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Review Article

Generating CNS organoids from human induced pluripotent stem cells for modeling neurological disorders

Andrew T Brawner[1], Ranjie Xu[1,2], Dingfeng Liu[1], Peng Jiang[1,2]

[1]Department of Developmental Neuroscience, Munroe-Meyer Institute, [2]Mary & Dick Holland Regenerative Medicine Program, University of Nebraska Medical Center, Omaha 68198, NE, USA

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Abstract: Understanding human brain development and disease is largely hampered by the relative inaccessibility of human brain tissues. Recent advances in human induced pluripotent stem cells (hiPSCs) have led to the generation of unlimited human neural cells and thereby facilitate the investigation of human brain development and pathology. Compared with traditional 2-dimensional (2D) culture methods, culturing the hiPSC-derived neural cells in a three-dimensional (3D) free-floating manner generates human central nervous system (CNS) organoids. These 3D CNS organoids possess the unique advantage of recapitulating multi-regional or region-specific cytoarchitecture seen in the early human fetal brain development. The CNS organoids are becoming a strong complement to the animal model in studying brain development and pathology, and developing new therapies to treat neurodevelopmental diseases. Further improvements to the long-term maintenance and neural maturation of the organoids may allow them to model neurodegenerative diseases. In this review, we will summarize the current development of hiPSCs to generate CNS organoids for modeling neurological disorders and future perspective.

Keywords: Cerebral organoids, CNS organoids, human pluripotent stem cells, 2D cell culture, 3D cell culture, biomaterials, neurological diseases

Introduction

Due to its uniqueness, complexity, and its highly developed and enlarged neocortex, the human brain is an immense challenge in the study of its development and pathology. In comparison to other organ systems of the human body, the unique features of the human brain distinguish humans from other animal species. When it comes to modeling human brain development and pathology, animal models have limits in their efficacy due to profound morphological and physiological differences [1, 2]. A decade ago, the emergence of induced pluripotent stem cell (iPSC) technology presented new possibilities of studying human brain development and disease using unlimited human neural cells. Human induced pluripotent stem cells (hiPSCs), reprogrammed from terminally differentiated somatic cells, such as skin fibroblasts, highly resemble human embryonic stem cells and can be cultivated to differentiate into any cell type in the body, including neural cells [3-5]. Moreover, hiPSCs can be derived from patients with specific neurological disorders, leading to the generation of disease- and patient-specific organoid models. Recently, research has increasingly been directed in developing and refining the 3D culturing of hiPSC-derived neural cells. 3D culture of cells enables them to employ their intrinsic ability of self-signaling and assembling to form small collections of brain tissue that can not only model differentiation and lineage progression of different neural cell types, but can also model the formation of cytoarchitecture and neural circuits of the human brain.

An organoid is defined as an artificial *in vitro* 3D structure grown from stem cells possessing the cells and structure specific to a particular organ system [6, 7]. Organoids can be generated with the appearance, activity, or gene markers of tissue reflective of the human CNS at early developmental stages. As a group, we will refer to

these organoids as CNS organoids in this review. The generation of CNS organoids is both the results of refinements to the specification of cell fate from hiPSC-derived neural cells and the neural cells' own innate ability to self-organize and form intercellular structure with appropriate function. This ability to self-organize is attributed to the convergence of specific intercellular signaling, mechanical inputs, and the simultaneous activation of the autonomous genetic programs [8]. CNS organoids have been generated to model specific CNS regions, such as the prefrontal cortex, occipital lobe, the hippocampus, cerebellum, and the retina, as well as model with multi-regional brain identity [9-12]. Combining with hiPSC technology, CNS organoids can be generated from patient-derived hiPSCs, providing a powerful tool to investigate pathogenesis of neurological disorders. In this review, we will introduce an overview on how CNS organoids are generated and describe alterations in their cultivation that have improved the organoids' viability and lifespan. Moreover, we will also discuss areas of interest for researchers to improve in the future in order to utilize the CNS organoids as a model to study neurodevelopmental and neurodegenerative diseases.

