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Organoids for modeling prion diseases

Ryan O Walters[1], **Cathryn L Haigh**[1,*]

[1]Prion Cell Biology Unit, Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 903 South 4th Street, Hamilton, MT 59840, USA.

Abstract

Human cerebral organoids are an exciting and novel model system emerging in the field of neurobiology. Cerebral organoids are spheres of self-organizing, neuronal lineage tissue that can be differentiated from human pluripotent stem cells and that present the possibility of on-demand human neuronal cultures that can be used for non-invasively investigating diseases affecting the brain. Compared with existing humanized cell models, they provide a more comprehensive replication of the human cerebral environment. The potential of the human cerebral organoid model is only just beginning to be elucidated, but initial studies have indicated that they could prove to be a valuable model for neurodegenerative diseases such as prion disease. The application of the cerebral organoid model to prion disease, what has been learned so far and the future potential of this model are discussed in this review.

Keywords

Prion; PrP; Cerebral organoid; Stem cells; iPSC

Introduction

Neurodegenerative diseases affecting humans have proven difficult to model in vitro. There are various reasons for this, including the difficulty of obtaining ethically sourced live human brain tissue in sufficient volumes for wide ranging investigations. The development of a new technology, human cerebral organoids, offers a model that can overcome some of the existing limitations on examining human tissue in culture. This model, and variations thereof, have been utilized for investigation of an increasing number of neurodegenerative diseases including prion diseases. Herein, we focus primarily on the human cerebral

*Corresponding: cathryn.haigh@nih.gov.

Declarations

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical approval

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organoid protocol developed by Lancaster and Knoblich (Lancaster & Knoblich, 2014), as this is the model thus far investigated in the context of prion research, but also consider possible new research directions as these models are expanded and refined.

Prion diseases

Prion diseases or the transmissible spongiform encephalopathies are a group of protein misfolding disorders characterized by a conformational change of a native protein (PrP^{C}) to a misfolded conformer (prions or PrP^{Sc}). PrP^{Sc} can recruit and template further misfolding of PrP^{C}. This templated misfolding allows disease to spread through the brain and also accounts for the transmissibility of the diseases. The precursor of the misfolded isomer is the prion protein, PrP^{C}, encoded by the prion protein gene (*PRNP*), which is highly expressed in the central nervous system but can be found throughout the body. It is typically found at the cell surface attached to the plasma membrane by a GPI-anchor. Although there is still considerable debate about the true function of PrP^{C}, it has been implicated in multiple cellular roles including copper homeostasis and neuroprotection (Linden, 2017). There is currently no treatment for prion diseases, and they are universally fatal.

Prion diseases in humans have three etiologies: sporadic, genetic or acquired. Of these, sporadic disease is the most common (>85% of cases) followed by genetic disease (10–15% of cases). Acquired disease can occur through several transmission routes including from surgical instruments, human-derived products (such as blood or hormones) and ingestion of contaminated meat. Despite the notoriety of these diseases that arose following the transmission of bovine spongiform encephalopathy to humans through consumption of contaminated meat (Ironside et al., 2017), the lowest occurrence is due to acquired disease. While less prion diseases arise due to transmission, the transmissible nature of prions is advantageous for laboratory studies. As a prion is able to imprint its misfolded structure onto a normal PrP^{C}, it can spread through tissue cultures and transmit a bona fide disease to animal models. This allows the disease development to be studied without the need for gross genetic modification of animals.

Sporadic prion diseases include sporadic Creutzfeldt–Jakob disease (sCJD), sporadic familial insomnia and variably protease-sensitive prionopathy. sCJD is the most common form of prion disease in humans with an average survival of only 6 months and a usual age of onset between 50 and 80 years (Baldwin & Correll, 2019). Biochemical hallmarks of prion disease include spongiform degeneration (vacuolation), PrP^{Sc} deposition, neuronal loss and gliosis (Geschwind, 2015). Different subtypes of sCJD have been observed that demonstrate different clinical presentations. Two factors define the different subtypes: firstly, a polymorphism at codon 129 of *PRNP* that can be either a methionine or a valine and, secondly, the molecular mass of the protein core of PrP^{Sc}. Careful analysis of these factors has led to two systems of disease classification where different subtypes may display similar or diverse clinical and biochemical features (Baiardi et al., 2021; Collinge et al., 1996; Hill et al., 2003; Parchi et al., 1999). The difference between the classification systems arises from the definition of subtype 1, which is further subdivided into two subtypes based upon molecular mass and disease duration in the Collinge system (Collinge et al., 1996). Diagnosing prion disease in human patients can be difficult due to the heterogeneity

of the clinical presentation of the different subtypes. The primary diagnostic tools in the clinic, along with the clinical picture, are the electroencephalogram, magnetic resonance imaging and cerebro-spinal fluid (CSF) 14–3-3 analysis (Knight, 2020; Wieser et al., 2006). Recently, a highly sensitive and specific test for the presence of PrP^{Sc} in the CSF, the real-time quaking-induced conversion assay (RT-QuIC) has also become an addition to the diagnostic tools available (Cramm et al., 2016; Groveman et al., 2017; Orrú et al., 2015; Orrú et al., 2016). However, a diagnosis of subtype can only be confirmed post-mortem by looking at the electrophoretic mobility of PrP^{Sc} from autopsied brain.

