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Building Models of Brain Disorders with Three-Dimensional Organoids

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Disorders of the nervous system are challenging to study and treat due to the relative inaccessibility of functional human brain tissue for research. Stem cell-derived 3D human brain organoids have the potential to recapitulate features of the human brain with greater complexity than 2D models and are increasingly being applied to model diseases affecting the central nervous system. Here, we review the use of human brain organoids to investigate neurological and psychiatric (neuropsychiatric) disorders and how this technology may ultimately advance our biological understanding of these conditions.

Introduction

Understanding the biological basis of nervous system diseases remains a major scientific challenge (Insel and Landis, 2013). This is largely due to the complexity of the human brain, which contains vast numbers of specialized cell types with diverse functions and intricate connections. Additionally, the symptoms and severity of neuropsychiatric disorders vary widely among individuals due to the interplay of genetic predisposition, developmental histories, psychosocial factors, and environmental exposures (Demjaha et al., 2012; Geschwind and Flint, 2015). In the absence of neurobiological diagnostic indicators, many neuropsychiatric disorders are defined by behaviors and self-reported symptoms, posing a fundamental challenge of disease categorization (Smoller et al., 2018; Taber et al., 2010). Despite progress in neuroscience, brain disorders are still difficult to treat and have a profound negative impact on human health and society at large (The Lancet Neurology, 2017; Vigo et al., 2016). What are the barriers to a better pathophysiological understanding of nervous system diseases, and which technological developments will accelerate progress?

A range of techniques have been used to investigate brain disorders, each carrying strengths and weaknesses (Figure 1). Neuropathological analysis of post-mortem tissues has led to the discovery of histological hallmarks, and this approach redefined our classification of many brain disorders. For instance, the loss of neuromelanin-rich substantia nigra dopaminergic neurons in Parkinson's disease is considered pathognomonic and inspired therapeutic strategies based on modulation of dopamine (Hornykiewicz, 2010). Furthermore, amyloid plaques and neurofibrillary tangles were identified in brain tissue from Alzheimer's disease patients (Perl, 2010), and decreased hippocampal volume has been identified in patients with schizophrenia (Harrison, 1999). In these latter cases, however, neuropathological observations have provided limited insights into disease mechanisms. Functional neuroimaging techniques can non-invasively reveal both the structure and activity of brain networks over time (Linden, 2012). Nonetheless, these modalities frequently rely upon surrogate markers such as blood flow or glucose uptake to make inferences, are limited by spatial resolution, and are not diagnostic for neuropsychiatric disorders (Braun et al., 2018). Neuropharmacological approaches have revealed that modulation of specific channels or neurotransmitter pathways has therapeutic effects on neurological and psychiatric conditions. However, many efficacious therapeutics (such as lithium for bipolar disorder) were serendipitously discovered rather than rationally designed (Mitchell and Hadzi-Pavlovic, 2000), and mechanistic insights are still elusive, even after decades of study (Alda, 2015). Rodent models allow for the study of environmental and genetic contributors to behavior and enable manipulation of specific cell populations and circuits (Gordon, 2016; Nestler and Hyman, 2010). However, about 75 million years of evolutionary time separate mice and humans (Waterston et al., 2002), which likely contributes to the low overall success of translating findings from rodent studies into the clinic. Non-human primates are evolutionarily similar and effectively model many human cognitive, behavioral, and social traits. They are, however, resource intensive and limited by availability and ethical considerations (Izpisua Belmonte et al., 2015).

A series of recently developed techniques hold promise to accelerate the investigation of nervous system disease mechanisms and contribute to the development of novel therapies. First, the genomics revolution has led to the identification of specific copy number and gene variants that confer increased risk of brain disorders (Geschwind and Flint, 2015; Hyman, 2008; Sullivan et al., 2012). Second, the swift pace of genome engineering tool development with CRISPR-Cas9 has enabled rapid, precise, and multiplexed genome modifications to investigate gene-function relationships in cellular, rodent, and primate models (Heidenreich and Zhang, 2016; Jennings et al., 2016). Third, patient-derived somatic cells can now be converted into human induced pluripotent stem (hiPS) cells capable of differentiating into a wide variety of cellular lineages in culture. These advances in cellular reprogramming make possible the *in vitro* generation of neurons and other brain cell types from an individual's unique genetic background (Dolmetsch and Geschwind, 2011; Hoffman et al., 2018; Soliman et al., 2017). Lastly, the bioengineering of stem cell-derived, self-organizing, three-dimensional (3D) cell cultures—also known as organoids—introduces more degrees of developmental freedom and models brain structure in remarkable and surprisingly complex ways (Clevers, 2016; Kelava and Lancaster, 2016; Lancaster and Knoblich, 2014; Paşca, 2018).