Generation of CNS organoids from human pluripotent stem cells

The generation of CNS organoids is the *in vitro* model recapitulation of *in vivo* fetal CNS development. This capacity to model *in vivo* development includes the establishment of region-specific neural identity with the recapitulation of discrete 3D structural organization [6]. The practical suitability of this developmental model is supported by the qualitative results of comparing gene expression pattern models, phenotypic cell markers, and single-cell RNA sequencing. These techniques are able to search and match gene markers for the fetal brain's development in tandem with the CNS organoid's development [13]. The culturing of CNS organoids is through the transformation of 2D hPSC colonies into 3D cell agglomerates with hPSCs committing to the neural cell fate pathway, followed by the discrete 3D localization of specific neural cell types; mimicking the developing fetal brain. In 2008, Dr. Hans Clevers pioneered the use of serum-free floating culture of embryoid-like aggregates with quick reaggregation (SFEBq). The process involves embryonic stem cells (ESCs) being harvested from growth factor free 2D culture then passaged to 3D culture to allow for a spheroid reaggregation in serum-free media. Through subsequent replating upon an adhesion plate, a continuous neuroectoderm-like epithelium (NE) forms. When cultured in 3D, the NE demonstrates the presence of stratified cortical tissue with regional identity selectively-controlled by the addition of specific patterning factor small molecules [6, 14].

Subsequent research in 2013 by Lancaster et al., introduced their protocol of organogenesis. Unlike SFEBq method, following the initial development of neuroectoderm, the EBs are embedded into the extracellular matrix (ECM), matrigel. The act of embedding EBs into the matrigel allows for the growth of larger neuroepithelial buds with the matrigel serving as a basement providing an apicobasal orientation for the formation of an expanded, continuous, and uniform layer of NE for corticogenesis than if left in 3D culture [6, 15, 16]. As an alternative, not embedding the EBs will simplify the process and create spheroids in which corticogenesis is more readily visible with expression of neuronal markers of defined superficial and deep cortical layers radiating out from the VZ [17]. However, this also results in organoids that possess sparse populations of NPCs characteristic of outer radial glia (oRGCs). oRGC populations are a hallmark of the embryonic human cerebral cortex and are considered important to the evolutionary increase in cortical size and complexity [16, 18]. After the embedding, large buds of this continuous NE protrude from the EBs, encircling and encompassing fluid-filled cavities structurally similar to ventricles. These NE buds then outwardly generate stratified cortical tissues containing cortical progenitors for deep and superficial cortical-layer neurons and Cajal-Retzius cells. Upon the formation of the germinal zone, the newly differentiated outer radial glia cells (oRG) self-organize, expand, and form various brain structures [9]. This region of oRG cells models the *in vivo* ventricular (VZ) and subventricular zone (SVZ) of the fetal brain from where most cortical neurons originate [15]. These zones contain the neural progenitor cell (NPC) populations that are responsible for the output of neuronal cell which are responsible for subsequent