Genetic prion diseases include genetic Creutzfeldt–Jakob disease (gCJD), Gerstmann–Straüssler–Scheinker syndrome (GSS) and fatal familial insomnia (FFI). Familial disease is usually caused by an autosomal dominant point mutation within *PRNP*; however, insertion and deletion mutations have also been identified. E200K and D178N mutations, known to cause gCJD and FFI, respectively, demonstrate the highest penetrance, with up to 100% of carriers developing the disease within their lifetime (Minikel et al., 2016). As the cause of genetic prion disease is known, aspects of these diseases can also be modeled in the laboratory by creating a system containing the mutation such as a cell line or transgenic animal.

Models of human prion disease

In vivo models—Animal models have been used extensively to model prion diseases *in vivo*. This has been reviewed elsewhere (Brandner & Jaunmuktane, 2017; Moreno & Telling, 2017), so we will only briefly mention the humanized models. Transgenic mice have been created expressing human PrP or chimeric human-animal PrP (Brandner & Jaunmuktane, 2017; Telling et al., 1994). Humanized mice recapitulate some of the differences in subtype pathogenicity of human prion disease, including the influence of the *PRNP* codon 129 genotype on disease incubation time (Asante et al., 2002; Asante et al., 2006; Asante et al., 2015; Collinge et al., 1996). Humanized mice have also been used for studying genetic disease. In these models, the primary sequence of the transgene appears to heavily influence the propensity to develop spontaneous disease. For example, a study by Asante et al. considering P102L and E200K mutations in human PrP (129MM) found no development of spontaneous disease (Asante et al., 2009). However, when using chimeric human-mouse PrP where the region between amino acids 96 to 167 of mouse PrP was replaced with the corresponding human sequence (resulting in a 9 amino acid difference from mouse PrP (Telling et al., 1994)), Friedman-Levi et al. demonstrated that the E200K mutation could cause spontaneous disease in mice (Friedman-Levi et al., 2011). Humanized mice are extremely valuable for investigating human disease, especially discerning subtype characteristics (Asante et al., 2002; Asante et al., 2015); however, prion disease in humanized mouse models progresses very slowly. This results in extremely long experimental timelines associated with significant costs.

In vitro models—Using an *in vitro* model system addresses several of the concerns found in using *in vivo* animal models such as cost, difficulty and ethical reduction of animal numbers. Many different types of cell cultures have been used over the years in attempts to model propagation of human PrP^{Sc}. Unfortunately, technical difficulties, including the

substantial species barrier between the infecting human prions and the cell line and long generational times to disease possibly caused by slower conversion kinetics of huPrP, have made propagating human prions extremely difficult (for a review, see (Pineau & Sim, 2021; Priola, 2018)). Mouse adapted human prions have been propagated in cell lines including RK13 (rabbit kidney epithelium) overexpressing mouse PrP, GT1–7 (mouse hypothalamic GnRH neuronal), N2a (mouse neuroblastoma) and OBL-21 (mouse olfactory bulb) cell lines (Arjona et al., 2004; Haigh et al., 2011; Lawson et al., 2008; Lewis et al., 2009). However, while the RK13 line in particular has demonstrated permissibility to propagating different species prions (Courageot et al., 2008), no propagation is seen when human PrP is expressed within these cells (Lawson et al., 2008). Primary cell cultures offer some advantages over immortalized cell lines such as the ability to study disease pathology in different cell types and the lack of a need for serial passaging, allowing the cultures to propagate different prion strains for longer periods of time (Pineau & Sim, 2021). Cerebellar granular primary cell cultures from transgenic mice expressing human PrP with methionine at *PRNP* codon 129 were able to propagate human prions from a sCJD subtype 1 (the PRNP 129 genotype is not stated) with detection of protease resistant PrP at 28 days (Cronier et al., 2007). Interestingly, using primary murine glial cells from mice expressing human PrP with methionine at codon 129, Wälzlein et al. demonstrated no propagation of subtype 1 (129MM) sCJD prions but clear propagation of subtype 2 (129MM) and vCJD prions (Wälzlein et al., 2021). PrP^{Res} was detectable from 120 days post infection but declined after 150 days, likely due to the cultures reaching the end of their natural healthy lifespan.

Organotypic slice cultures, slices of brain tissue cultured *in vitro*, are more difficult than monolayers to culture but offer several advantages over 2D culture as they maintain a full complement of different brain cells and the structure of the brain regions. A study from 2008 initially described the methodology of this culture system adapted for prions, termed POSCA (Prion Organotypic Slice Culture Assay), and the feasibility of its use in studying prion propagation which in this model happens at an accelerated timescale (Falsig & Aguzzi, 2008). This model also shares an advantage of primary cell culture, the ability to create organotypic slices from mice expressing human PrP (Pineau & Sim, 2020).

While all these systems have produced valuable information on disease processes, they all have one limitation in common and that is the animal cell background. To add to the understanding of human disease, a fully human culture system was desirable. However, as alluded to above, it has proved difficult to propagate human prions in human cell cultures. To date, only one study to date has successfully propagated human prions in an immortalized cell line, SH-SY5Y human neuroblastoma cells (Ladogana et al., 1995). The SH-SY5Y cell line is known to suffer consistency problems due to the mixture of different cell phenotypes (neuroblast-like and epithelial-like) and the presence of adherent and suspension cells, which often results in only the adherent cells being passaged over time (Kovalevich & Langford, 2013). The prion-SH-SY5Y model system was never adopted as a human cell model beyond the original study, likely due to the aforementioned cell line limitations.