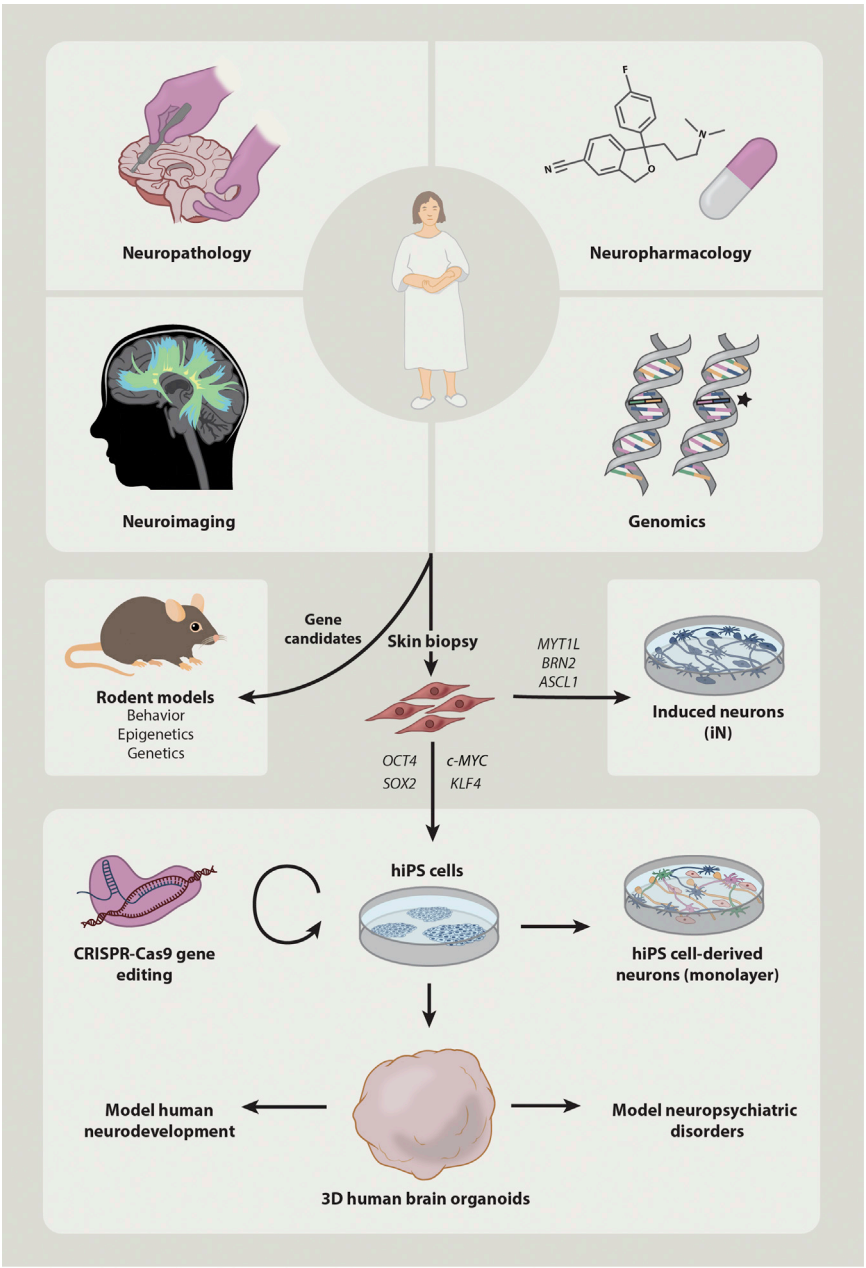


Figure 1. Investigating Neuropsychiatric Disorders

Neurobiological mechanisms of brain disorders can be investigated with a variety of approaches in humans and model organisms. Somatic cells from patients can be directly reprogrammed into induced neurons (iNs) or human induced pluripotent stem (hiPS) cells. Using genome engineering techniques such as CRISPR-Cas9, disease-associated mutations can be introduced or corrected in hiPS cells, which can subsequently be differentiated into monolayer (2D) neuronal cultures. Alternatively, hiPS cells can be differentiated into brain organoids which are 3D aggregates of neural tissue that resemble human brain regions. Subsequently, 2D and 3D cultures can be applied to the investigation of brain disorders.

Disease Modeling with Human Stem Cell-Derived Neurons in 2D

Human stem cells differentiated into monolayer neuronal cultures have been used to investigate neurobiological mechanisms of many neuropsychiatric disorders. In an early study, hiPS-derived monolayer neurons generated from Rett syndrome patients exhibited decreased number of glutamatergic synapses (Marchetto et al., 2010). This phenotype was rescued by applying Insulin Growth Factor-1 (IGF1), a peptide being actively explored for therapeutic potential in clinical trials of neurodevelopmental disorders (Vahdatpour et al., 2016). Additionally, changes in activity-dependent dendrite retraction (Krey et al., 2013) and neurotransmitter production (Paşca et al., 2011) were identified in neurons derived from Timothy syndrome patients that carry mutations in an L-type calcium channel. Neurons derived from hiPS cells that carried a TALEN-induced mutation of *DISC1* previously linked to psychiatric disorders exhibited defective synaptic vesicle release (Wen et al., 2014).

Neurons derived from patient hiPS cells have also been used to model highly heritable yet idiopathic psychiatric disorders. In one study, predominantly glutamatergic neuronal cultures derived from schizophrenia patients exhibited decreased neuronal connectivity and neurite number, and some molecular and cellular phenotypes could be rescued with an anti-psychotic drug (Brennand et al., 2011). Studies of hippocampal dentate gyrus-like neurons derived from patients with bipolar disorder revealed mitochondrial abnormalities and neuronal hyperexcitability compared with controls (Mertens et al., 2015b). Furthermore, neuronal hyperexcitability was rescued by lithium, but only for neurons derived from patients whose bipolar symptoms responded to lithium, demonstrating a potential role for hiPS cell-derived cultures in predicting drug-responsiveness (Mertens et al., 2015b). Many of these studies utilized differentiation protocols to generate cortical neurons, though human stem cells can also be differentiated into other disease relevant neuronal subtypes (Tao and Zhang, 2016) such as serotonergic neurons (Lu et al., 2016), dopaminergic neurons (Kriks et al., 2011), GABAergic interneurons (Maroof et al., 2013; Nicholas et al., 2013), and spinal motor neurons (Du et al., 2015).

While stem cell-derived neurons grown in monolayer have led to promising findings, they lack many of the distinguishing features of the human brain, which has limited their utility in

modeling many disease-relevant neurobiological phenomena. Neurodevelopment and synaptic connectivity are choreographed by cell-cell and secreted ligand-receptor interactions, and these important signaling dynamics are perturbed when neurons are grown in monolayer. Precise patterns of synaptic connectivity across specialized brain regions are the basis of higher-order brain functions, but monolayer cultures typically contain neurons modeling a single brain region in isolation. Astrocytes, microglia, and oligodendrocytes carry out important regulatory functions in the brain yet are not typically represented in monolayer cultures. Furthermore, many stages of neuronal maturation take place on timescales many times longer than the typical monolayer culture can be maintained, limiting the emergence of important late-stage developmental properties such as gliogenesis, axonal myelination, and certain neuronal electrophysiological and synaptic properties (Clowry et al., 2010; Dehaene-Lambertz and Spelke, 2015; Rubenstein, 2011; Silbereis et al., 2016).

Developing 3D Human Brain Organoids

The limitations of stem cell-derived monolayer cultures spurred efforts to develop more sophisticated *in vitro* models that could better recapitulate the structural and functional complexity of the human brain. Relying upon the innate self-organizing capacity of aggregated cells, hiPS and human embryonic stem (hES) cells can be grown and differentiated into 3D human brain organoids with structural features resembling the developing brain (Muguruma et al., 2015; Nakano et al., 2012; and reviewed in Paşca, 2018). The effect of single gene mutations or genetic backgrounds on brain organoids can be respectively modeled by introducing specific genetic modifications into stem cells or by deriving hiPS cells from patients and controls. Undirected organoids are generated in the absence of inductive cues, often while grown in extracellular matrix such as matrigel, and stochastically give rise to cells resembling those found in multiple brain regions ranging from retina to hindbrain (Lancaster et al., 2013). Alternatively, specific combinations and timing of exogenously applied morphogens or signaling molecules can guide the *in vitro* neurodevelopmental specification of 3D aggregates of stem cells into directed organoids (Mariani et al., 2015; Paşca et al., 2015; Qian et al., 2016). Directed organoids resembling multiple brain regions can be generated with different protocols in parallel and subsequently fused into assembloids that model brain regional interconnectivity (Birey et al., 2017). Organoids continue to mature over many months (Sloan et al., 2017), grow to several millimeters in diameter, and contain a diversity of neural progenitors including outer radial glia cells, neuronal subtypes, astrocytes, and oligodendrocytes (Birey et al., 2017; Camp et al., 2015; Kadoshima et al., 2013; Pollen et al., 2015; Qian et al., 2016; Quadrato et al., 2017; Sloan et al., 2017).

