neuroscience today. Organoids are an appealing approach to explore human brain specific circuit function, as they are more homogenous than human samples, easier to generate, and tractable for genetic manipulations. However, there are significant barriers to the study of circuit development and function in organoids that currently limit their use for these purposes.

From the earliest studies of organoids, the cells that compose them have been known to be electrically active, with functional synapses that can be inhibited with tetrodotoxin (10,11,28,77). This activity has been assayed in various ways, including patch clamp recording, extracellular electrode recording, and calcium imaging (10,28,78,79). These studies have identified important general principles of organoid activity, showing that there is an increase in electrophysiological activity and synchrony, and a strengthening of connections over time (78). Organoids have also been shown to exhibit evoked activity in response to stimuli – for example Quadrato et al. showed that organoids containing cells expressing markers of photoreceptors were responsive to light stimulation (28).

However, major barriers exist to using organoids to study circuit function. As undirected organoid protocols generate structures with heterogenous neuronal populations, it is unclear if the connections identified in these structures will reliably recapitulate relevant connections in the human brain. While directed organoid protocols are more homogenous, the majority of neural circuits connect disparate brain regions, and thus modeling these connections would rely on the creation of assembloids of two distinct regions. This approach has been adopted by a number of groups to look at circuit activity in thalamocortical circuitry and intracortical circuitry (13), but in these protocols each component of the assembloid is developed separately before they are fused, raising questions about how accurately this process recapitulates the integrated development found in the human brain. Finally, thus far assembloids have primarily been composed of two different brain regions, but it is well known that higher order brain regions, particularly the cortex, make a large array of connections with distinct brain regions (80). This complexity may be difficult to ultimately accurately model in organoid models.

Finally, an important goal of circuit neuroscience is to assess the impact of circuit dysfunction on neural output and ultimately behavior. Similarly, sensory experience is a key component of shaping circuit development *in vivo*. As organoids are fundamentally disembodied, groups attempting to address these questions have relied primarily on xenotransplantation models of organoids into the brain of traditional animal models. One paper by Revah et al. transplanted human cortical organoids into the somatosensory cortex of newborn athymic rats at a timepoint in development when cortical circuits are still developing (81). They found that these organoids not only survived in the rat brain and the cells composing these organoids were more mature transcriptionally, morphologically, and functionally than neurons in purely in vitro organoids. In fact, samples from fetal brain tissue were transcriptionally more similar to the transplanted organoids than in vitro organoids. These transplanted organoids were found to receive inputs from both cortical regions as well as subcortical regions such as the thalamus. Not only was optogenetic stimulation of these inputs found to be able to stimulate activity in the transplanted organoid cells, but so was whisker deflection, suggesting that cortical organoids are integrated into the somatosensory circuit. Conversely, neurons from the organoid extended axons throughout the brain to key target regions including both cortical (auditory, motor, and somatosensory) regions and subcortical regions (striatum, hippocampus, and thalamus) and activation of these neurons can drive reward seeking behaviors. While there is far to go in characterizing the relevance of xenotransplantation models to human circuit development, these studies set the stage for exciting future work.

Organ-organ interactions

Finally, while progress is being made on developing more sophisticated organoid systems to model the complex interactions between cell types in the human brain, these systems remain unable to model more complex organ interactions that occur in vivo. A key example of this interaction is the relationship of the placenta as a neuroendocrine and immune organ during fetal development which is increasingly recognized as essential in shaping in the developing brain (82).

Summary

Since their initial introduction more than 10 years ago, human cerebral organoids have become a key method to model the early phase of human neurodevelopment and assess the potential impacts of genetic and environmental perturbations during this sensitive period. They have already begun to be applied to a wide range of neurodevelopmental disorders and have shed light on some key pathologies of neonatology such as neonatal hypoxemia and drug exposure. As organoids become more complex and representative of the unique features of human brain development (such as with the integration of vascular and immune cells), they will become an increasingly powerful tool to explore early human brain development.

Take Away Points

- Research into human neonatal brain development and disease is limited by a number of factors, including lack of translational validity of animal models and lack of human tissue

- Cerebral organoids are an important method of studying early human brain development, and has been used in models of both key genetic and environmental disruptions
- Limitations of organoid models include their lack of complex vascular and immune components, as well as their extracorporeal nature and lack of organ/organ interactions
- New technologies addressing these limitations will be helpful in further organoid modeling of conditions important in neonatology

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** Anton-Bolanos et al. This study uses a novel organoid model which includes cells from multiple human donors. The authors show that using this method they can identify donor specific responses to multiple different drug compounds. This model sets the stage for further use of organoids as a drug testing model, as well as starting to assess interindividual variation in drug response.

** Schafer et al. This study develops a novel model whereby human organoids and human stem cell derived microglia are implanted into a mouse brain. This approach allowed researchers to study neuro immune interactions that were previously not accessible in classical organoid models.

** Crouch et al. This study describes a protocol for co-culture of organoids and human vascular cells, addressing current limitations of organoid vasculature.