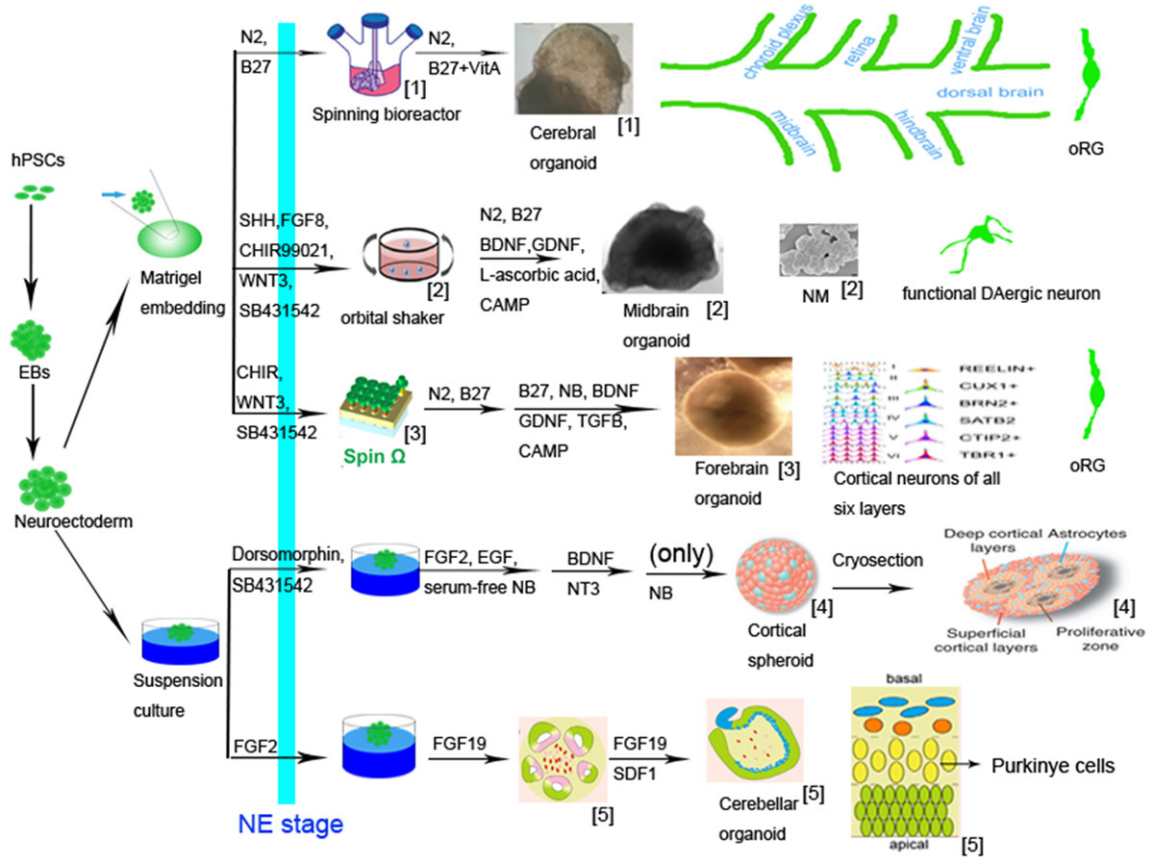

Figure 1. Differentiation of hiPSCs to CNS organoids. Neural differentiation of hiPSC colonies are initiated by culturing them in a free-floating 3D manner to form EBs. The hiPSC-derived neural progenitor cells in the EBs spontaneous assemble neuroepithelium-like structure. Based on time-specific alteration of environment, by embedding the EBs into the extracellular matrix, such as Matrigel, or applying various morphogens and neurotrophic factors, the neural progenitor cells in the neuroepithelium-like structure will further acquire brain regional identities, differentiate to neurons and glial cells, and form structures resembling different brain regions. NB: Neurobasal medium. [1] (Lancaster et al., 2013); [2] (Jo et al., 2016); [3] (Qian et al., 2016); [4] (Pasca et al., 2015); [5] (Muguruma et al., 2015).

increased brain size seen for humans compared to other animals [1]. With the original hPSCs having adopted the neural identity and committed to the neural lineage as NPCs, they migrate outward and self-organize into cerebral organoids. It is from these NPC populations outwardly migrating through the organoid tissue to the cortical plate, that cortical neurons are generated and localized similarly to the fetal brain neocortex [12, 13]. Discrete regionalization of the brain regions of the forebrain, midbrain, and the hindbrain can be detected in these organoids. Examining the expression of regional identity markers demonstrates the presence of forebrain markers, *BF1* and *Six3* and/or hindbrain markers, *Krox20* and *Isl1* [15]. Initial tests successfully demonstrated the presence of both in their respective regions. Further testing also demonstrated that the forebrain markers were still highly expressed after long-term culture, while the expression of the hindbrain markers decreased. These levels of expression over time mirror the greater expansion of the forebrain in early human brain development [9, 15].