Importantly, organoids are accessible and versatile tools for studying brain development and function, as they can be manipulated and probed with a variety of techniques (Figure 2). Early-stage organoids contain a ventricular zone-like layer with radial glia (vRG), intermediate progenitors, and outer radial glia (oRG), which can be investigated as a model of corticogenesis with immunofluorescence, optical clearing, birth-dating, or live imaging. The contribution of environmental factors to neurodevelopment can be modeled by exposure of organoids to chemicals or viruses, such as the Zika virus that can cause congenital microcephaly (Qian et al., 2016). The electrophysiological properties and network connectivity of neurons within more mature organoids can be investigated with optogenetics, patch clamping, single electrodes, and multielectrode arrays. These techniques can also be applied to stem cell-derived monolayer neuronal cultures, though they lack organoids' greater architectural complexity and cellular diversity. Human organoids lack sensory inputs or motor outputs, but advances in mouse transplantation (Mansour et al., 2018) open possibilities to study the functional integration of human neurons within the circuits of a living brain that can respond to complex environmental stimuli and regulate behavior. Combining brain organoids with non-neuronal cell types such as microglia-like cells in assembloids (Abud et al., 2017; Lin et al., 2018) can model neuroimmunological interactions that might exacerbate or protect against neuronal pathology. Furthermore, single-cell RNA sequencing has been applied to organoid cultures to investigate the transcriptional diversity of human brain cell types and underlying gene regulatory networks (Camp et al., 2015; Quadrato et al., 2017; Sloan et al., 2017).

Brain organoids model complex features of brain development, and a diverse array of tools and techniques have been applied to brain organoids in efforts to better understand nervous system diseases. In this review, we will describe the impact of human brain organoid technology on the investigation of disease with a focus on neurobiological mechanisms and organoid-based experimental strategies used to uncover them (Table 1).

Models of Neurodevelopmental Disorders

Congenital Lissencephalies

Miller-Dieker syndrome (MDS) is a severe lissencephaly variant in which the surface of patients' cerebral cortices are smooth. Most frequently, it is caused by a deletion of chromosome 17p13.3, including *LIS1* and *YWHAE*. Defective neuroproliferation (Yingling et al., 2008) and radial migration (Moon and Wynshaw-Boris, 2013) are prominent cellular phenotypes previously associated with lissencephalies. Although mouse models replicate disorganization of cortical layers seen in patients (Toyooka et al., 2003), the fact that the mouse brain is naturally lissencephalic illustrates the need for alternative models of human brain features relevant to disease. Prominent neuroepithelial loops developed on the surface of hiPS cell-derived control forebrain organoids but were significantly reduced in organoids generated from MDS patients (Iefremova et al., 2017). Additionally, MDS organoids were smaller than controls, suggesting an underlying proliferative defect (Figure 3). Re-expression of *LIS1* or *YWHAE* individually using a doxycycline-inducible system could partially rescue these phenotypes. The microtubules of vRG in MDS organoids were disrupted and truncated, in contrast to the well-organized and fully extended microtubules in control organoids. Neurogenesis can be dysregulated by disruptions in the proliferative niche (Marthiens et al., 2010), and vRG in MDS organoids had less frequent symmetrical cell divisions associated with stem cell renewal. The β-catenin/Wnt signaling axis within the MDS organoid proliferative niche was impaired, and pharmacological activation of β-catenin rescued defects in vRG division planes, apical membrane length, and

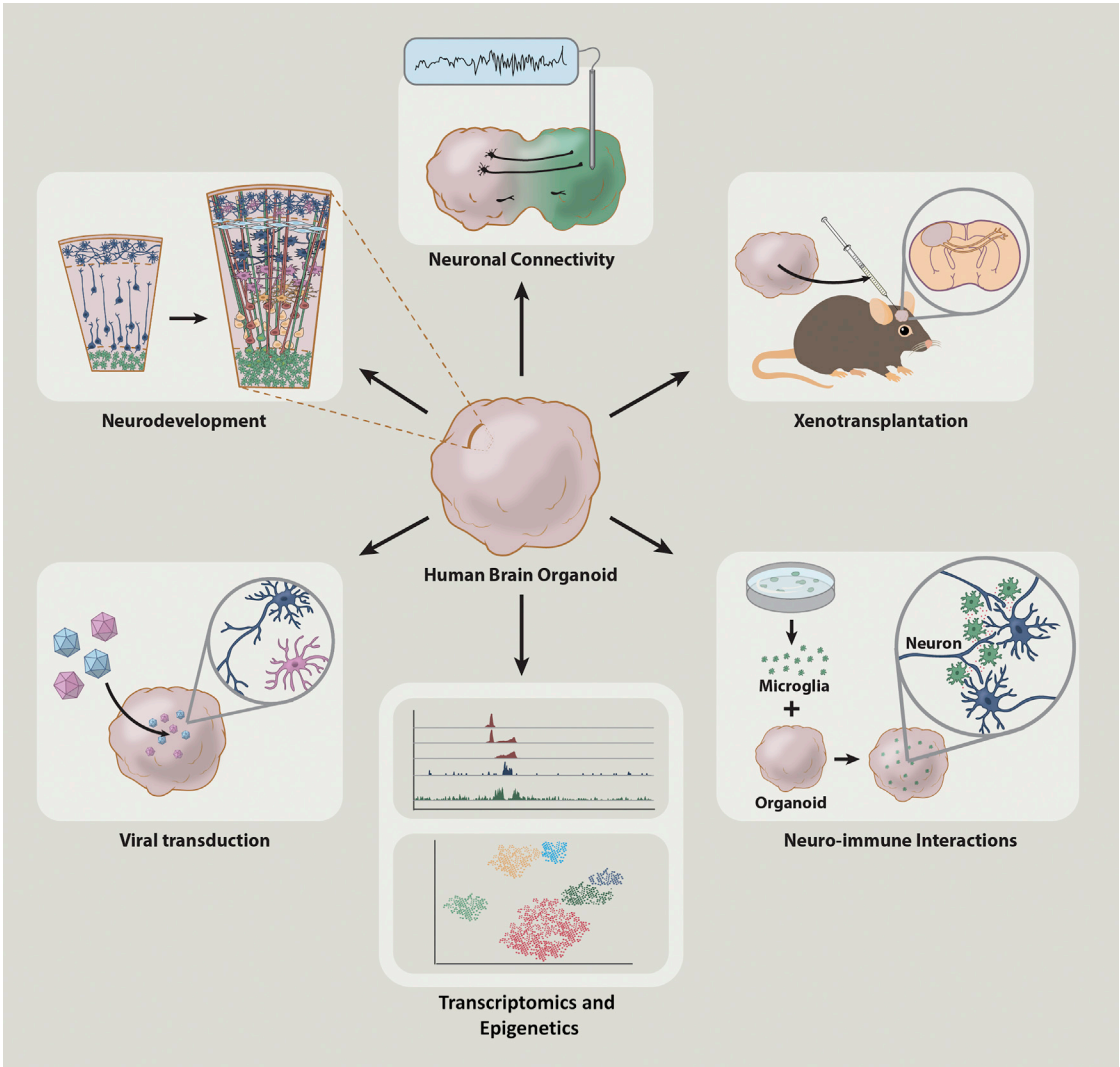


Figure 2. Human Brain Organoids Are Versatile and Accessible Cellular Models
Human brain organoids can be investigated with many approaches and experimental techniques (clockwise from top) including neuronal connectivity (Deng et al., 2018; Mariani et al., 2015), xenotransplantation (Mansour et al., 2018), multi-lineage assembloids (Abud et al., 2017; Lin et al., 2018), transcriptomics (Quadrato et al., 2017) and epigenetics (Luo et al., 2016), viral transduction (Qian et al., 2016), and neurodevelopment (Bershteyn et al., 2017; Birey et al., 2017; Iefremova et al., 2017).

neuroepithelial organoid looping macrostructures (Iefremova et al., 2017).