In a landmark study, Krejciova et al. generated the first fully human model of infection since the SH-SY5Y model (Krejciova et al., 2017). The authors used human-induced pluripotent

stem cells (iPSCs), which are reprogrammed from somatic cells, often dermal fibroblasts, back to an immature state that demonstrates many of the characteristics of embryonic stem cells and, most importantly, pluripotency; the capacity to differentiate into any cell type. Using the iPSCs, Krejciova et al. were able to differentiate astrocyte progenitor cells and astrocyte cultures that showed maturation stage dependent functionality (Krejciova et al., 2017). When exposed to human prions from vCJD and sCJD brain homogenates, mature astrocyte cultures, but not the astrocyte progenitor cells, demonstrated propagation of the human prions and accumulation of protease-resistant PrP at 3 and 8 days post-infection. The cultures continued to demonstrate propagation up to 28 days post-infection. Furthermore, propagation was dependent on the *PRNP* codon 129 genotype. In agreement with the known susceptibility of human prion genotypes to preferentially propagate specific *PRNP* codon 129 genotypes and subtypes (Fernández-Borges et al., 2017; Klemm et al., 2012), *PRNP* codon 129MM astrocytes readily propagated MM1 sCJD, VV2 sCJD and vCJD but 129VV astrocytes only propagated VV2 sCJD. The authors additionally showed that the propagated prions were infectious by passaging the infected astrocyte prions back into naïve astrocytes. This important advance demonstrated that human prions could be propagated in culture with retention of the biochemical characteristics of the infecting inoculum.

Since the cells were grown as monolayers, the astrocyte model still lacked 3D structure. Three-dimensional models of neuronal tissue are being used to more accurately model the cytoarchitecture of the brain during disease (Slanzi et al., 2020). Murine 3D models derived from undifferentiated and differentiated neural stem cells have been shown to propagate various prion strains (Collins & Haigh, 2017; Giri et al., 2006; Herva et al., 2010; Iwamaru et al., 2017; Iwamaru et al., 2013). These studies raised the possibility that human prion infections could be modeled in 3D cultures of human brain cells. The opportunity to study neurodegenerative disease in this context presented in 2013, when a study was published describing a 3D model of human brain tissue that recapitulated features of human cortical development (Lancaster et al., 2013). The authors termed the cultures ‘human cerebral organoids.’

Human cerebral organoids

The human cerebral organoid model developed by Lancaster et al. (Lancaster et al., 2013) involved generation of self-assembling 3D structures of multiple cell types differentiated from human embryonic stem cell and human iPSCs. This is achieved in four distinct stages via the use of additives to the media, the first stage initiates the formation of aggregates of iPSCs known as embryoid bodies (Lancaster & Knoblich, 2014). The embryoid bodies (EB) are then moved to a minimal media formulation for neural induction while kept in suspension allowing formation of neural ectoderm around the surface of the EB. Organoids are then embedded within a Matrigel matrix to allow expansion of the neuroepithelium before being transferred into agitated culture for maturation and long-term maintenance (Lancaster & Knoblich, 2014). Here, they can continue to grow and develop for several months to over a year (a summary of the progression of differentiation is shown in Figure 1). Human cerebral organoids offer several benefits such as scalability and ease, like more traditional cell model systems, while retaining much of the diverse cell types and network physiology seen in *in vivo* models (see Table 1 and below).

Organoid development recapitulates fetal human brain development in several ways. For example, they develop a similar tissue architecture and neuronal polarization. Organoids form layers during development including a ventricular zone, subventricular zone, deep layer neurons and finally upper layer neurons comprised of polarized neurons similar to a developing human brain (Chiaradia & Lancaster, 2020). Additionally, Cajal–Retzius cells may be contributing to cortical plate formation in similarity with what is seen *in vivo* (Chiaradia & Lancaster, 2020; Kadoshima et al., 2013; Lancaster et al., 2013). Organoids also contain the multiple cell types found in the developing human brain. Excitatory glutamatergic neurons make up the bulk of the neuronal cell types in organoids (Chiaradia & Lancaster, 2020), but inhibitory GABAergic interneurons are also present (Giandomenico et al., 2019; Velasco et al., 2019). Organoids can additionally contain astrocytes of healthy morphology, with a smaller amount of reactive astrocytes after 30 weeks in culture (Giandomenico et al., 2019; Qian et al., 2020; Sloan et al., 2017), as well as oligodendrocytes (Madhavan et al., 2018; Marton et al., 2019). This regional specificity and cellularity closely resembles what is seen in the developing brain and provides an improved experimental model of human brain tissue when compared to traditional 2D monocultures.

One of the most important stages of neuronal development is the establishment of cell–cell interactions and longer distance network formation. The organoids develop cell–cell interactions and electrophysiological signaling within the neuronal layers that is seen in the developing mammalian brain allowing for a multitude of signaling studies to be investigated using this model (Fair et al., 2020; Yakoub, 2019). For example, Watanabe et al. showed that organoids recapitulated corticogenesis and many metrics such as membrane potential, capacitance and resistance were found to be similar to what is observed in human fetal cortex (Watanabe et al., 2017). Additionally, spontaneous network activity has been recorded which further recapitulates the embryonic brain environment (Lancaster et al., 2013). Finally, organoids have also been shown to mimic the transcriptional expression of a developing brain for 24 weeks after generation at which point the maturing organoid core begins to become hypoxic and necrosis will begin (Chiaradia & Lancaster, 2020; Giandomenico et al., 2019; Tanaka et al., 2020). These properties are unique to organoid development when compared with more traditional cell models and recapitulates far more of the relevant *in vivo* environment from which these diseases emerge.

