Review Article

Applications of Brain Organoids for Infectious Diseases

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Abstract

Brain organoids are self-organized three-dimensional aggregates generated from pluripotent stem cells. They exhibit complex cell diversities and organized architectures that resemble human brain development ranging from neural tube formation, neuroepithelium differentiation, neurogenesis and gliogenesis, to neural circuit formation. Rapid advancements in brain organoid culture technologies have allowed researchers to generate more accurate models of human brain development and neurological diseases. These models also allow for direct investigation of pathological processes associated with infectious diseases affecting the nervous system. In this review, we first briefly summarize recent advancements in brain organoid methodologies and neurodevelopmental processes that can be effectively modeled by brain organoids. We then focus on applications of brain organoids to investigate the pathogenesis of neurotropic viral infection. Finally, we discuss limitations of the current brain organoid methodologies as well as applications of other organ specific organoids in the infectious disease research.

Introduction

The brain and central nervous system are susceptible to viral, bacterial, fungal, or parasitic infections.[1] The brain is particularly sensitive to infections due to insufficient host defense mechanisms. There are many pathogenes that cause familiar infectious diseases, such as the common cold and flu, which affect the respiratory and digestive systems but not the brain. However, many viruses, such as SARS-CoV-2, Zika virus (ZIKV) and human immunodeficiency virus (HIV), show neurotropic properties and cause severe illnesses in the central neural system.[2–12] Neurotropic virus infections can cause acute or chronic brain dysfunction and result in huge economic burdens on our society due to the challenges in providing treatment to patients. This is largely due to unique features of the brain, which consist of many different cell types, complex structures and intricate neuronal networks.[13–15] Infection by neurotropic viruses can irreversibly disrupt the complex structural and

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functional architecture of the brain and induce local immune responses, which can leave patients with a poor prognosis.[16]

It is very difficult to study the impact of infectious diseases on the human brain directly because of ethical and technical constraints. Many studies on infectious disease have relied on autopsy samples. The limited access to primary human brain tissues and a lack of robust *in vitro* human disease models has precluded most mechanistic studies. Traditionally, researchers investigate the effect of neurotropic virus infections by using immortalized cell lines or animal models. However, most immortalized cell lines are of cancer origin and primary human cells cultured in a monolayer do not have any tissue structure and exhibit limited cell diversity, which is not fully representative of the complexity of the human brain.[17] These disadvantages of monolayer cell culture limit our understanding of the effect of neurotropic viruses in complex tissues and on different neural cell types. Mice and humans share evolutionarily conserved neural architecture made up of similar types of brain cells.[18] Mouse models can also be genetically modified and disease phenotypes can be studied within an intact organism to understand disease mechanisms and progression. However, it is increasingly clear that there are important differences between these species.[19,20] For example, cell cycle length of neural progenitors is much longer in humans than in mice and humans show a greater complexity of different cell types.[21] More importantly, some viruses do not infect mouse cells efficiently due to the lack of necessary cellular components, such as the ACE receptor, which is critical for SARS-CoV-2 infection.[22,23] Although mouse models have allowed us to learn a great deal about neurotropic viruses, it is also important to have human-derived models to study mechanisms of neurotropic virus-induced neural disorders. Human pluripotent stem cell-derived brain organoids represent the most promising model, which can provide a powerful *in vitro* platform for mechanistic studies and identifying novel therapeutic interventions.[7]

In this review, we first introduce recent advances in brain organoid technologies, and their applications as model systems and discovery tools. We then discuss applications of brain organoids for modeling infectious diseases involving neurotropic viruses. Finally, we discuss the limitations of current brain organoid methodologies for modeling infectious diseases and opportunities for further advances.

Current brain organoid methodologies

Methodologies to induce neural differentiation from pluripotent stem cells, such as embryonic stem cells (ESCs), in three dimensions (3D) have been pursued since the early 1980s. When cultured in suspension, mouse ESCs follow intrinsic developmental trajectories to form small spherical cell aggregates called embryoid bodies (EBs), mimicking certain features of the developing embryo with the capacity to differentiate into cell types of all three germ layers.[24–27] The formation of organized neuroectoderm from EBs was first reported by using mouse ESCs in 1996[28] and then human ESCs in 2001.[29] These early studies show that EBs can differentiate into the neural lineage when they are cultured on an adhesive coating substrate and in the presence of fibroblast growth factor 2 (FGF2). The 3D EBs grown on the substrate transform into two-dimensional (2D) neural rosettes, with primitive neural stem cells radially organized around a lumen, structures reminiscent of the embryonic neural tube.[30] Although the neural rosettes exhibit high levels of self-organization and recapitulate important features of very early embryonic development of the nervous system, further differentiation of these primitive neural stem cells into neural stem cells with regional identities responsible for generating different brain regions, for example forebrain, midbrain, hindbrain and spinal cord, is lacking and there is no subsequent formation of the cytoarchitectural signatures of the nervous system.[31] One important step toward the generation of 3D brain organoids was the discovery in 2002 that EBs could be directed to form a homogeneous population of primitive neuroectoderm in suspension by using conditioned media from a human hepatocellular carcinoma cell line.[27] By manipulating Wnt or Shh pathways, it was later shown that EBs could be further patterned into distinct cortical subregions with neural stem cells showing either dorsal or ventral telencephalic identities, respectively.[32,33] While recapitulating many early spatial and temporal features of early cortical development, further neurodevelopmental events such as neuronal subtype differentiation and spatial organization into discrete layers were not observed. Subsequently, several studies found that extracellular matrix (ECM), such as Matrigel, plays an important role in promoting the formation of polarized neural tube-like buds from neuroepithelial tissue.[34–37] The first human cerebral organoid protocol, now often referred to as an unguided protocol, was established in 2013 by embedding human ESC (hESC)- or induced pluripotent stem cell (hiPSC)-derived EBs in Matrigel droplets.[38] Around the same time, Kadoshima et al. took a different approach and used a TGFβ inhibitor and a Wnt inhibitor to promote telencephalic differentiation, which was the first guided brain organoid protocol from human ESCs.[37] Following these initial studies, brain organoids that mimic the embryonic human cerebral cortex have been further characterized and many key developmental signatures have been observed in this model. For example, cortical organoids can recapitulate the structural organization of neural progeni-