The developing human cortex contains an expanded outer proliferative zone containing abundant oRGs that are lacking in rodents (Lewitus et al., 2013; Pollen et al., 2015). In a study of hiPS cell-derived telencephalic organoids from control and MDS patients, oRG cells remained in mitosis for much longer periods of time in MDS organoids, implicating impaired oRG division in lissencephaly pathogenesis (Bershteyn et al., 2017) (Figure 3). Control and MDS organoids were cultured for 5 weeks and subsequently plated on matrigel to observe neuronal migration with live imaging (Bershteyn et al., 2017). Unlike control neurons, MDS neurons did not sustain saltatory migration, traveling with less linearity and overall slower speeds. This migration phenotype was rescued using a clever genetic strategy involving the replacement of a 5.3 Mb sequence containing *LIS1* and *YWHAE* within a ring chromosome (Bershteyn et al., 2014, 2017).

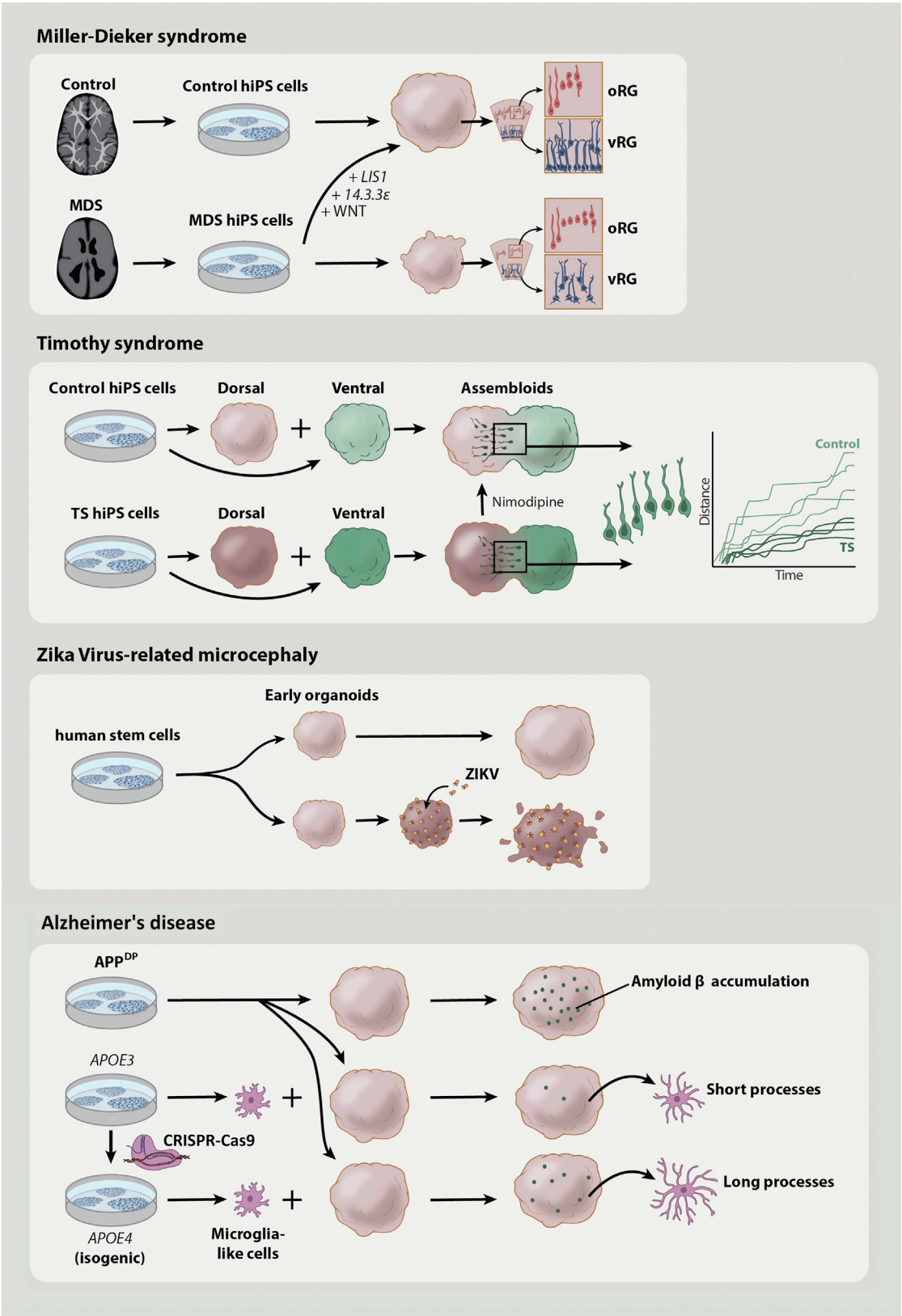
Disordered Cortical Folding

The biophysical forces driving the development of brain organoid surface convolutions were studied using a thin microfabricated compartment that constrained vertical growth as a model of brain gyrification (Karzbrun et al., 2018). hES-derived telencephalic organoids carrying isogenic heterozygous mutations in *LIS1* associated with congenital lissencephaly exhibited fewer surface convolutions and alterations in cell cycle-dependent nuclear motion and swelling (Karzbrun et al., 2018). A separate study investigated the role of *PTEN* implicated in human macrocephaly (Butler et al., 2005) in the development of mouse and human organoid surface features (Li et al., 2017b). While *PTEN* knockout organoids derived from both mouse and human

Table 1. Selected Studies Investigating Neuropsychiatric Disorders Using Human Stem Cell-Derived 3D Organoids