Organoid models and neurodegenerative disease

The value of cerebral organoids for modeling human brain diseases was quickly realized and applied to several diseases including other protein misfolding disorders. A disease phenotype that has been difficult to recapitulate in animal and cell models is amyloid deposition, tau hyperphosphorylation and neurodegeneration, which is seen in Down syndrome (DS) or Alzheimer's disease (AD). Transgenic mice serve as the primary model for investigation into AD and have shown the ability to recapitulate several aspects of the disorder such as age-dependent amyloid plaque formation and cognitive defects. Some models, such as the knock-in of human microtubule-associated protein tau (MAPT), can mimic the widespread neuronal loss that accompanies AD in humans (Saito et al., 2019), but each of these phenotypes is largely tied to a single transgenic mutation and so recapitulating them all within a single model requires multiple knockouts followed by expression of

human genes (Duyckaerts et al., 2008). Using cerebral organoids grown from patient iPSCs alleviated this barrier to an extent by providing a model utilizing all of the human genes necessary to recapitulate a disorder such as AD, and indeed, Gonzalez et al. were able to show structures similar to amyloid plaques and neurofibrillary tangles when using a cerebral organoid model (Gonzalez et al., 2018). An earlier study was able to demonstrate an effect of beta amyloid and gamma secretase inhibitors on amyloid generation and tau pathology resulting from familial AD mutations (Choi et al., 2014). Another study in 2016 using cerebral organoids showed the above pathologies as well as endosomal abnormalities to be age-dependent (Raja et al., 2016). The cellular complexity of cerebral organoids is a great opportunity to model multi-modal diseases such as AD.

Another disease that has been a subject of scrutiny in organoid models is Parkinson's disease (PD). As PD primarily affects dopaminergic neurons of the substantia nigra pars compacta, studies using human organoids to consider mutations linked with PD (such as LRRK2 G2019S) have focused on a different organoid differentiation protocol that produces mid-brain organoids. These studies have shown that the mid-brain organoids can re-produce aspects of genetic Parkinson's disease including, alpha-synuclein deposition, thioflavin-T positivity, and reduced tyrosine hydroxylase (Kim et al., 2019; Smits et al., 2019). The ability to differentiate organoids that correspond to different brain regions offers increased utility of the organoid model and demonstrates that organoid models can be applied across a spectrum of diseases.

Organoids in prion research

Genetic prion disease—The first studies to approach looking at prion diseases in 3D iPSC-derived cultures considered the influence of mutations within the prion gene on the development of a disease phenotype. A study using iPSCs generated from a human donor with a Y218N *PRNP* mutation, which is associated with the development of GSS, showed cell death and increased Tau phosphorylation without any detection of diseased isoforms of PrP (Matamoros-Angles et al., 2018). In another study, *PRNP* E200K and an eight-octarepeat insert mutation (both associated with development of genetic CJD) were used as negative controls for beta-amyloid, phospho-tau and neuronal morphology when comparing phenotypes with organoids generated from donors with mutations causing familial AD or DS (Gonzalez et al., 2018). The authors of this latter study did not comment on identification of any PrP-associated phenotype. We also generated organoids from two donors carrying the E200K *PRNP* mutation (Foliaki et al., 2020) and, likewise, found no endogenous pathology in these organoids for over a year post-differentiation, although neuroelectrophysiological dysfunction was later demonstrated (Foliaki et al., 2021). Together this indicates that *PRNP* mutation is not sufficient to cause a prion disease phenotype within organoids and that there is not continuous production of misfolded PrP. This instead raises the question of what might trigger PrP misfolding in the aging brain.

Prion infections—In 2019, we demonstrated that human cerebral organoids were able to propagate human prions and recapitulate some disease features (Groveman et al., 2019). This study used iPSCs that were heterozygous at *PRNP* codon 129 (129MV) to generate cerebral organoids that were grown for 5 months before exposure to human prions. Five

months of age was selected as the time of infection because at this point organoids are populated with more mature cells including astrocytes and oligodendrocytes (Renner et al., 2017). Organoids were exposed to brain homogenate from patients who died of sCJD subtypes MV1 and MV2 for 1 week with serial dilution. At 7 days, the organoids were washed and cultured in a fresh vessel. In contrast to the astrocyte model, after removal of the infectious inoculum, organoids required 3–4 weeks to clear residual inoculum, likely due to the complexity of the 3D structure permitting residual protein to evade destruction for longer than when readily washed away in 2D culture. After approximately 5 weeks, the organoids started to accumulate *de novo* prions, with a greater accumulation in the organoids receiving the MV2 subtype inoculum. The accumulation in the organoids that received the MV2 inoculum was sufficient that protease-resistant PrP^{Sc} could be observed by western blotting and PrP deposits could be visualized by immunohistochemistry. The organoids exposed to the MV1 inoculum showed no protease-resistant PrP^{Sc} or tissue deposition; however, they did show seeding activity in the highly sensitive real-time quaking-induced conversion assay (RT-QuIC) (Groveman et al., 2019; Orrú et al., 2016) suggesting a low level of infection was present.