In general, two different approaches for culturing CNS organoids in 3D culture have emerged: i) self-patterning organoids and ii) extrinsically patterned organoids. Self-patterned organoids are cultured without externally added morphogens that would direct differentiation to favor one brain regional identity over another, such as the addition of Sonic hedgehog (SHH) to induce NPCs to develop a ventral cell fate. Self-patterned organoids will generate a more "complete" mini-brain that exhibits neural tissue with multiple brain regional identities. The ben-

efit to this self-directed approach is the generation of a systemic model where inter-regional interactions as well as larger-scale impacts, such as the overall size reduction of cortex tissue in microcephaly can be better observed. The immediate drawback is that these self-patterned CNS organoids can be of inconsistent individual quality with how well distinct regions are modeled [15]. By comparison, extrinsically-patterned CNS organoids are generated in the presence of morphogens. Extrinsically-patterned CNS are directed to assume specific regional identities with a higher level of uniform accuracy and can be used to better model region-specific neural activities [16]. A recent study showed the use of extrinsically patterned ventral and dorsal forebrain organoids to model the migration of GABAergic interneurons from the ventral to the dorsal forebrain and subsequent integration of these interneurons into functional microcircuits [19].

Depending on the morphogens and neurotrophic factors provided in the cultures, organoids patterned with cerebral, hippocampus, adenohypophysis, forebrain, and the optic cup region identities can be derived (**Figure 1**). Organoids with a separate, specific regional identity can be selectively generated by the addition of specific patterning factors like fibroblast growth factors (FGFs) and agonists of SHH, Wnt, and BMP signaling pathways to the organoid cultures at the neuroepithelium differentiation stage [6]. For example, a midbrain-region organoid can be derived by promoting the differentiation of EB neuroectodermal tissue toward a floor plate through the simultaneous addition of three small molecules; the dual-SMAD inhibition factors (Noggin and SB431542) and a Wnt pathway activator (CHIR99021) (**Figure 1**) [21-23]. The EBs were then patterned toward a mesencephalic fate with the addition of SHH and FGF8 [20]. By day 7, neural markers for midbrain dopaminergic (mDA) progenitor neurons, FOXA2, OTX2, CORIN, and LMXIA were detected. The mDA neurons were cultivated to self-organize into 3D human midbrain-like organoids (hMLO) around 2 mm in diameter, midbrain-distinct cell layers with functional mDA neurons able to synthesize dopamine (**Figure 1**) [20].

In the generation of cerebellar organoid, cerebellar neural cells are differentiated with the initial treatment of the EBs with insulin and FGF2, which suppresses differentiation to forebrain cells. This is then supplemented with the use of V-bottom well plate rather than a typical U-bottom to aid in re-aggregation and application of the TGFβ signaling inhibitor SB431542. Subsequent treatment with FGF19 and SDF1 induces the NE to mimic the dorsal-ventral and apicobasal orientation of the cerebellar plate (**Figure 1**). With this two-step protocol, the cerebellar plate neuroepithelium plate (CPNE) begins to form. The CPNE is where neural progenitors of the GABAergic neurons and Purkinje cells of the cerebellar cortex will be generated in the organoids [24]. Disease-specific organoids generated from patient-derived hiPSCs would be effective for the study of genetic cerebellar diseases, such as Dandy-Walker syndrome [24, 25]. Currently, other CNS regions observed in organoids include the hippocampus, adenohypophysis, forebrain, and the optic cup [6, 16, 26, 27].