tors and contain a large ventricular zone (VZ) formed by SOX2-expressing ventricular radial glia cells (vRGs).[9,37,39,40] An inner subventricular zone (iSVZ) layer can also be identified by TBR2 (EOMES)-expressing intermediate progenitor cells. A well-defined outer SVZ (oSVZ) can be observed in cortical organoids expressing outer radial glia (oRG)-specific markers, such as HOPX and PTPRZ1.[9,39–41] Another important aspect of the human brain is cell diversity, including many different subtypes of neurons and glial cells. Several studies have investigated the cellular composition of brain organoids using large-scale single-cell RNA-sequencing and revealed a broad diversity of cell types in cerebral organoids, including several types of cortical neurons as well as glial cells, such as astrocytes.[42] These data suggest that brain organoids can recapitulate some of the cell diversity of the human brain and provides information about the molecular developmental trajectories of cell types and cellular composition. Examples of unguided and guided organoid strategies are illustrated in Figure 1, and interested readers can consult several additional reviews on general topics of brain organoids.[43–48]

Unguided methodologies: self-patterned cerebral organoids

The unguided method relies mainly on the intrinsic capacity of human pluripotent stem cells to generate neural tissue without the use of patterning factors. Based on this principle, Lancaster et al. developed a cerebral organoid culture method with minimalistic media. The first step of this method is to culture pluripotent stem cells in a floating manner to allow the formation of EBs. EBs are then differentiated into neuroectodermal tissue, which is further developed by embedding in Matrigel.[47] Embedding neuroectodermal progenitors in Matrigel can enhance the formation of neural rosettes within the 3D structure.[38] Subsequently, the neuroectodermal progenitors differentiate into neural progenitors, which will generate neurons and other cell types. In this method, the human pluripotent stem cells differentiate in a stochastic way, which give rise to progenitors and neural cells of a variety of brain regions, such as forebrain, midbrain, hindbrain, retina, choroid plexus and mesoderm.[38,49] Large scale single-cell transcriptomic analysis has further revealed that cerebral organoids derived from unguided methods contain many different types of cells, such as astrocytes, oligodendrocyte precursor cells, inhibitory neurons, excitatory neurons, neural progenitors and retinal cells.[42] Due to self-patterning-based development, the unguided method generates highly heterogeneous cerebral organoids that vary across individual organoids, different batches and hiPSC lines in terms of both morphology and brain region composition.[42,50–52] Although unguided cerebral organoids provide a valuable model to study interactions among different brain regions, the heterogeneity and variability limits quantitative analyses and mechanistic studies.

Guided methodologies: Brain region-specific organoids

During brain development, neural stem cells acquire regional identity depending on the location of the cells along the anterior-posterior (AP) and dorsal–ventral (DV) axes of the neuroectoderm. For example, the early neuroectoderm is patterned by a variety of caudalizing signals along the AP axis, including WNT signaling and FGF8.[53] The patterning effect relies on the concentration, combination and timing of these secreted molecules.[45–47] For example, manipulation of BMP and TGF-β signaling by small molecules, such as dorsomorphin and A-83, have been shown to induce broad neuroectodermal identity, whereas inhibition of TGF-β and WNT signaling mainly induces telencephalic identity.[37,54] Several molecules have been used to guide the identity along the DV axis, including WNT, BMP and sonic hedgehog (SHH). For example, activation of WNT signaling at the later stage of telencephalic organoid culture can enrich cerebral cortex tissue[9,39–40] and the combination of BMP and WNT treatment enhances the medial pallium fate to generate hippocampal tissue.[55] On the other hand, the ventral fate largely relies on SHH, secreted by the floorplate and the notochord.[56] Activation of SHH is critical for ventral fates of the telencephalon,[57–59] midbrain and hypothalamus.[9,60]

Based on the extensive knowledge learned from cumulative studies of brain development, Sasai's group developed a series of 3D differentiation protocols by using combinations of growth factors or small molecules targeting specific signaling pathways.[37,54,55,61–63] A large number of protocols have since been developed to generate brain region or subregion-specific organoids from hESCs or hiPSCs in the field by other investigators (Figure 1). In general, a "molecular cocktail" is supplemented to EBs to guide neural stem cell specification with regional identity, including neocortex, hippocampus, midbrain and cerebellum. For example, to generate midbrain organoids, Qian et al. applied SHH agonists (recombinant SHH and Purmorphamine), FGF-8, SMAD inhibitors (SB431542 and LDN193189), and a GSK3β inhibitor (CHIR99021) to induce floor plate differentiation of hiPSCs to acquire midbrain identity.[9] These brain organoids recapitulate key features of the regional human brain development, such as structural organization, neurogenesis, cell diversity and gene expression. More importantly, these models are more reproducible and can be subjected to quantitative analyses. Thus, the brain region-specific organoids provide valuable human cell models to study neurotropism and pathogen-host interactions and to