As with all cellular prion models, validation that the PrP seeds and PrP^{Res} detected were due to *de novo* production rather than persistence of the initial inoculum is imperative. Production of *de novo* PrP^{Sc} is supported by the loss of seeding activity at 3–4 weeks followed by its increase to higher levels than detected after the initial inoculum was removed. Additionally, in organoids infected with the MV2 subtype inoculum we saw a shift to di-glycosylated dominant protease resistant species. This indicated cellular PrP within the organoid was being converted, as it is unlikely that the original, mono-glycosylated sCJD inoculum would become modified in this way. To investigate this further PrP^{Res} production could be analyzed by the use of amino acid isotopic-labeling to show that radio labeled PrP^{C} synthesized by the cell converts into PrP^{Res}. The gold standard, which remains to be demonstrated, would be to show a lack of propagation in PrP knock-out organoids.

Additionally, the Groveman et al. study also demonstrated that organoids can be used to model neuronal dysfunction and death. Over the course of the experiment, the organoids were monitored weekly for changes in cellular metabolism and periodically analyzed for evidence of cell death (Groveman et al., 2019). The organoids exposed to MV1 inoculum demonstrated changes in their metabolism throughout the infection period, initially showing a decline but later increasing their metabolism over the control organoids. The MV2-inoculated organoids showed no such change in their metabolism from their baseline readings or compared with the metabolism of the control organoids (Groveman et al., 2019). Cytokine assays also showed an increase in cytokine secretion in the MV1-inoculated organoids. This started around 90 days post-infection with a large increase in chitinase 3-like-1, a cytokine that is also increased in brain tissue from people who died of MM1 and VV2 subtypes of sCJD (Llorens et al., 2017). These results showed that several changes that occur in the human brain during disease were also happening within the organoids as they developed infection and that there may be subtype-specific differences in the presentation of infection.

The potential to investigate the influence of different subtypes offers some interesting opportunities. The different sCJD subtypes show a phenomenon called neuronal selective vulnerability, wherein the different subtypes damage the cells within different brain regions disproportionately (Jackson, 2014). This results in greater lesions in some brain regions over others, which produces the lesion profiles characteristic of the subtype (Baiardi et al., 2021). Using the cerebral organoids and different infecting subtypes, it will be possible to examine which neuronal subsets are most damaged in response to which subtypes and investigate common versus disparate cell death mechanisms. This analysis is made more powerful by developments in organoid technologies that allow regional specification (Bagley et al., 2017). Comparing different regions will allow the neuronal responses to the different subtypes to be monitored over the course of infection.

Prion-infected organoids for therapeutic screening

Being able to infect organoids and monitor prion propagation over time also allows for investigation of how propagation can be slowed or halted. Prion disease therapeutics remain absent despite years of study. Many drug candidates that appeared promising in animal or traditional cell culture models failed to show efficacy when introduced to humans (Qian & Tcw, 2021). A human model was required to understand the failings in the translation of these proposed therapeutics and the organoid–prion infection model presents a new option for drug screening (Groveman et al., 2021). The study by Groveman et al. showed the capacity of the organoids to act as a drug screening model by assessing the efficacy of pentosan polysulfate (PPS), which has been shown to exhibit anti-prion activity in numerous model systems (Caughey & Raymond, 1993; Doh-ura et al., 2004; Farquhar et al., 1999; Groveman et al., 2021). Even though this compound has not been used extensively in humans due to its inability to pass the blood–brain barrier (requiring intra-cranial delivery), its potent inhibition of prion replication in animal and cell models renders it prototypical for developing new treatment systems *in vitro* (Groveman et al., 2021). In a treatment paradigm representing prophylactic administration, organoids were treated with PPS for 7 days before exposure to infected inoculum and for a further 14 days throughout infection and for 1 week after the infectious inocula was removed from the media. The prophylactically treated organoids showed both a reduction of RT-QuIC seeding activity and a reduction in protease-resistant PrP^{Sc}. In an alternative approach more closely representing therapeutic treatment once infection is established, organoids were infected for 63 days before treatment with PPS for 28 days (ending at 91 dpi). These organoids also showed a reduction in RT-QuIC seeding activity and PrP^{Sc}. Additionally, after PPS treatment was stopped at 91 dpi, organoids were cultured for a further month to 120 dpi and maintained the reduction in RT-QuIC seeding activity and no PrP^{Sc} detection (Groveman et al., 2021). This study illustrates the potential the organoid model to be used as a tool for investigations into therapy and even drug discovery.

The organoid model holds promise of being a versatile model for testing anti-prion therapeutics. The Groveman et al. manuscript considered only two possible therapeutic paradigms: prophylactic and therapeutic; however, many variations upon this theme could be tried. Variations might include: duration of treatment, combinations of treatments and testing toxicity (both in the context of brain cells and other tissues that can be differentiated from

iPSCs such as liver or kidney organoids). A specific and powerful advantage to the prion organoid therapeutic model is that it permits study of therapeutic efficacy in different *PRNP* codon 129 backgrounds and with different subtypes. As only very few patients present with sCJD each year and their molecular subtyping will not be confirmed until post-mortem examination, clinical trials of new compounds are unlikely to be able to judge the efficacy of a putative therapeutic in different subtypes. However, using organoids this can be achieved in the laboratory by generating organoids from donor cells with different *PRNP* codon 129 genotypes and infecting these organoids with inoculums of patient brain tissue identified as different subtypes. An additional advantage to screening putative therapeutics in organoids is that they provide a means to monitor neuroelectrophysiological function. When considering the benefit of a potential therapy, the capacity to maintain or even restore neuronal function increases the likelihood of benefit for the individual. These attributes of organoids offer therapeutic screening paradigms with much greater flexibility than existing models.