The CNS organoids can be cultured for long term for up to one year and can model fetal neocortex tissue at thirteen weeks of age with the organoid possessing a diameter up to 4 mm [15, 28]. This long-term culture is achieved primarily through the implementation of agitation culture method, by using spinning bioreactors or orbital shakers. The use of the spinning bioreactor or orbital shakers is to overcome the inherent lack of vasculature tissue, while an intrinsic component to any *in vivo* tissue, is lacking within or around the CNS organoid. This lack of vasculature tissue is due to hPSCs having been committed to the neural lineage pathway, they no longer possess the capacity to differentiate into any cell identity outside of the neural lineage [7, 9]. Generally, in 3D cultures, the external cells of the CNS organoid would block access for the internal cells to the nutrients and oxygen in the media [29]. As a result, the internal cells would die, compromising the CNS organoid's ability to further develop and model brain development. Using spinning bioreactors or orbital shakers partially ameliorates this limitation by the agitation of the organoids to facilitate the distribution of oxygen and nutrients throughout the organoids. A recent study of the Zika virus using CNS organoids further developed a miniaturized spinning bioreactor, named SpinΩ in the implementation of agitated culture. The SpinΩ device was produced using

Table 1. Modelling neurological disease by using CNS organoids

Organoids	Timing	Main cell types	Application	Advantages
Neuroepithelial cysts [26]	5 days	Retinal progenitor cells	Promising for transplantation approaches for the replacement of dysfunctional or lost RPE by rapidly generating pure human RPE cells	Directly placing hPSCs in neural induction conditions significantly enhanced the speed of getting cysts with renal identity
Forebrain organoids [16]	28 days	oRGCs, cortical neurons expressing markers found in all six layers of human cortex, and GABAergic neurons	Chemical compound testing and modeling ZIKV infection	Generated cost-effectively, with minimized heterogeneity and variability, and exhibiting a well-defined oSVZ-like region with a prominent oRGC-like NPC layer
Forebrain organoids [30]	31 days	oRGCs, layer-specific cortical progenitors and neurons	Modeling neuronal differentiation in autism spectrum disorders and also applicable to studies of the dorsal-ventral specification of the telencephalic region, and the inside-out pattern formation in the human fetal cortex	Studying the role of oRG progenitors in human corticogenesis with recapitulating the 3D context of the developing human cortex as well as the production and migration of GABAergic interneurons
Cerebral organoids [15]	30-40 days	oRGCs, cortical interneurons, functional cortical neurons	Modeling microcephaly	Developing a variety of regional identities organized as discrete domains capable of influencing one another
Cerebellar organoids [24]	35 days	Cerebellar neurons (Purkinje cells, GCs, interneurons, and DCN neurons)	Useful for modeling spinocerebellar ataxia and understanding inborn disorders of cerebellar development, such as Dandy-Walker syndrome	Demonstrating a set of electrophysiological analyses of human Purkinje cells and FGF19 and SDF1 promoting self-formation of polarized neural-tube-like structures
Cortical spheroids [17]	43 days	Functional cortical neurons and nonreactive astrocytes	A versatile platform for patterning and specification of various neuronal and glial cell types as well as for designing large-scale drug screening in vitro	Simple to be generated, containing astrocytes developing spontaneously, overlapping cortical developmental stages up to late mid-fetal periods
Hippocampal-Choroid plexus organoids [27]	35-42 days	Zbtb20+ Hippocampal neurons (Prox1+ DG neurons and KA1+ pyramidal-like neurons)	Applicable to human choroid plexus research in vitro and the analysis of human hippocampus-related disorders, including Alzheimer's disease and schizophrenia	Providing dorsomedial telencephalon-like 3D tissues that give rise to hippocampal DG and pyramidal-like neurons, facilitating recapitulating complex neural circuitry
Midbrain organoids [20]	35 days	Functional dopaminergic and neuromelanin producing neurons	Useful for studying NM granules and associated proteins from PD patient-derived iPSC lines, which may shed light onto the underlying pathophysiological mechanisms of PD	Generating human mDA neurons that readily produce NM granules

3D design and printing technology to fit over a standard 12-well culture plate. This device was able to individually deploy a spinning shaft into each well. As each unit only requires 2 mL of media, it is a 50-fold reduction in media consumption and saves on shelf space [16]. In the coming years, continued bioengineering advances in the cultivation of organoids will see exponential increases in their efficacy as models of the developing fetal brain.