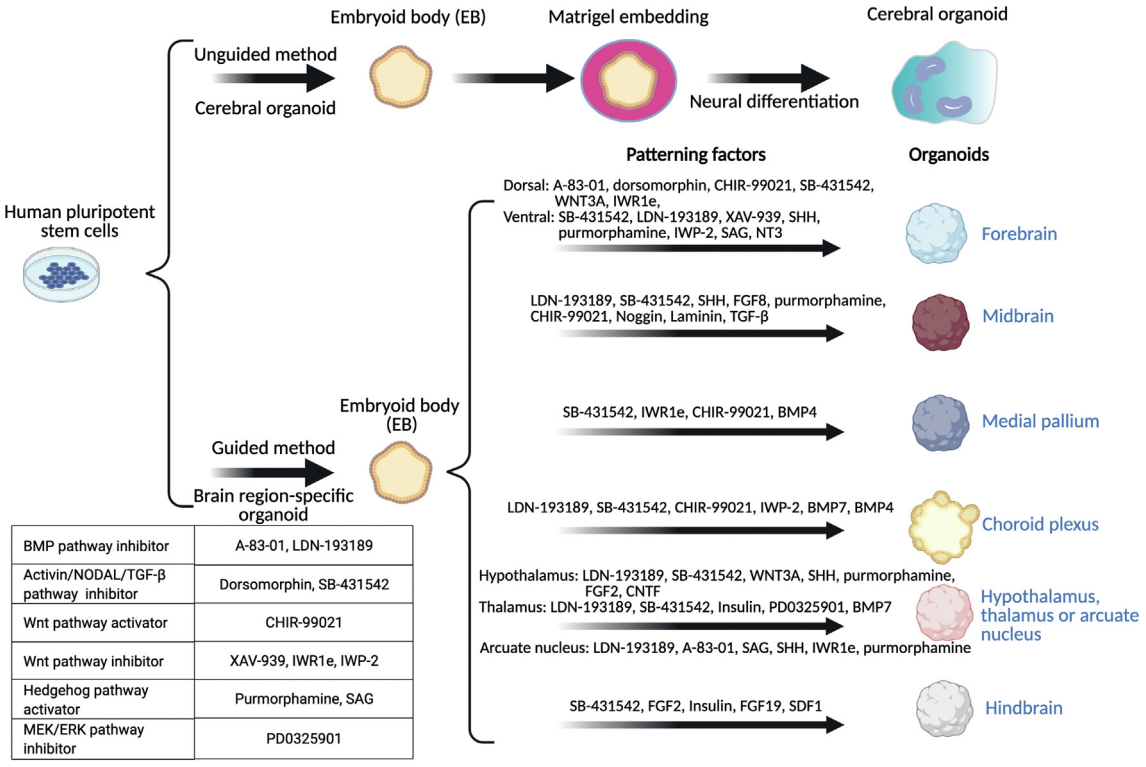

Figure 1. Unguided and guided approaches for generating brain organoids from human pluripotent stem cells. Unguided approaches (top) follow the intrinsic signaling and self-organizing capacity of hiPSCs to differentiate into cerebral organoids mimicking the early development of the human brain. Due to spontaneous differentiation, cerebral organoids often contain heterogeneous tissues resembling various brain regions. By contrast, guided approaches (bottom) use a combination of patterning factors, such as small molecules and growth factors, to generate brain region-specific organoids resembling one region of the developing human brain.

understand underlying mechanisms and develop therapeutic interventions.

Application of brain organoids for modeling neurotropic virus infection and understanding its impact on the brain

Identification of viral tropism

Viral tropism refers to the capability of an infectious virus to infect particular cells (cellular tropism), tissue (tissue tropism) or host species (host tropism).[64] Not all types of virus can infect humans. To understand the characteristics of a particular infectious virus and its impact on humans, the first important aspect is to know the host tropism of the virus. While not completely understood, it is clear that some viruses can evolve and jump the species barrier from animals to humans, such as influenza viruses and coronaviruses.[65,66] The next step is to identify tissue tropism and cellular tropism, which is critical for understanding the mechanism of viral infection, its pathogenesis and to develop targeted treatments. However, due to the limited access to primary human tissue, it is sometimes difficult to appreciate the full extent of the tissue and cellular tropism. For example, if a patient presents with neurological symptoms after a viral infection, it is difficult to know whether this is due to direct infection in the brain or secondary immune responses that impact brain function. Most current studies rely heavily on clinical data and examination of postmortem brains.[67]

As a new tool that has been established only in the last decade, human brain organoids provide an unprecedented opportunity to study susceptibility and cellular tropism of viral infection and its consequences in the nervous system. The hiPSC-derived forebrain organoids were the first to be applied to study the tropism of ZIKV[7,68] (Table 1). ZIKV is a mosquito-borne flavivirus, first identified in monkeys in Uganda in 1947 and later identified in humans in 1952 in the United Republic of Tanzania and Uganda (WHO, 2018). A sudden outbreak in over 70 counties in 2016 resulted in an epidemic. While ZIKV is capable of infecting adult humans and typically leads to mild symptoms, much attention has been drawn towards the co-occurrence of the 2016 ZIKV outbreaks and an increased incidence of newborns with microcephaly, a condition in which the head circumfer-

Table 1 Applications of brain organoids in the neurotropic virus research.

VIRUS	ORGANOIDS	MAJOR FINDINGS	REFERENCES
ZIKV	Forebrain organoids; Cerebral organoids;	Increase neural progenitor cell death; Increase stress response and antiviral response; Decrease cell proliferation and organoid growth;	4,6,9,90
CMV	Cerebral organoids	Increase cell apoptosis and decrease cell proliferation	90,110–112
SARS-CoV-2	Choroid plexus organoids; Midbrain organoids; Cortical organoids; Hypothalamic organoids; Cerebral organoids	Increase epithelial cell death; Increase cytokine transcription; Decrease cell adhesion and CSF transport transcription.	8,83–84,94,96
HSV-1	Cerebral organoids	Impaired neuronal differentiation and dysregulated cortical layer and brain regionalization; Increase cell death and non-neural differentiation genes transcription	90,152
HIV-1	Cerebral organoids	Increase inflammatory response and tumor necrosis factor (TNF-α) and interleukin-1 (IL-1β) expression	141

ence of an infant is significantly smaller than expected, often due to abnormal brain development.[68] Due to the increased reports of microcephaly coinciding with clusters of ZIKV outbreaks in Brazil, the WHO declared a public health emergency of international concern (PHEIC).[69] ZIKV has been detected in almost all types of body fluids, including salvia, semen, tears and urine.[70–73] More importantly, ZIKV was found in the microcephalic fetal tissue from women infected with ZIKV during pregnancy.[74,75] However, live infected fetal tissue is not accessible, postmortem tissues are highly variable in terms of sample quality and genetic background, and clinical studies cannot provide sufficient information to determine the causality between ZIKV infection and microcephaly. In an attempt to link ZIKV exposure and microcephaly, Tang et al. found that ZIKV is capable of infecting hiPSC-derived forebrain-specific cortical neural progenitors in monolayer cultures.[10] This specific tropism toward neural progenitors was soon confirmed using hiPSC-derived forebrain organoids, as well as cerebral organoids[7]. In addition, astrocytes were found to be a target of ZIKV in forebrain organoids.[68]