Cerebral organoids could additionally allow researchers to test compounds against prion infection established within organoids generated from donors with *PRNP* genotypes associated with genetic disease and rapidly screen potential drug candidates with a throughput that would not be feasible in animal models. Furthermore, in families suffering genetic prion disease, organoids could provide a model for personalized medicine in which a cohort of organoids could be grown from a patient unique to their specific genetics. The feasibility of various treatments could be tested in the patient-specific organoids before administering the best candidate to the patient.

An idea that arises when considering personalized medicine is whether it is possible to produce new neurons to replenish those that have been lost and the capacity to re-grow neuronal function. While there is precedent from mouse studies looking at degeneration associated with the E200K mutation that implanting neural stem cells alone and as part of a combination therapy could delay disease and symptom onset (Frid et al., 2018; Frid et al., 2020), it is too soon to know if neurons or neuronal precursors generated within organoid cultures could be used this way. One hint of possibility comes from a case study generating personalized dopaminergic progenitors for the treatment of Parkinson’s disease. In this study, the dopaminergic precursors were grafted into the putamina of the patient who showed limited improvements in motor assessment and quality of life 24 months following the graft (Schweitzer et al., 2020). While clearly not an immediate application of organoid technology, the application to personalized medicine is certainly in interesting area to watch for future developments.

Other potential applications of the organoid model to prion disease—The use of organoids for studying prion disease is still in its foundation phase (this is summarized in Table 2). Generally, as organoids are grown in cell culture similarly to other in vitro systems, most experimental paradigms applied to investigation prion infection in cell culture are likely to be transferrable to the organoid system. This potentially permits the study of many pathways that cannot be directly interrogated in humans. For example, to date the only infection protocol published is an overlay technique, where the infectious inoculum is included in the media surrounding the organoids. However, many other protocols exist for prion infections, including steel wires and exosomes (Fevrier et al., 2004; Flechsig et al.,

2001; Vella et al., 2007; Zobeley et al., 1999). The trial of these for infecting organoids may be able to provide information about the modes of cell–cell spreading as well as further avenues for preventing cell to cell spread.

Other aspects of organoid biology may be useful for the investigation of prion disease both in the context of genetic and infectious disease. A simple, but critical, aspect of organoid maintenance is that organoid culture systems are maintained in highly defined media (Lancaster & Knoblich, 2014). A strong confidence in the composition of the cellular environment provides opportunities to make precise changes to media components for investigation of their influence on prion conversion and development of infection. Many co-factors have been implicated both in the establishment of infection at the prion uptake stage and the on-going propagation of prions including glycosaminoglycan sulfation (Lawson et al., 2010), RNAs (Adler et al., 2003), and lipids (Kazlauskaite & Pinheiro, 2005). While these pathways are unlikely to offer themselves as something that could be readily manipulated to counter disease, they could provide valuable insight into pathogenic processes.

Limitations of the organoid model—When discussing the applications of the organoid models we cannot fully appreciate the value without understanding the limitations. Some limitations have already been considered, but others should also be given due consideration when interpreting experimental results. A clear variation from the human brain is that the organoid model has no vascularization or blood–brain barrier. New models, both variations of the organoid differentiation protocol and 3D-printed stand-alone models (Bose et al., 2021; Kaisar et al., 2017; Pellegrini et al., 2020; Pham et al., 2018), are attempting to create a blood–brain barrier like environment or introduce a pseudo-vasculature into the organoid. These are discussed further below. Beyond the biological improvement this also improves diffusion within the core of the organoid, which both limits its overall growth and can become necrotic once an organoid has reached a certain size (Pellegrini et al., 2020).

A second potential weakness is that there are no non-neuronally derived cells, such as immune cells. Microglia are a population of immune cells found within the brain. They have been shown to have a significant impact on the progression of prion disease; if they are prevented from proliferating, disease incubation is extended (Zhu et al., 2016), but if they are absent the disease progresses significantly faster (Carroll et al., 2018). Microglia are derived from the yolk sack, infiltrating the brain tissue during development. Therefore, organoid differentiation from neuroepithelium is naturally devoid of microglia that are derived from mesoderm. Protocols have been developed to induce microglia to develop during organoid differentiation (Ormel et al., 2018) and also to introduce microglia back into the organoid after development (Abud et al., 2017). These protocols are continuing to be developed and are also being applied to co-incubation with epithelial tissue as needed for the blood–brain barrier. It is reasonable to expect that they will become highly developed and widely available within a few years.

One of the strengths of the organoid model of prion infection is also a specific limitation. Organoids contain differentiated neuronal lineage cells that permit aspects of neuronal function and health to be monitored, but the differentiated cells within the organoid are no

longer growing. This means that the culture does not keep reproducing itself in the same way as an infected immortalized cell line, which can produce generations of infected cells by passing on infection to the daughter cells. The limitation that is encountered here is inherent to all differentiated or ex vivo models, that each new infection must be established in a new batch of organoids using fresh inoculum (Krance et al., 2020). This introduces error through batch-to-batch variability. Conversely, terminal differentiation does remove the limitation that in growing cell cultures the accumulation of prions is influenced by the rate of cell division, which may alter the apparent efficacy of anti-prion therapeutics tested in such systems (Ghaemmaghami et al., 2007).