Disease	Cohort and Cell Lines	Organoid Type	*In Vitro* Stage	Unique Experimental Features	Phenotypes and Rescue
Miller-Dieker syndrome (Iefremova et al., 2017)	two patients (Δ17p13.3); two controls (gender/age-matched) [hiPS cell]	forebrain	4 weeks	doxycycline-inducible overexpression of *LIS1* or *YWHAE*	smaller organoids with fewer neuroepithelial loops, fewer symmetric vRG divisions, disrupted cortical niche; rescue with gene re-expression or by β-catenin activation
Miller-Dieker syndrome (Bershteyn et al., 2017)	three patients (Δ17p13.3, two lines each); three controls (four control lines total) [hiPS cell]	forebrain	5 weeks	introduction of a 5.3 Mb extrachromosomal DNA containing 17p13.3	mitotic defect in oRG, apoptosis of progenitors, cell migration defect; rescue with introduction of extrachromosomal fragment
Autosomal recessive primary microcephaly (Li et al., 2017a)	one patient (*ASPM* mutation, three lines); one control [hiPS cell]	undirected	~3 months	patient versus control organoids	smaller organoid size
Autosomal recessive primary microcephaly (Lancaster et al., 2013)	one patient (*CDK5RAP2* mutation; four lines); one control [hiPS cell]	undirected	~3 weeks	electroporation-mediated overexpression of *CDK5RAP2* and shRNA-*CDK5RAP2*	altered vRG morphology, reduced organoid size; rescue by shRNA for *CDK5RAP2*
Autism Spectrum Disorder (Mariani et al., 2015)	four patients with idiopathic ASD and macrocephaly; four familial controls (two to three lines per patient and control) [hiPS cell]	forebrain	6 weeks	lentiviral-mediated expression of shRNA-*FOXG1*	transcriptome dysregulation including *FOXG1* upregulation, increased GABAergic neuron production; rescue by shRNA knockdown of *FOXG1*
Timothy syndrome (Birey et al., 2017)	three patients (*CACNA1C* mutation, seven lines); five controls (six lines) [hiPS]	dorsal forebrain, ventral forebrain, assembloids	~2 months	forebrain assembloids; cell type specific labeling (*Dlxi1/2b::*eGFP)	increased calcium following electrical depolarization, increased saltation frequency and shorter saltation length of GABAergic neurons; rescue by pharmacological modulation of L-type calcium channels
ZIKV-associated microcephaly (Qian et al., 2016)	two controls (two lines each) [hiPS cell]	forebrain	~3 months	ZIKV strains: MR766 and FSS13025 (99% amino acid similar to Brazilian ZIKV)	reduced organoid size, reduced ventricular thickness with increased ventricular lumen, non-cell autonomous apoptosis
Retinitis pigmentosa (Deng et al., 2018)	two patients (*RPGR* mutation); two controls [hiPS cell]	retinal	36 weeks	CRISPR-Cas9 genome correction of *RPGR* in hiPS cells	decreased number of rods and cones, shorter cilia, gene expression changes, increased cell death; rescue following *RPGR* gene correction
Leber congenital amaurosis (Parfitt et al., 2016)	one patient (intronic *CEP290* mutation); one control [hiPS cell]	retinal (with optic cup)	21 weeks	splice-correcting ASO for *CEP290*	defect in RPGR localization in cilia, number and length of cilia; rescue with ASO
Brain tumor (Ogawa et al., 2018)	one H9 line [hESC]	undirected	8 months	CRISPR-Cas9 recombination of HRasG12V into the *TP53* genomic locus	tumorigenesis, tumor invasiveness after xenotransplantation into the mouse CNS
Alzheimer's disease (Park et al., 2018)	ReNcell VM cells (immortalized hNPCs), hiPSC-derived hNPCs	neural cell line in microfluidic chamber	9 weeks	viral transduction of hNPCs with an APP variant carrying multiple FAD mutations; co-culture with the human immortalized microglia SV40 cell line	increased Aβ aggregation and greater microglia recruitment in FAD cultures; reduction in microglia migration speed with anti-CCL2 neutralizing antibodies; microglia induce neuronal toxicity in FAD cultures

Relevant experimental features are highlighted, including the type and number of independent human cell lines, the specific type of brain organoid differentiation, the duration of organoid culture for disease phenotyping, and key disease-related phenotypes identified, including any phenotypic rescues. hiPS cell, human induced pluripotent stem cell; hESC, human embryonic stem cell; hNPC, human neural progenitor cell; vRG, ventral radial glia; oRG, outer radial glia; ASO, anti-sense oligonucleotide; APP, amyloid precursor protein; FAD, familial Alzheimer's disease.



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embryonic stem cells were larger than respective species controls, only those derived from human stem cells exhibited neuroepithelial overgrowth and an easily apparent increase in surface convolutions. Examination of progenitors along organoid curvatures revealed more dividing cells, suggesting rapid proliferation in these areas contributed to physical buckling and the formation of surface folds. Viral transduction of wild-type *PTEN* rescued these phenotypes, and activation or inhibition of AKT could similarly modulate neuroepithelial proliferation and organoid surface complexity (Li et al., 2017b), linking the PTEN/AKT signaling pathway to the genesis of human brain organoid macrostructures.

Autosomal Recessive Primary Microcephaly

Patients with autosomal recessive primary microcephaly (MCPH) exhibit mild intellectual disability and grossly normal cortical architecture but have dramatically decreased brain volume (Faheem et al., 2015). MCPH has been linked to several genes encoding centrosome-related proteins that are thought to regulate human cortical neurogenesis (Faheem et al., 2015). The most frequent known causes of MCPH are mutations in *ASPM* encoding a mitotic spindle protein (Bond et al., 2002). Mice with truncated *ASPM* genes have only slightly smaller brains than controls (Pulvers et al., 2010), driving interest in exploring non-rodent models (Johnson et al., 2018) that might better recapitulate disease processes. hiPS cell-derived undirected brain organoids from a patient with biallelic *ASPM* mutations exhibited significantly reduced organoid diameter, disordered neuroepithelia, and a reduction in progenitor pools (Li et al., 2017a). When cultured for nearly 3 months, organoids carrying *ASPM* mutations had fewer neurons with calcium activity, and neuronal activity was less synchronized (Li et al., 2017a). Mutations in *CDK5RAP2* have also been identified in MCPH patients, although Cdk5rap2 loss-of-function mouse models did not show obvious brain morphological phenotypes (Barrera et al., 2010). Undirected brain organoids generated from an MCPH patient with compound heterozygous *CDK5RAP2* mutations exhibited premature neuronal differentiation and smaller overall organoid size compared to control organoids. Overexpression or RNAi-mediated knockdown of *CDK5RAP2* by electroporation was able to respectively rescue and reproduce these phenotypes (Lancaster et al., 2013).

Seckel Syndrome

Defective neurogenesis has been observed in organoid models of other neurodevelopmental conditions. Patients with a mutation in the centrosomal-P4.1-associated protein (*CPAP*) develop Seckel syndrome, which is characterized by dwarfism and congenital microcephaly. Undirected brain organoids derived from a Seckel syndrome patient exhibited premature differentiation of neural progenitors (Gabriel et al., 2016). Patient-derived organoids also contained apical neural progenitors with increased length and number of cilia compared to organoids derived from control patients, providing additional clues about CPAP's role in neurogenesis (Gabriel et al., 2016).