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was responsible for the world-wide COVID-19 pandemic in 2020 that had resulted in 3.8 million deaths by June, 2021 (WHO, 2021). Infection of SARS-Cov-2 is associated with acute respiratory distress syndrome as well as other comorbidities, such as hypertension, diabetes and chronic obstructive pulmonary disease.[76] However, many patients infected by SARS-CoV-2 developed neurological symptoms, such as dizziness, headache and myalgia.[77–79] Other neurological symptoms also include peripheral nervous system and central nervous system manifestations, such as impaired olfaction, impaired gustation, neuralgia, acute cerebrovascular disease, ataxia and seizures.[80–82] Whether this array of neurological symptoms was a result of SARS-CoV-2 neurotropism or indirect systemic immune responses was difficult to know. hiPSC-derived brain organoids, including region-specific organoids that modeled the choroid plexus, cerebral cortex, hippocampus, hypothalamus, and midbrain, were employed to investigate the neurotropism of SARS-CoV-2 and its functional consequence after infection[8,83] (Table 1). In these brain organoids, neurons and glial cells were sparsely infected by SARS-CoV-2, probably due to the low expression of the viral receptor ACE2 in the cells.[8,83,84] Surprisingly, choroid plexus epithelial cells showed relatively high and productive infection by SARS-CoV-2 (Figure 2).[8,83] A subsequent study also showed dysregulation of choroid plexus in postmortem adult human brain tissue.[85]

Many emerging or re-emerging infectious viruses can infect the brain and spinal cord, causing meningitis, encephalitis and microcephaly.[86] For example, West Nile virus has been a threat in the United States for a number of years.[87] Using hiPSC-derived neural stem cells and neurons, West Nile virus shows neurotropism and high infection rates in these cells, leading to massive cell death.[88] However, little is known about cellular tropism of other neurotropic viruses such as Japanese Encephalitis, Eastern Equine Encephalitis, Tick Borne Encephalitis, Deer Tick, and Powassan.[86] Human brain organoids offer critical advantages for investigating the tropism of these neurotropic viruses in a physiologically relevant model system.

Identification of pathophysiology of viral infection at cellular and structural levels

As tissue and cellular tropism can be accurately reflected in brain organoids, this model system provides a previously unavailable opportunity to understand the pathology associated with viral infection in relevant human neural cell types at both cellular and structural levels. For example, some babies born to ZIKV-infected mothers exhibited thinner cortical layers, the hallmark of microcephaly.[74,89] In forebrain organoids, infection of ZIKV results in a reduced progenitor cell layer, as well as a reduction in cortical layers, structural features resembling the microcephalic brain.[68] The preferential infection of radial glia cells by ZIKV results in decreased cell proliferation and increased

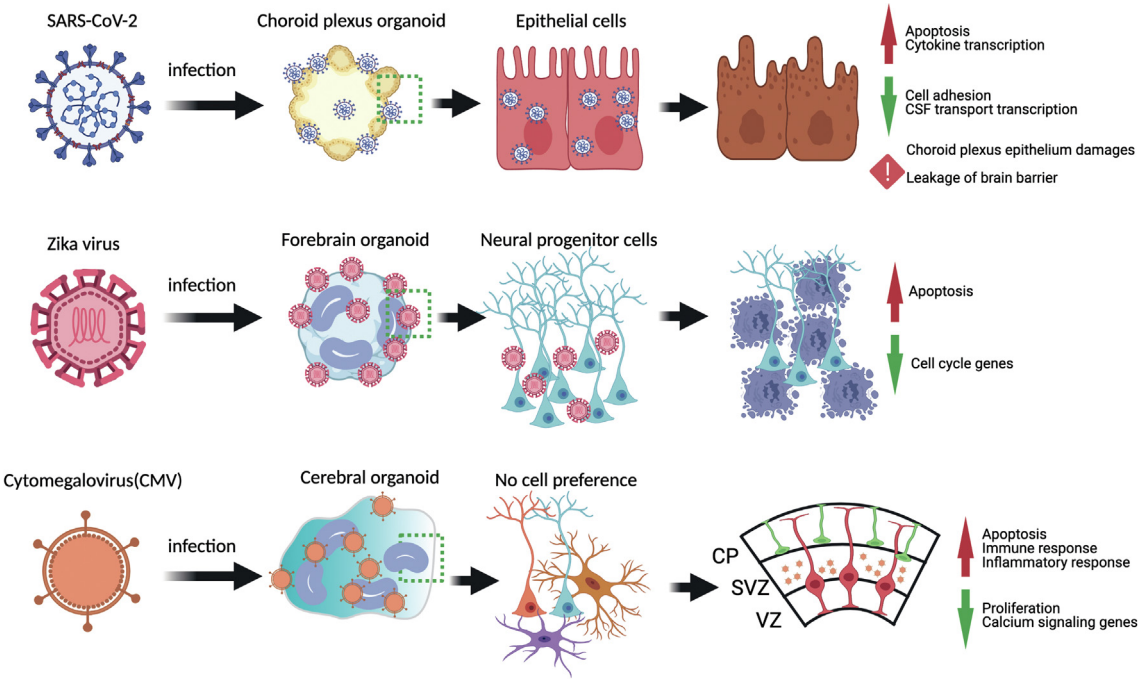

Figure 2. Examples of the impact of neurotropic viruses on different biological processes in brain organoids. SARS-CoV-2 (top) illustrates neurotropism and productively infected choroid plexus epithelial cells. SARS-CoV-2 infection of choroid plexus organoids induces extensive cell death, choroid plexus epithelium damage, and leakage of the blood–brain barrier. SARS-CoV-2 infection also leads to upregulation of cytokines and downregulation of cell adhesion and cerebrospinal fluid (CSF) transporter-related genes. ZIKV (middle) shows preferential infection of radial glia cells in forebrain organoids, inhibition of cell proliferation and expression of cell cycle genes, and an increase of cell death in both a cell-autonomous and non-cell-autonomous fashion. CMV (bottom) infection induces brain organoid malformation, including reduced growth and abnormal structure of cortical layers. CMV infection also increases cell death, immune responses, and inflammatory responses while decreasing cell proliferation and calcium signaling-related gene expression.