Modeling neurological disease using hiPSC-derived CNS organoids

Human iPSC-derived CNS organoids have been demonstrated to be an effective model for studying the neurodevelopmental diseases, such as microcephaly, macrocephaly, and autism [15, 30]. Due to the current growth limitations for CNS organoids, it is most effective as a model for neurodevelopmental disorders in the fetal brain that would emerge in the first trimester [9, 31]. The first example of CNS organoid being used for disease was in the initial study performed by Lancaster et al. for the modelling of microcephaly. Mice were unusable as a model for microcephaly as human patients with microcephaly possess compound heterozygous truncating mutations in CDK5RAP2 protein, which binds and activates the CDK5 gene, while CDK5 mutant mice die at birth and exhibit profound brain disorganization [29]. In the subsequent differentiation of the patient-derived hiPSCs, the CNS organoid revealed very few progenitor regions, a larger degree of neuronal outgrowth, and smaller neuroepithelial tissues similar to the microcephalic brain. In the examination of the hypoplasia pathogenesis, it was observed that there was an increase of $BrdU^+$/DCX^+ cells in the patient's organoids indicating premature neurogenic non-proliferative divisions. The study concluded that the loss of CDK5RAP2 led to premature neural differentiation so that the founder population failed to fully expand, thus leading to microcephaly [15].

Another recent application of the CNS organoid as a neurodevelopmental disease model is in the study of the ZIKA virus. Previous studies had established the ZIKA and other flaviviruses activate the transcription of Toll-like receptor 3 (TLR3) in human skin fibroblasts. TLR3 is also implicated in many neuroinflammatory and neurodegenerative disorders, whose effects are visible even in NPCs. It was determined by qRT-PCR that TLR3 is upregulated in organoid and neurospheres after ZIKA infection. In subsequent testing, neurospheres inoculated with ZIKA were significantly different than those that were inoculated with ZIKA and a TLR3 competitive inhibitor. Although they are appeared to be cell death and disruption of the developing neuroepithelium in those organoids treated with the TLR3 inhibitor, the ZIKA-mediated apoptosis and organoid shrinkage was attenuated compared to those inoculated without the TLR3 inhibitor. The activation of TLR3 in early brain development likely triggers apoptosis by inhibiting SHH and Ras-ERK signaling in NPCs. This early loss of NPCs limits the extent to which the fetal brain can grow and develop, inducing a similar phenotype to genetic microcephaly (**Table 1**) [10, 16].

Both microcephaly and ZIKA virus infection impact brain development during the key period of corticogenesis. Researcher employing CNS organoids to study the induction of folding of cortical tissue, identified the PTEN-AKT regulatory pathway in the development of an expanded VZ/SVZ region [32]. Deleting PTEN results in the expanded VZ/SVZ region with more proliferating NPCs and further lead to the generation of CNS organoids with increased overall size and larger surface area characterized by continuous cortical folds. These PTEN mutant organoids demonstrated a marked increase in susceptibility to infection by ZIKA resulting in increased cell apoptosis compared to control CNS organoids. Similar efforts in studying dysfunction during corticogenesis with CNS organoids includes pathology of lissencephaly with its patients demonstrating smooth surface brains due to defective radial cell migration during corticogenesis [33].