A further related consideration when producing organoids is that their cellularity and gene expression may vary across batches, within batches and across different iPSC lines. Inter- and intra-batch variations have been identified as a major consideration when modeling Alzheimer's pathology in organoids (Hernández et al., 2021), and, for studies of gene mutations, the best consistency across iPSC lines is achieved using genetically matched, isotype controls. Single cell RNA sequencing analysis has shown that despite the variations, the cell developmental trajectories and terminal cells produced within organoids follow the complex cellular diversity of the cortex (Velasco et al., 2019). Many variations on the culture protocols are under investigation for producing greater uniformity of cultures, especially in the context of greater scale and automation for cultures (Louey et al., 2021). However, experiments should be conducted with the potential impact of this increased variability considered in the assay design and controls utilized.

A final consideration is that, like other dementias, prion diseases primarily affect older people. Human organoids are not similar to a mature or elderly brain but better represent the development of neonatal brain and, therefore, may contain cell types that respond differently to prion infection than aging brain cells. Already ways to adapt the organoid system to more accurately reflect the aging brain are underway by utilizing manipulation of known cellular factors associated with aging (reviewed in (Grenier et al., 2020)). As the model develops further, it may become possible to interrogate the influence of prion infection on aging cells.

Future developments

As we have alluded to, many refinements are being developed to address some of the potential drawbacks of the cerebral organoid model such as the addition of scaffolding, air interfaces, and vascularization for better nutrient diffusion, as well as for improving their overall usefulness. As cerebral organoid protocols have progressed, they have also specialized. Protocols now exist for producing mid-brain, ventral forebrain, dorsal forebrain, cerebellar, thalamic and hypothalamic brain organoids (Bagley et al., 2017; Huang et al., 2021; Jacob et al., 2021; Muguruma et al., 2015; Qian et al., 2016; Smits et al., 2019; Watanabe et al., 2017; Xiang et al., 2019) and this list of regions is not exhaustive. Regional specification is an exciting development for prion diseases where different subtypes and different genetic mutations preferentially attack some regions more than others. As these protocols have evolved, they have also addressed the intrinsic properties of the cells themselves and new methods can now improve neuronal myelination by enhancing the specification and survival of oligodendrocytes (Shaker et al., 2021).

Although generalized network activity and neuronal communication have been shown in cerebral organoids, steps are being taken to further recapitulate what is seen *in vivo.* One such study implemented an air–liquid interface to both improve organoid core survival and increase axonal outgrowth (Giandomenico et al., 2019). The authors found their culture system exhibits active neural networking, long-range axonal projection and growth cone turning in addition to expressing RNA indicating varied neuronal identity. Furthermore, most organoid models consider only one brain region, but the human brain displays circuitry between regions. Bagley et al. were able to demonstrate that organoids differentiated to represent dorsal or ventral forebrain could merge and GABAergic interneurons would migrate from the ventral forebrain organoid into the dorsal tissue (Bagley et al., 2017). The combination of these systems for improved neuronal communications with new developments in electrophysiology that permit recording of neuronal activity within the organoid 3D structure (Tasnim & Liu, 2021) offers the potential to better understand neuronal function, or disease-associated dysfunction, over long periods of time in culture.

Finally, as we have already mentioned, one of the primary difficulties with organoid cultures has been their lack of vascularization, which, aside from the lack of a biologically important system, results in less diffusion of nutrients to the organoid core. A potential solution to this is the use of an internal scaffold where channels of medium allow nutrients to reach the interior of the organoid and diffuse to the core. This has been investigated using scaffolds constructed from poly-(lactic-co-glycolic acid) (PLGA) fibers or carbon fibers to study mid-brain organoids (Tejchman et al., 2020). The scaffolds were shown to improve neuronal survival. Another approach to the problem of vascularization was demonstrated by Pham and colleagues where iPSCs from a patient were used to differentiate organoids while at the same time also performing a separate differentiation into endothelial cells. After a month, the organoids were re-embedded into Matrigel and coated with the corresponding endothelial cells. They were then either grown *in vitro* for 3–5 additional weeks or transferred into a mouse model for 2 weeks, resulting in vascularization of the organoid (Pham et al., 2018). Additionally, when the organoid was transplanted into a mouse model, the authors report detection of CD31-positive blood vessel within the center of the organoid. This demonstrated that organoids could be vascularized with a patient's own tissue and provides a step over one of the largest hurdles in organoid development and one of the largest steps toward recapitulating the *in vivo* brain.

Summary

Human cerebral organoids present an exciting and powerful model system to study neurological disorders such as AD, PD and prion disease. They readily differentiate into neuronal cell types such as neurons, astrocytes and oligodendrocytes and form regions of specialization. Their cellularity and complexity facilitate detailed study of human neuronal function and the development of pathologies, including several diseases that have thus far been difficult to model using animal or 2D monoculture systems. Cerebral organoids are relatively simple to create and can be grown from human donors carrying genetic mutations allowing researchers to use them to study both the underlying genetics of disorders as well as the progression of infectious disease. Throughout this review, we have attempted to give a detailed account of what can be learned from organoid studies and examine the limitations

to their application. The speed of developments in this area will undoubtably offer further exciting opportunities that are yet to be revealed and as the methods of organoid creation continue to be refined and expanded so will their usefulness to prion research.