cell death (Figure 2).[9] Subsequently, many studies have shown that infection with ZIKV could reduce the growth of the brain organoids generated by unguided and guided methodologies.[4,6,9,90] Interestingly, cell death was found not only in infected neural progenitors, but also in uninfected neurons, suggesting both cell-autonomous and non-cell-autonomous effects. Herpes simplex virus (HSV) is another prevalent neurotropic virus that may induce newborn microcephaly[90,152] (Table 1). Using brain organoids as a model, recent studies found that HSV-1 mostly targets neuroepithelial cells and induces cell death.[90,152] Consequently, HSV-1-infected brain organoids show impaired neuronal differentiation and cortical layer formation.[90,152] Compared to ZIKV infection of brain organoids, apical accumulation of N-cadherin was reduced in HSV-1-infected neural progenitors.[152] It suggests that HSV-1 infection specifically disrupts neuroepithelial integrity of brain organoids. Together, these studies provide an explanation of a potential connection between ZIKV or HSV-1 infection during pregnancy and microcephaly and demonstrate that brain organoids can be a powerful tool to understand ZIKV and HSV-1 pathology.

COVID-19 patients frequently present with neurological symptoms.[77–79] Extensive studies have been performed on the effect of SARS-CoV-2 infection in the brain by using hiPSC-derived monolayer neural progenitor cells, neurons, astrocytes and microglia as well as 3D brain organoids.[8,12,83,84,91–96] Upon SARS-CoV-2 exposure, choroid plexus organoids are particularly susceptible to SARS-CoV-2 infection due to the high expression level of ACE2, which is critical for viral entry.[8,12,83] After SARS-CoV-2 exposure, infected cells tend to die and the tight junctions between choroid plexus epithelial cells may incur progressive damage, leading to leakage across this important barrier that normally prevents the entry of some cytokines, immune cells, and pathogens into the cerebrospinal fluid (Figure 2).

Human cytomegalovirus (CMV) is a ubiquitous and highly adapted human pathogen that establishes lifelong latency in infected individuals.[97] CMV infection is one of the major causes of birth defects and childhood disorders in the United States.[98] CMV infection during pregnancy can result in viral transmission to the developing fetus and consequently lead to birth defects

in newborns.[97] Most children (60–90%) with symptomatic CMV infection and 10% to 15% children with asymptomatic CMV infection could develop one or more neurodevelopmental disorders, including intellectual disability, hearing loss, microcephaly or cerebral palsy.[97,99,100] Current studies indicate that the number of children with neurological disorders related to *in utero* CMV infection is much higher than the other better-known childhood disorders, such as fetal alcohol syndrome, Down syndrome or spinal bifida.[101] The neurotropism of CMV is evident from the predominant symptoms of the central nervous system in newborns. However, CMV-induced neuropathogenesis in the developing brain is poorly understood. Many studies have employed animal models to investigate the pathogenic mechanism of CMV infection and its neurotropism in the developing brain, but species-specific differences are a major barrier to understand the mechanism of CMV infection in the human brain.[97,102] Moreover, most studies on the effect of CMV infection in the brain are performed with cultured human brain cells, such as neural progenitor cells,[103,104] astrocytes,[105] brain microvascular endothelial cells,[106–108] neuronal cells,[108] microglia/microphages[109,110] and oligodendroglial cells.[111] Brain organoids have also been used to investigate the effect of CMV infection.[112–114] Infection of brain organoids with the clinical-like CMV strain TB40/E results in the impairment of organoid growth and development of cortical structures, mimicking CMV-induced microcephaly in newborns (Figure 2). Further analysis on the TB40/E strain-infected brain organoids found that TB40/E infection results in aberrant formation of the outer neural progenitor and cortical layers.[112] Compared to mock-infected organoids, TB40/E-infected organoids exhibit a much lower rate of proliferation and a higher rate of apoptotic cell death.[112] Together, these studies suggest that brain organoids provide a powerful platform to explore viral pathology.

Identification of pathological mechanisms at the molecular level

hiPSC-derived brain organoids have emerged as a powerful model to study human brain development and have been used to investigate the infection mechanism of ZIKV, SARS-CoV-2 and CMV in the brain. Mechanisms underlying ZIKV infection and pathogenesis in the brain have been studied extensively. Several pathways have been identified to explain the mechanism of ZIKV infection and its functional consequence at the molecular level. hiPSCs differentiate into neural progenitor cells and were infected efficiently by ZIKV exposure. Global transcriptome analysis of infected human neural progenitor cells showed a dysregulation of cell-cycle related pathways and apoptotic pathways.[10] Using forebrain cortical organoids, Yoon et al showed that the ZIKV-encoded NS2A protein disrupts cortical neurogenesis with reduced proliferation, premature differentiation of radial glial cells, and aberrant positioning of newborn neurons by degrading adherens junction proteins.[115] In addition, it was shown that ZIKV infection activates toll-like receptor 3 (TLR3) in hESC-derived cerebral organoids, resulting in the dysregulation of a network of genes involved in antiviral defense pathways, response to interferon, neuronal development and apoptotic pathways.[4] In contrast, a recent study found that HSV-1-infected brain organoids show different gene signatures from ZIKV-infected brain organoids. GO term analysis of molecular signatures from HSV-1-infected organoids revealed enrichment in the regulation of developmental and cellular processes, as well as activation of multiple non-neural pathways.[90]