While ideal for modeling neurodevelopmental diseases, CNS organoids are also used for studying early stage conditions for neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease. This is made possible through the use of patient-derived iPSCs which possess the appropriate genetic background for the neurodegenerative disease at question. Due to their maturity, CNS organoids are limited in their ability to model late stages of neurodegenerative diseases. Current studies mainly focus on examining the occurrence of known

pathological markers associated with the diseases. Studies showed that Alzheimer's disease patient-derived organoids were capable of recapitulating key Alzheimer's pathology such as the aggregation of misfolded amyloid-β plaques and neurofibrillary tangles [34-36]. In the same vein, hMLOs generated from Parkinson's disease patient-derived hiPSCs in 3D culture possessed neuromelanin-like (NM) granules, which were absent in 2D culture, suggesting not only the maturation of mDA cells, but the appearance of the NM granules in the hMLOs is important for studying pathology of Parkinson's disease [20]. In addition, these CNS organoids offer novel opportunity to evaluate and screen for therapeutic drugs and determine their efficacy and safety for the neurodegenerative diseases. Future work would be to develop protocols able to effectively induce aging in the CNS organoids in order to model late-stage brain structure and activity.

Future perspective and challenges

CNS organoid models have demonstrated a strong potential to be an effective means of studying human brain development and pathology. The efficacy of CNS organoids as models is constantly improving with continual refinements to protocol methods, but CNS organoids can still be developed with regards to (i) the degree they will be able to model the targeted region of the CNS, (ii) the absence of certain tissues and cells in the organoids, and (iii) their use in modeling neurodegenerative diseases that emerge later in life.

The first issue is due to the CNS organoid's self-organization ability based upon autonomous intra- and intercellular electrochemical and mechanical signals [37]. The microenvironment in which the organoids grows is inherently incomplete by the loss surrounding fetal tissues and organ systems that the native fetal brain would interact with [1]. The loss of this larger environmental interaction results in the organoids developing with the only the autonomous electrochemical interaction and what signaling can be provided by researchers. This overall loss of environmental interaction results in both whole-brain and region-specific CNS organoids' individually varying in how well they successfully model the *in vivo* fetal brain. This inconsistency requires researchers to cultivate a plurality of organoids in any assay so as to compare any and all end result variations for morphological and cellular content. This is necessary to ensure that the object of research within the model is more likely to be consistently observed. Thus, an immediate improvement would be to construct an environment more-closely resembling the brain environment that the CNS organoids would develop within. A possible improvement is to use a cell culture medium that not only promotes cell survival and differentiation, but that can simultaneously also promote the functional maturation of the neurons. BrainPhys neuronal medium is a great candidate because this medium was developed in order to combine these two roles resulting in mature human neurons more physiologically active and stable during long-term culture as compared to artificial cerebrospinal fluid, and traditional media, such as DMEM/F12, and Neurobasal medium [38].

To further create this environment, another focus is developing an effective 3D scaffolding that is able to mimic native biochemical signaling providing a higher degree of spatio-temporal control over and improving the viability of the organoids [39]. *In vivo*, ECM are essential in maintaining soluble growth factors, ion channel organization, and the heterogeneity of molecular composition further enabling the integration and differentiation of stem cells in the embryonic brain [40]. As of now, studies have been focusing on developing an artificial ECM able to model fetal brain ECM, but also be dynamically tunable with the culture's physical and chemical properties. The synthetic ECM needs to be able act as a 3D scaffolding for the CNS organoid to grow around [41]. As a result, there is a need for the advanced 3D printing technology able to manufacture it. A recently developed integrated tissue-organ printer that can fabricate stable, human-scale tissue constructs. This printer is designed to be able to create tissue incorporating microchannels for the diffusion of nutrients able to exceed the 100-200 um requirement for cell survival in engineered tissue [42]. This is compounded with the use of a new bioink made of a biopolymer, gellan gum-RGD (RGD-GG), which was found to have a profoundly positive effect on the proliferation of neural cells and formation of networks [43]. Another advance is the creation of a specially-designed combination hydrogel scaffold which

is constructed from the extracellular proteins and carbohydrates found in the brain environment via the use of 3D printing [44]. This hydrogel system has been shown to be able to better mimic the brain microenvironment. In addition, CNS organoids grown in biodegradable ECM show more signs of cells differentiation compared to scaffolding without the ability to degrade. This is also an example of a mechanism allowing a greater degree of control over the microenvironment by making the ECM biodegradable in a photo-activated manner [37].