Idiopathic Autism Spectrum Disorder

Autism spectrum disorder (ASD) refers to a group of heterogeneous developmental conditions characterized by deficits in language and social-emotional reciprocity as well as restriction in interests or repetitive patterns of behavior. Twin studies point to genetic heritability as a significant ASD risk factor (Hallmayer et al., 2011), and dozens of specific mutations and copy number variations (CNVs) have been linked to a minority of ASD cases with widely varying penetrance and effect sizes (Geschwind, 2011; Lord et al., 2018; State and Šestan, 2012). Few studies have attempted to study the far more common cases of idiopathic ASD. In a cohort of four ASD patients with associated macrocephaly, mutations previously associated with ASD could not be identified by whole-genome sequencing (Mariani et al., 2015). Dorsal telencephalic organoids were generated from hiPS cells derived from these patients and unaffected familial controls. ASD organoids exhibited dysregulation of transcripts involved in cell proliferation, neuronal differentiation, and synaptic assembly compared with controls. An expanded analysis including patient phenotypes revealed correlations between the degree of gene expression change, macrocephaly, and symptom severity. Transcriptomic data additionally suggested an increase in progenitors and neurons of the GABAergic lineage in ASD organoids, a finding that was verified by an increase in immunoreactivity and electrophysiological features characteristic of GABAergic neurons. Additionally, *FOXG1*, an important regulator of forebrain development, was among the most upregulated genes in ASD organoids (Mariani et al., 2015). In fact, *FOXG1* mutations were previously identified in patients exhibiting a neurodevelopmental syndrome resembling ASD (Kortüm et al., 2011). Interestingly, stable expression of an shRNA directed against *FOXG1* was able to rescue the aberrantly high production of GABAergic neurons seen in ASD patient organoids (Mariani et al., 2015). In a separate study, telencephalic organoids carrying a heterozygote mutation of the ASD-linked chromatin remodeling factor *CDH8* (chromodomain helicase DNA-binding protein 8) also showed dysregulation of genes involved in GABAergic interneuron differentiation (Wang et al., 2017). The observation that both an idiopathic and non-idiopathic ASD organoids have altered GABAergic interneurons is consistent with a hypothesis that ASD is the result of imbalance between excitation and inhibition in the brain (Rubenstein and Merzenich, 2003).

Figure 3. Cellular and Molecular Phenotypes Revealed Using Human Brain Organoid Models of Neuropsychiatric Disorders

Miller-Dieker syndrome (MDS) patients have smooth cortical surfaces as detected by magnetic resonance imaging (MRI), and cellular biopsies were obtained from affected patients to generate hiPS cells and subsequently human brain organoids. Organoids from MDS patients had reduced growth rates and fewer symmetric vRG divisions, and oRGs had prolonged mitosis. These phenotypes could be partly rescued with expression of *LIS1* or *YWHAE* genes or activation of Wnt signaling (Bershteyn et al., 2017; Iefremova et al., 2017). Ventral inhibitory neurons within assembloids derived from Timothy syndrome (TS) patients exhibited alterations in saltatory migration that could be corrected with the L-type channel blocker, nimodipine (Birey et al., 2017). To investigate the effect of microglia on amyloid beta (Aβ) clearance in Alzheimer's disease, microglia-like cells derived from hiPS cells were cocultured with 2-month-old organoids overexpressing amyloid precursor protein (APP). Organoids containing microglia-like cells derived from a patient with a low-risk gene variant (APOE3) had fewer Aβ aggregates than organoids containing isogenic microglia-like cells carrying a high-risk gene variant (APOE4) generated with CRISPR-Cas9 (Lin et al., 2018). To investigate ZIKV-associated microcephaly, ZIKV strains added to early-stage organoids caused cell death and decreased organoid size (Cugola et al., 2016; Garcez et al., 2016; Qian et al., 2016).

Timothy Syndrome

Monogenic causes of ASD provide an opportunity to investigate specific gene-function relationships that disrupt human brain development. Patients with a missense mutation in a gene encoding the L-type calcium channel *CACNA1C* exhibit Timothy syndrome, which is characterized by ASD and epilepsy (Birey et al., 2017) (Figure 3). Initial investigation of hiPS cell-derived neurons in monolayer identified calcium signaling defects, activity-dependent gene expression differences, and abnormalities in cortical specification with increased production of upper-layer neurons (Krey et al., 2013; Paşca et al., 2011). Within human brain organoids resembling the dorsal or ventral forebrain, Timothy syndrome-derived neurons exhibited calcium signaling defects (Birey et al., 2017). Viral transduction of ventral organoids with a Dlxi1/2b::eGFP expression vector fluorescently labeled GABAergic interneurons. Live imaging of Timothy syndrome forebrain assembloids revealed a cell-autonomous increase in saltation frequency coupled with a decrease in saltation length of GABAergic interneurons, resulting in overall delayed migration. This phenotype was rescued by blocking the activity of L-type calcium channels using nimodipine (Birey et al., 2017).

Sandhoff Disease

Sandhoff disease is a GM2 gangliosidosis caused by genetic disruption of *HEXB* encoding a component of the beta-hexosaminidase lysosomal enzyme and is characterized by developmental regression, neurodegeneration, and childhood death (Bley et al., 2011). Mutant mice have been used to model Sandhoff disease, though ganglioside degradation pathways differ in mice and humans (Lawson and Martin, 2016; Sango et al., 1995). Undirected brain organoids were generated from hiPS cells derived from an infantile Sandhoff disease patient with megalencephaly carrying compound heterozygous *HEXB* mutations and isogenic control hiPS cells in which a *HEXB* mutation was corrected with CRISPR-Cas9 (Allende et al., 2018). Sandhoff disease brain organoids accumulated excess GM2 ganglioside versus isogenic controls, which is the histopathological hallmark of this disease. AAV-mediated overexpression of macaque *HEXA* and *HEXB* rescued hexosaminidase activity and GM2 accumulation in Sandhoff disease organoids. Organoid proliferation and overall size increased in Sandhoff disease organoids, correlating with increased expression of transcription factors involved in neuronal differentiation identified by whole-organoid RNA sequencing (Allende et al., 2018).

Tuberous Sclerosis Complex

Tuberous sclerosis complex is a multisystem condition associated with epilepsy, ASD, intellectual disability, and psychiatric disorders and is caused by heterozygous germline mutations in *TSC1* or *TSC2* (Curatolo et al., 2015). TSC1 and TSC2 protein form a heterodimeric complex that negatively regulates mechanistic target of rapamycin complex 1 (mTORC1), a kinase that controls cellular proliferation and metabolism (Saxton and Sabatini, 2017). Patients commonly have focal regions of disorganized and dysmorphic neurons and glia called cortical tubers that could be the result of haploinsufficiency or loss of heterozygosity causing mTORC1 hyperactivation (Martin et al., 2017). Using CRISPR-Cas9, mutations of *TSC1* or *TSC2* were introduced in hES cells that were subsequently differentiated into cortical organoids (Blair et al., 2018). *TSC1* and *TSC2* heterozygous organoids showed no alterations in neuronal or glial differentiation, but homozygous knockout organoids had reduced or delayed expression of neuronal markers with a concomitant increase in the glial lineage. Neurons and glia in *TSC1* and *TSC2* homozygous knockout organoids were dysmorphic, similar to cellular phenotypes observed in patients' tubers. Organoids carrying a *TSC2* conditional allele and generated from patient-derived fibroblast lines also showed that biallelic inactivation of *TSC2* was required to replicate the cellular phenotypes. These findings are consistent with loss of *TSC1* and *TSC2* heterozygosity causing cellular phenotypes observed in cortical tubers. Addition of rapamycin to early-stage $TSC2^{-/-}$ organoid cultures to suppress overactive mTORC1 signaling restored the glial differentiation bias, and addition of rapamycin in late stage organoids reversed cellular hypertrophy (Blair et al., 2018).