SARS-CoV-2 is another infectious virus, which exhibits neurotropism and induces many neurological disorders. SARS-CoV-2 productively infects choroid plexus organoids and increases cell death.[8,12,83] In these organoids, global transcriptome analysis showed that SARS-CoV-2 infection increases the expression level of genes involved in viral responses, RNA processing, response to cytokines, cytoskeletal rearrangement, and cell death. On the other hand, SARS-CoV-2 infection downregulates genes related to ion transport, transmembrane transport cilium, and cell junctions.[12,83] Upon SARS-CoV-2 exposure, brain organoids show a dysregulation of genes enriched in cell division, organelle fission and metabolic processes.[94] SARS-CoV-2 infection of brain organoids shows both cell-autonomous and non-cell-autonomous effects. SARS-CoV-2-positive cells show enrichment of genes involved in viral transcription and enzymatic processes, such as cytochrome C to oxygen and NADH to ubiquinone. Conversely, SARS-CoV-2-negative cells show a mitochondrial catabolic state with upregulation of alcohol metabolism, cholesterol synthesis and cell death. The hypermetabolic state is unique to SARS-CoV-2 infected cells.[94]

hiPSC-derived brain organoids also provide an opportunity to screen potential factors mediating infection of neurotropic viruses, such as CMV. For example, several receptors involved in TB40/E infection of organoids, such as epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor alpha (PDGFRα), and integrins (α3, α5, and β3) have been knocked down to investigate the importance of these receptors for CMV infection.[112] Brain organoids treated with EGFR- or PDGFRα-specific siRNA exhibit much lower levels of TB40/E infection, suggesting that both EGFR and PDGFRα are important mediators for CMV infection in the organoids.[112] Together, hiPSC-derived brain organoids allow us to investi-

gate the mechanism of infection and pathogenesis of neurotropic viruses in early human brain development.

Application of brain organoids for screening of treatment strategies for infectious diseases

Although there is a rapid progress in the preclinical development of antiviral drugs, testing the safety and efficacy of drugs in humans can take a significant amount of time. Effective and larger scale screenings of small molecule therapeutics are urgently needed. For instance, ZIKV-infected brain organoids have been used to facilitate drug screening.[116–118] Previous studies on ZIKV-infected brain organoids exhibited thinner neural progenitor and neuronal layers, and an overall reduction in organoid size, which are consistent with microcephalic features in newborns.[3,4,6,9,10] A high-throughput compound screening was designed using a caspase-3 activity assay in 2D cultures infected with ZIKV and identified emricasan and niclosamide as positive hits.[117] In this study, treatment with a combination of antiviral and neuroprotective compounds could effectively reduce ZIKV-induced cell death in human neural progenitors and astrocytes both in 2D and in brain organoids.[117] Another high-throughput compound screening study targeting ZIKV infection identified hippeastrine hydrobromide (HH) and amodiaquine dihydrochloride (AQ) as potential inhibitors of ZIKV infection.[118] Further validation found that HH suppresses viral propagation when administered to adult mice with active ZIKV infection, highlighting its therapeutic potential.[118]

The SARS-CoV-2 outbreak spread rapidly in 2020 and, although many patients presented with several neurological symptoms including such as dizziness, headache and myalgia,[77–79] there is no approved antiviral treatment for COVID-19-related neurological symptoms. Using hiPSC-derived brain organoids as a model system, Mesci et al. found that Sofosbuvir, an FDA-approved anti-hepatitis C drug, can rescue the cell death and impaired synaptogenesis induced by SARS-CoV-2 infection in cortical neurons.[93] It suggests Sofosbuvir could be a potential treatment to alleviate COVID-19-related neurological symptoms. Taken together, these studies suggest that the compound screening platform based on brain organoid models are effective and can lead to the identification of new therapeutic targets. Brain organoids derived from patient-specific iPSCs would be useful for precise drug selection and prognosis prediction in future.

Brain organoids may also be useful to evaluate the safety of existing treatments for viral infections for specific patient populations. One such example are the antiretroviral drugs that are used to combat or prevent infections of the HIV.[119] Although combinational antiretroviral therapy has been highly effective in suppressing the viral load in people living with HIV (PLWH), up to 50% of PLWH have some mild to moderate cognitive impairment.[120,121] Recently, questions have been raised about the possibility of neurotoxicity arising from the drugs themselves[122,123] and cell-type specific toxicity and dysregulation of cellular processes has been observed *in vitro* using rat primary neurons and human iPSC-derived co-cultures of microglia, astrocytes and neurons.[124–126] Because continuation of antiretroviral therapy throughout pregnancy is necessary to prevent vertical transmission of HIV to the fetus and maintain the health of the mother, it is critical to understand whether any antiretroviral drugs may selectively impact neural progenitors or the developing brain. Some longitudinal studies of children exposed to antiretroviral drugs but not infected with HIV have reported transient developmental delays,[127] while other studies have reported no detectable differences in cognitive outcomes following perinatal exposure to antiretroviral drugs.[128,129] Given the difficulties of controlling confounding variables in observational studies, brain organoid models could provide another avenue to generate data on the safety and off-target effects of therapeutic drugs on the developing human brain.

Limitation of current brain organoid models and future improvements

Recent advancements in human iPSC-derived brain organoids offer a promising opportunity to investigate human brain neurodevelopment, neurotropic infectious diseases, and perform high-throughput drug screening. However, like all model systems, there are many limitations of brain organoids.

The size of brain organoids generated from both unguided and guided methods is much smaller than the human brain, partially due to the insufficient supply of growth factors, nutrients and oxygen to the deeper regions of the organoids, which constrains the overall size.[38,130] Because neural progenitor cells have a high metabolic demand and are mostly located in the innermost regions of the organoids, they are the first to succumb to the diffusion limit and continuous neurogenesis cannot be sustained in long-term organoid cultures. Sliced brain organoid culture and agitation have been used to promote neuronal self-organization and growth factor diffusion, and inclusion of a circulatory system may promote cell survival by enhancing metabolite exchange.[9,38,39] Current brain organoid culture methodologies lack vascular cells, which are critical for the establishment of blood vessels in the brain. Pham et al. tried to incorporate endothelial cells in the brain organoids, but endothelial cells alone could not form a functional circulatory system within the organoids.[131] The blood brain barrier (BBB) is even