Until recently, protocols could only be composed of cells derived from the neural lineage, so tissues composed of cells such as microglia and vasculature tissue that had not originated from the neuroectoderm were absent. The lack of vasculature tissue still poses the most immediate issue in organoid development with the lack of an effective means for nutrients and oxygen to be transported to the interior of the organoid [45]. As a result, cells on the interior are progressively less able to sustain themselves as the organoid grows and matures. This goes until the eventual cell death compromises the structural support of the organoid. This interior degradation presents the most immediate limit to the organoid's ability to continue modeling postnatal brain development and ageing beyond the early fetal brain development [9, 15]. Therefore, by developing a means to engender the vascularization throughout the organoid, it can let development of the CNS organoid go past the current early fetal brain developmental stages. Current work in increasing vascularization include neoangiogenesis, where the scaffolding is seeded with endothelial cells that will become new blood vessels, microfluidic devices to allow uniform distribution of flow and mass transfer, and the creation of collagen constructs small enough to avoid diffusion limitation but then are covered with endothelial tissue to create larger perfusion structures [39, 40, 46].

Microglia are derived from primitive macrophages that migrate from the embryonic yolk sac during early development via the circulatory system [2, 45]. Microglia not only perform the functions of the immune system in the brain, but also restore and maintain the homeostasis of the brain microenvironment. The morphology of a microglia cell demonstrates its various abilities to perform these roles by having its surface covered with a large quantity of membrane receptors of varying types able to respond to a large and diverse variety of chemical signals [47]. Recent studies have demonstrated the successful differentiation of hiPSCs to microglia-like cells [48-50]. Particularly, a very recent study showed the integration of these hiPSC-derived microglia-like cells into brain organoids [49]. These microglia-like cells were able to mature, ramify processes, and transform into an amoeboid form in response to injury in the organoids [49]. The generation of CNS organoids with microglia creates a model more effectively able to model the *in vivo* fetal brain and opens new avenues of research into studying microglia's possible roles in disease pathology.

CNS organoids mimic the slow chronology of native human neurodevelopment. Thus, in order to model late-onset neurodegenerative diseases, effective means of inducing accelerated aging are needed. Generating aged CNS organoids can potentially be achieved through i) as aforementioned, integrating vasculature tissue into organoids to maintain them for long term. ii) integrating microglia and further inducing them to release inflammatory cytokines. Somatic cells' aging is affected by the presence of inflammatory cytokines, so accelerated aging could be achieved by directly adding inflammatory cytokines to the culture or integrating microglia cells into the CNS organoids and then inducing release of cytokines from these microglia [51, 52]. iii) altering the culture environment. Along with using media such as BrainPhys neuronal medium, an additional way of promoting aging would be to apply oxidative stress to the organoid culture as oxidative damage is one of the primary determinants of aging [53]. In addition, forced expression of progerin has also been used to induce aging phenotypes in iPSC-derived neurons to reproduce features of Parkinson's disease such as Lewy-body-precursor inclusions and enlarged mitochondria [54]. Further refinements to the culture protocols will enable CNS organoids to more effectively model neurodegenerative disorders.

The CNS organoid offers an effective complementary model to studying human development and disease pathology. As issues such as vascularization and functional cell maturation

are overcome, the CNS organoid becomes an increasingly effective model for studying the pathology behind not only complex neurodevelopmental disorders, but also neurodegenerative disorders such as Alzheimer's disease [36, 49].

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Peng Jiang, Department of Cell Biology and Neuroscience, Rutgers University, Piscataway, NJ 08854, USA; E-mail: jiang@dls.rutgers.edu

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