Models of Zika Virus-Associated Microcephaly

The dramatic increase in congenital microcephaly cases observed in Brazil from 2014 to 2015 coincided with an outbreak of Zika virus (ZIKV), a member of the flavivirus family (Fauci and Morens, 2016). Other flaviviruses are not known to be teratogenic, and experimental evidence linking ZIKV infection to altered brain development was initially lacking. In an early laboratory study, ZIKV exhibited high infectivity in hiPS cell-derived human neural progenitor cells (hNPCs) grown in monolayer, causing attenuated hNPC growth and death (Tang et al., 2016). Another early study demonstrated that exposure of undirected human cerebral organoids to ZIKV caused grossly restricted growth and caspase-mediated cell death while exposure to DENV2, a flavivirus that causes Dengue fever, did not (Garcez et al., 2016). In forebrain organoids exposed to ZIKV at 2 and 4 weeks, hNPC proliferation was reduced, non-cell autonomous cell death was observed, and organoid ventricular zones and neuronal layers were thinner (Qian et al., 2016). A ZIKV strain isolated from the recent Brazilian epidemic caused even greater perturbation of neurogenesis and apoptosis in human and chimpanzee undirected organoids than the older and more highly passaged ZIKV-MR766 strain (Cugola et al., 2016) (Figure 3). Interestingly, hNPCs infected by ZIKV were less likely to exhibit horizontal division planes that are essential for hNPC symmetric expansion (Gabriel et al., 2017), indicating that premature differentiation contributes to ZIKV-mediated progenitor depletion.

The biological mechanism behind these phenotypes was subsequently investigated by examining the constituent proteins encoded by ZIKV. In fetal neural stem cell monolayer cultures, ZIKV-NS4A and ZIKV-NS4B were found to suppress the Akt-mTOR pathway, thereby causing defective neurogenesis (Liang et al., 2016). In forebrain-directed organoids, overexpressing the ZIKV-NS2A protein by electroporation caused destabilization of adherent junctions and disruption of vRG (Yoon et al., 2017). These organoid phenotypes recapitulate a post-mortem analysis of a ZIKV-infected microcephalic fetus in which ZIKV envelope glycoprotein was identified in the ventricular zone and vRG scaffolding was disorganized (Onorati et al., 2016). Importantly, the overexpression of the respective Dengue virus proteins,

DENV-NS4A, DENV-NS4B, and DENV-NS2A, had no effect on neurogenesis, suggesting ZIKV-derived proteins have unique teratogenic properties among the flavivirus family (Liang et al., 2016; Yoon et al., 2017).

Flaviviruses have been shown to activate the Toll-like-Receptor 3, TLR3, in human fibroblasts (Hamel et al., 2015; Tsai et al., 2009), and similarly, ZIKV-mediated toxicity might result from activation of intrinsic immune processes. Undirected cerebral organoids infected with ZIKV upregulated TLR3, while treatment with a direct competitive inhibitor of TLR3 rescued ZIKV-mediated apoptosis and suboptimal organoid growth (Dang et al., 2016). RNA sequencing of ZIKV-infected forebrain organoids demonstrated upregulation of genes involved in innate immunity, including cholesterol 25-hydroxylase, an enzyme that generates 25-hydroxycholesterol (25HC) that can block viral fusion with cell membranes (Watanabe et al., 2017). Culturing brain organoids with exogenously applied 25HC effectively reduced ZIKV replication. However, 25HC may have intrinsic toxicity, and increased ZIKV-induced cell death was not rescued by 25HC (Watanabe et al., 2017).

Early studies using non-neuronal cell cultures indicated that ZIKV preferentially uses the cell membrane-localized AXL receptor tyrosine kinase to gain intracellular entry required for infectivity (Hamel et al., 2015; Savidis et al., 2016). Single-cell sequencing of human undirected brain organoids revealed high expression of AXL in radial glia and minimal expression in excitatory and inhibitory neurons, leading to the hypothesis that AXL mediates the apparent selective tropism of ZIKV for neural progenitors (Nowakowski et al., 2016). However, genetic elimination of AXL in undirected brain organoids did not alter ZIKV infectivity or ZIKV-mediated organoid growth restriction (Wells et al., 2016). Consistently, a separate study demonstrated that pretreatment of forebrain organoids with a small molecule inhibitor of AXL, R428, or AXL blocking antibodies had a modest effect on ZIKV-mediated defects in neurogenesis (Watanabe et al., 2017). ZIKV was shown to use AXL to enter human glia (Meertens et al., 2017) suggesting AXL might mediate ZIKV entry in non-neuronal or progenitor cell types that are not represented in all types of organoids. Another study showed that hiPS cell-derived microglia-like cells had high ZIKV infectivity rates but were resistant to ZIKV-mediated toxicity (Muffat et al., 2018). When ZIKV-infected microglia-like cells were co-cultured with hiPS cell-derived undirected brain organoids, they could invade and effectively transmit ZIKV to other cell types (Muffat et al., 2018).

Since human brain organoids model ZIKV replication and the resulting disruption of neurogenesis that underlies ZIKV-mediated microcephaly, they are being used to test potential therapeutic agents. Among thousands of compounds screened in monolayer hNPC cultures, emricasan was the most potent suppressor of ZIKV-mediated caspase activity in cell culture. When tested in forebrain organoids, emricasan was neuroprotective but did not suppress ZIKV replication (Xu et al., 2016). In a second high-content chemical screen in hiPS cell-derived hNPCs, several compounds inhibited ZIKV replication without negatively affecting cell proliferation (Zhou et al., 2017). Among these, hippeastrine hydrobromide (HH) and amodiaquine dihydrochloride dihydrate showed the highest efficacy. When tested on hiPS cell-derived forebrain organoids infected with ZIKV, HH rescued ZIKV-mediated apoptosis, reduced progenitor proliferation, and disrupted organization of ventricular and neuronal zones. Furthermore, HH reduced ZIKV in organoids to undetectable levels and suppressed ZIKV infection in an immune-deficient mouse model (Zhou et al., 2017).