more critical for protecting the brain from pathogen infection. The BBB is a physical barrier that neurotropic viruses must pass in order to infect the brain.[132] Upon viral infection, a neuroimmune response is mounted and certain peripheral immune cells crosses the BBB to respond to invading pathogens.[133] Current approaches of viral infection in brain organoids mainly focus on inoculating organoid culture media with virus. Although these approaches allow researchers to study viral pathology in the organoids, it is not clear if the infection accurately models viral infection *in vivo* due to the lack of physical interaction between BBB cells and brain organoids. Since BBB is a fundamental component of the central nervous system, a lot of effort has been spent on developing an *in vitro* organoid model with BBB. Cho et al. developed spheroids with BBB by co-culturing primary human astrocytes and human brain vascular pericytes with two different human brain endothelial cell (EC) types: primary human brain microvascular ECs (HBMECs) and immortalized human cerebral microvascular EC line D3 (hCMEC/D3).[134] The spheroids with BBB exhibit key BBB features, such as structural organization and functional activity.[134] Co-culture between BBB spheroids and brain organoids could potentially serve as a useful model to study the pathology of viral infection through the BBB. Alternatively, brain organoids transplanted into the adult mouse brain have successfully integrated and developed a functional vasculature system within the organoids (Figure 3).[135] Organoid grafts have shown promising results, such as enhanced neuronal differentiation and maturation, integration of microglia, gliogenesis, and projection of axons to multiple regions in the host mouse brain.[135] The combination of brain organoids and an *in vivo* physiological environment provides an opportunity to study infectious diseases in human neural cells within vascularized and mature brain organoids. For example, recent studies on ZIKV and CMV using brain organoids *in vitro* have shown increased cell death and slower cell cycle kinetics in ZIKV or CMV-infected organoids. However, the consequences of ZIKV or CMV infections *in vivo* could be more extensive due to changes in the local environment following cell death that could lead to secondary pathology. In the organoid transplantation model, infected organoids or healthy organoids could be transplanted into infected or healthy animals (Figure 3). In this way, it would be possible to dissociate some of the effects of direct infection vs. microenvironmental influences on human cells within an *in vivo* microenvironment.

Brain organoids, particularly those generated with unguided protocols, rely on intrinsic mechanisms to self-organize and as a result, there is considerable heterogeneity and inconsistency from batch to

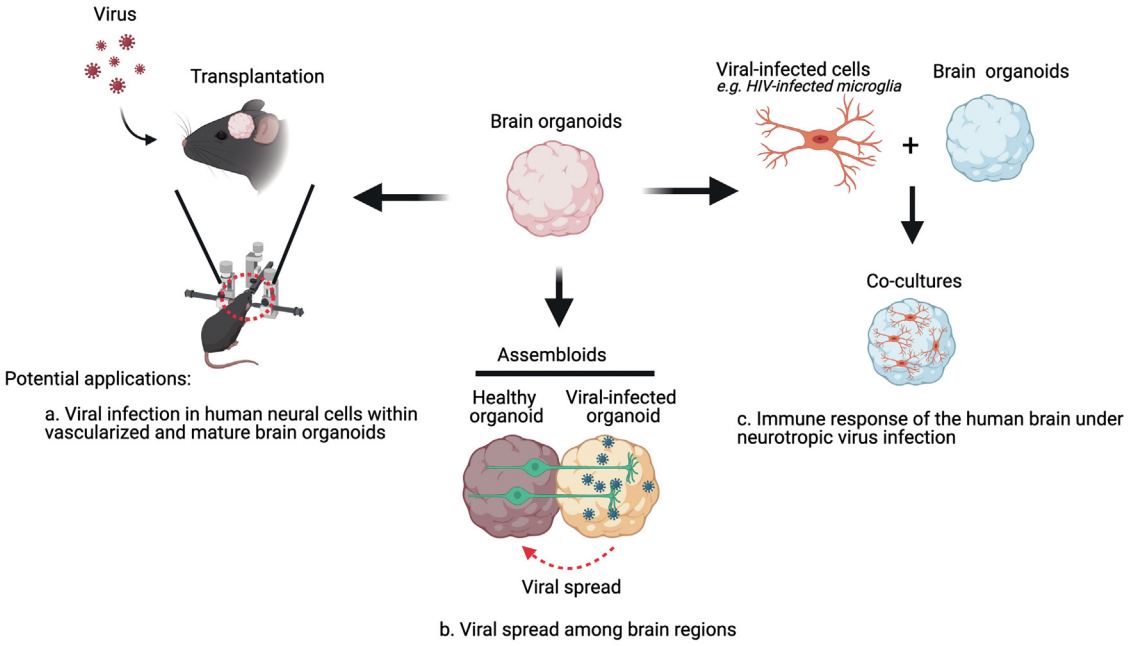

Figure 3. Next generation of organoids for infectious disease research. Transplantation of brain organoids (left) into immunodeficient mice allows organoids to develop a functional vasculature system and enhance neuronal maturation and gliogenesis. Mouse and human brain organoid chimeras allow the study of viral infection in human neural cells within vascularized and mature brain organoids. Co-cultured viral-infected neural cells with brain organoids in vitro (right), such as co-culture of HIV-infected microglia with brain organoids, allow the investigation of immune responses within brain organoids. Region-specific brain organoids generated by guided methods can be used to make "assembloids", which represent different brain region connections (bottom). Fusion between healthy and viral-infected brain organoids can be used to investigate the mechanism of viral spread among brain regions.

batch, even when similar conditions and procedures are used. An unguided protocol can lead to regional differences within a single organoid, which could provide an opportunity to explore these differences, but the heterogeneity restricts the utility of these organoids to investigate disease pathogenesis in a robust, quantitative and reproducible manner.[136] However, the development of brain region-specific organoid methods (guided method), fused organoids and 'assembloids' provides a path to investigate interbrain-region crosstalk. Several groups have developed fusion organoid methods that allow the study of interactions between brain regions. In this method, hiPSCs were differentiated into region-specific organoids separately and then fused together to form organoids with multiple brain regions, including cortical-subpallium assembloids and cortico-spinal-muscle assembloids.[57–59,137] Organoid fusion is a promising new strategy to better model the regional complexity of the human brain and identify potential region-specific differences in viral infections. For example, recent studies confirmed the neurotropic property of SARS-CoV-2 and revealed that although the ACE2 receptor is expressed at very low levels in most brain cells, it is highly expressed in choroid plexus cells. In choroid plexus-specific organoids, SARS-CoV-2 exposure induced extensive cell death in an autonomous and non-autonomous manner.[8,12,83] Considering our limited knowledge of the primary and secondary effects of SARS-CoV-2 within the brain, infected organoids fused with other region-specific organoids could shed light on the mechanisms of virus-mediated pathogenesis within the brain.