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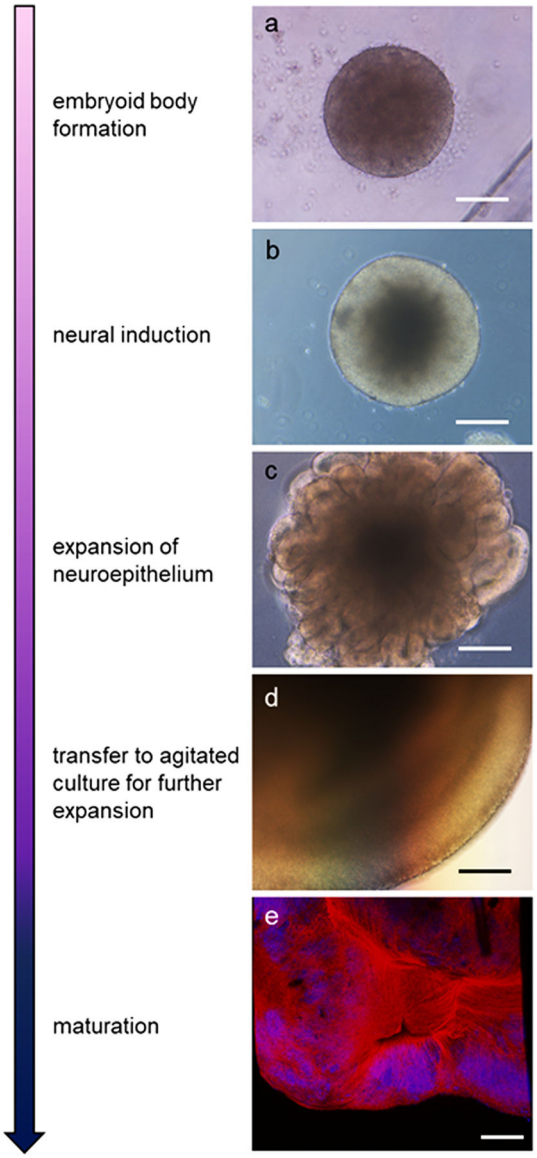

Fig 1.

Organoid developmental stages and morphology. Arrow indicates increasing maturity. Bright-field images show organoid morphology at various developmental stages including; a) fully formed embryoid body beginning neural induction, b) appearance of bright neuroepithelium, c) expansion of neuroepithelial buds, and d) further expansion and structuring of the organoid. e) Detection of mature neurons. Immunofluorescent staining shows neurofilament light chain (red) and nuclei (blue; DAPI) at approximately 45 days old. Scale bar = 100 μm

Table 1

Comparison of human/humanized models of infection

	Cerebral Organoids	Transgenic Animal	Primary Cell Culture	Organotypic Slice
Cost of Generation	+	+++	+	++
Cost of Maintenance	+	+++	+	+
Difficulty of Generation	++	+++	+	+
Difficulty of Maintenance	+	++	+	+
Cell Diversity	+++	+++	+	+++
Experimental Timeframe	++	+++	+	+
Time to Maturity	+	++/+++(*)	+	+
Scalability	+++	+	+++	++
Proximity to Humans	+++	++	+	++

+ minimal

++ average

+++ high

(*) depending on the infection subtype

Table 2

Summary of studies using prion mutation or infections of organoids to date. N/a = not applicable.

Organoid protocol	PRNP mutation	Prion infection	Experimental timeframe	Key findings	Ref.
Spherical neural masses	Y218N	[1]Y218N and CJD[2]	Differentiated for 3, 6 or 9 weeks	*PRNP* Y218N neurons demonstrate hyperphosphorylation of Tau and neurofibrillary degeneration without evidence of misfolded PrP	(Matamoro s-Angles et al., 2018)
Cerebral organoids (Lancaster & Knoblich, 2014)	E200K 8-octerepe at insertion	N/a	110 days in culture	No pathological changes reported	(Gonzalez et al., 2018)
Cerebral organoids (Lancaster & Knoblich, 2014)	N/a	PRNP 129M/V organoids MV1 and MV2 subtype inoculums	5 months from starting differentiation to infection Up to 6 months to analysis	Organoids become infected with and propagate prions. PRNP 129M/V organoids showed a preference for propagation of the MV2 subtype but greater pathological changes associated with the MV1 subtype.	(Groveman et al., 2019)
Cerebral organoids (Lancaster & Knoblich, 2014)	E200K	N/a	Up to and including 1 year	No pathological changes reported	(Foliaki et al., 2020)
Cerebral organoids (Lancaster & Knoblich, 2014)	N/a	PRNP 129M/V organoids MV2 subtype inoculum	5 months from starting differentiation to infection Up to 4 months to analysis	Organoids could demonstrate the therapeutic efficacy of PPS[3] when it was administered prophylactically, before, during and for a short time after infection, and therapeutically, administered after infection was established.	(Groveman et al., 2021)
Cerebral organoids (Lancaster & Knoblich, 2014)	E200K	N/a	Up to 10 months	Electrophysiological dysfunction with disturbed excitatory to inhibitory balance	(Foliaki et al., 2021)

[1]Forebrain neuronal cultures only were tested for uptake of infection and found negative

[2]CJD type was not specified.

[3]Pentosan polysulfate