In addition to neural cells derived from an ectoderm lineage, the human brain is also composed of a variety of non-neural cells derived from mesoderm and endoderm lineages. The non-neuronal cells in brain organoids, such as astrocytes, could potentially affect viral infection. However, most current protocols drive cells into the neuroectoderm lineage directly by using dual-SMAD inhibition at the EB stage.[138] Non-neural cells, such as microglia, are therefore largely absent in the resulting organoids. As a result, most brain-specific organoids cannot recapitulate interactions among neural and some non-neural cells. Using an unguided organoid protocol, Ormel et al. found that brain organoids without dual-SMAD inhibition innately contain mesodermal progenitors, which are able to differentiate into microglia.[139] Alternatively, a co-culture system of microglia with brain organoids can also be employed to study the interaction between microglia and neural cells in physiological and pathological conditions. For example, a recent study employed a microglia and brain organoid co-culture system to study the human microglia response to ZIKV infection in brain organoids[140] and supports the idea that an integrated model could be used to investigate the immune response of the human brain to neurotropic virus infection. Another study used a microglia and brain organoid co-culture system to study HIV-1 neuropathogenesis in the human brain.[141] Upon HIV infection, microglia-containing brain organoids exhibited increased inflammatory responses via release of tumor necrosis factor (TNF-α) and interleukin-1 (IL-1β), which is similar to the chronic inflammation observed in HIV-infected humans.[141] Microglia and brain organoid co-culture models offer a unique opportunity to investigate the functional consequences of virus-host interactions in the context of immune responses to pave the way for identification of new biomarkers and therapeutic targets.

There are also practical limitations for using brain organoids to model viral infection. Embedding EBs in Matrigel and supplementing culture medium with Matrigel could potentially interfere with viral infection and affect the application of brain organoids to model infectious diseases. The composition and maturation status of brain organoids will be different depending on the timing of culturing, therefore studies need to be matched with these developmental stages. Furthermore, the immaturity of neurons in the brain organoids largely limits the application of brain organoids in modeling viral infection in the adult human brain. For example, many neurological symptoms were detected primarily in adolescent and adult patients with COVID19.[142] The SARS-CoV-2 infection of early brain organoids cannot fully recapitulate the pathology of viral infection due to the immaturity of neurons. A recent study found that long-term maturation of human cortical organoids matches key early postnatal transitions.[143] They leveraged a directed differentiation protocol of human cortical organoids and can reach postnatal stages between 250 and 300 days by the analysis of the epigenetic clock and transcriptomics.[143] Brain organoids with mature neurons will provide a useful model to study the pathology of viral infection in the postnatal human brain.

Application of other organ organoids for viral infection studies

Over the past decade, there has been tremendous progress in hiPSC-derived 3D organoid technologies and many organ-specific organoids have been developed and characterized.[47,144–147] These hiPSC-derived organ-specific organoids have been extensively applied in the study of viral pathogenesis and contributed to the identification of therapeutic drugs.[148] For example, adenovirus (AdVs) are common viruses and can cause mild flu-like symptoms, more severe symptoms, such as diarrhea or pneumonia, or even result in death. Using human intestinal enteroids, Holly et al. identified cellular tropism of respiratory human AdV-5p, which preferentially

infects goblet cells. Although surprising, the targeting of a respiratory serotype of human AdVs for goblet cells demonstrates the power of organoid culture system to uncover aspects of adenovirus that were previously unattainable with standard cell lines.[149] hiPSC-derived colonic and lung organoids have also been used to identify SARS-CoV-2 inhibitors.[150] In this study, colonic organoids were used to investigate SARS-CoV-2 infection and consequently found that a variety of colonic cells, especially enterocyte, are permissive to SARS-CoV-2 infection.[150] By using lung organoids, SARS-CoV-2 entry inhibitors, such as imatinib, mycophenolic acid and quinacrine dihydrochloride, were identified and treatment by these inhibitors significantly reduced SARS-CoV-2 infection in both colonic organoids and lung organoids.[150] Liver organoids have been used to study the pathogenesis of hepatitis C virus (HCV) infection. Hepatocytes in liver organoids are polarized and different from 2D hepatocytes, which usually do not exhibit polarization.[151] The infection efficiency of HCV in liver organoids is much higher than in 2D hepatocyte cultures. Using liver organoids, the complex process of HCV infection of hepatocytes was observed by single-molecule imaging.[151] These recent studies further support the use of organoid culture systems to recapitulate the complexity of *in vivo* physiology in viral pathogenesis studies. Even more importantly, the capacity to scale-up organoid cultures for higher-throughput assays provides a powerful platform to perform drug screens for potential antiviral therapeutics.

Summary

Recent advancements in brain organoid technologies have contributed greatly to our understanding of human brain development. Using growth factors or small molecules with precise timing and combinations, researchers have guided human pluripotent stem cell aggregates to form region-specific brain organoids, which resemble the structural organization and neuronal diversity of specific brain regions. Brain organoid models provide a new opportunity to study viral pathogenesis such as tropism, cellular and molecular mechanisms of viral infection in relevant human cell types (Table 1). Although brain organoids show great promise for the studies of neurotropic viral pathology in the brain, limitations in reproducibility and maturity currently restrict their utility. Improvements in protocols to expand cellular diversity and extend maturity will allow researchers to continue studying the neurotropic viral infection in the human brain in ways that were previously impossible. Ultimately, brain organoids provide a complementary system, in combination with other organ-specific organoid models, that will help us better understand pathology of infectious diseases.

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