Models of Environmental Exposures

Since many human brain organoid models transcriptionally and epigenomically resemble the fetal brain (Camp et al., 2015; Luo et al., 2016), they are relevant for study of teratogens affecting central nervous system development. Prenatal toxic exposures can result in acquired brain disorders and is most frequently observed with fetal alcohol exposure, which increases the risk of microcephaly, ataxias, and intellectual disability. Exposing undirected brain organoids to ethanol caused premature progenitor differentiation, decreased neurite outgrowth, dysregulation of genes affecting signaling transduction pathways, and increased cell death (Zhu et al., 2017). Undirected brain organoids grown using microfluidics and treated with varying levels of nicotine exhibited disruption of cortical layers (Wang et al., 2018). Brief cocaine exposure increased reactive oxygen species in forebrain organoids, possibly mediated by CYP3A5 (Lee et al., 2017). Psychogenic and dissociative agents are being explored for treatment of psychiatric conditions, but the neurobiology of their activity is poorly understood. In one study, the active psychogenic compound in ayahuasca, 5-meO-DMT, altered peptides expressed in undirected brain organoids as detected by mass spectrometry (Dakic et al., 2017). Finally, forebrain organoids exposed to Bisphenol A, commonly found in household plastics, exhibited dose-dependent reduction in proliferation of hNPCs and ventricular-like zone thickness (Qian et al., 2016).

Models of Neuronal-Glia Interactions

Aicardi Goutieres

Mutation in *TREX1* (three prime repair exonuclease 1) is associated with Aicardi-Goutieres syndrome (AGS) which causes severe central nervous system inflammation and infantile encephalopathy. AGS shares clinical and diagnostic features with systemic lupus erythematous which has also been associated with TREX1 mutations and can result in debilitating neuropsychiatric symptoms (Rice et al., 2015). Dorsal telencephalic organoids generated from AGS patient-derived hiPS cells and isogenic *TREX1*-mutated hES cells were smaller in size than controls and contained more apoptotic neurons (Thomas et al., 2017). Consistent with its role in suppressing intracellular ssDNA, TREX-1-deficient monolayer neurons contained abundant extrachromosomal DNA. Interestingly, a large fraction of ssDNA was comprised of Long Interspersed Element-1 (LINE-1) retrotransposons. When LINE-1 reverse transcription was chemically inhibited, TREX-1-deficient monolayer neurons had more neurites and less cell body clumping, thereby linking aberrant LINE-1 activation to AGS neuronal pathology. Furthermore, treatment of organoids with conditioned media from TREX-1-deficient, hiPS cell-derived astrocytes caused reduction in organoid size and increased cell death, which was consistent with data from monolayer cultures showing that TREX-1-deficient astrocytes

contribute to neuronal toxicity via activation of a type 1 interferon-mediated response (Thomas et al., 2017).

Neuronal Injury

Microglia are immune surveillance cells within the central nervous system and impact diverse brain processes. One study developed protocols to generate human microglia-like cells from hiPS cells (MGLCs) and subsequently co-cultured MGLCs within undirected organoids to model their behavior within a 3D brain environment (Abud et al., 2017). Within 3 days, MGLCs embedded into the organoids and demonstrated tiling. Organoids were pierced to model neuronal injury, and MGLCs within the organoids changed their morphology, reflecting an activated state (Abud et al., 2017). Organoids co-cultured with MGLCs have also been used to examine ZIKV infectivity (Muffat et al., 2018), which is discussed above, and Alzheimer's disease-related processes (Lin et al., 2018), which are covered below.

Models of *DISC1* Gene Disruption

DISC1 encodes an intracellular hub protein that influences a wide range of molecular pathways and is associated with psychiatric diseases (Bradshaw and Porteous, 2012). DISC1 interaction domains and binding partners are thought to mediate its role in neurodevelopment and psychiatric disease. The paralogous proteins NDE1 and NDEL1 are cell cycle regulators also implicated in psychiatric disease (Feng and Walsh, 2004; Nicodemus et al., 2010) and were found to interact with DISC1's peptides 765–852 within its C-terminal coiled-coil region (Ye et al., 2017). The DISC1 765–852 peptide can have a dominant-negative effect by disrupting DISC1-NDE1/NDEL1 interactions. When overexpressed by plasmid electroporation in control hiPS-derived forebrain organoids, DISC1 765–852 caused a delay of cell cycle progression and reduction in vRG proliferation. Of note, the DISC1 765–852 domain is also lost in patients with the DISC1 t(1; 11)(q42; q14.3) translocation that co-segregated with schizophrenia in a Scottish family. Investigation of 20-day-old undirected brain organoids from a patient from an American family carrying a 4 base pair frameshift mutation in *DISC1* phenocopied the delay in cell cycle progression seen after electroporation of DISC1 765–852, implicating disrupted DISC1-NDE1/NDEL1 interactions in disease pathogenesis (Ye et al., 2017). In a later study, *DISC1*-mutated organoids were found to lack ventricle-like structures, have smaller and more disorganized rosette structures, and decreased progenitor proliferation (Srikanth et al., 2018). Addition of a WNT antagonist partially rescued these phenotypes (Srikanth et al., 2018), which is consistent with a prior report that *DISC1* mutations interfere with WNT signaling pathways (Srikanth et al., 2015).

Models of Retinal Diseases

Retinitis Pigmentosa

Retinitis pigmentosa (RP) is a genetically heterogeneous inherited disorder of retinal photoreceptors and is associated with night blindness, loss of visual acuity, and complete blindness (Ferrari et al., 2011). The most frequent cause of X-linked RP is a mutation in *RPGR* (Retinitis pigmentosa GTPase regulator) encoding a protein localized to the connecting cilium which separates photoreceptors' outer and inner segments. Timed application of BMP4 to 3D aggregates of stem cells generates retinal organoids with retinal epithelia containing rod and cone photoreceptors and allows for *in vitro* investigation of human retinal pathology (Kuwahara et al., 2017). RP patient-derived retinal organoids with *RPGR* mutations exhibited delocalization of rhodopsin (Deng et al. 2018), consistent with a previously described role for a complex containing RPGR in the transport of this photosensitive protein to the photoreceptor outer segment (Wang and Deretic, 2014). RP patient-derived retinal organoids also exhibited fewer rods and cones, shortened cilia, defects in photoreceptor electrophysiology and morphology, opsin mislocalization, and dysregulation of gene transcripts involved in intraflagellar transport. Correction of the *RPGR* mutation in RP patient-derived hiPS cells using CRISPR-Cas9 rescued both cellular and transcriptional phenotypes (Deng et al., 2018).

Leber Congenital Amourosis

One of the earliest-onset and more severe inherited retinal disorders is Leber congenital amaurosis (LCA), which causes congenital blindness. A frequent cause of LCA is an intronic mutation that causes mis-splicing of *CEP290* (centrosomal protein of 290 kDa), which encodes a protein that regulates protein trafficking between the inner and outer segments of photoreceptor neurons. Relative levels of *CEP290* mis-splicing increased when LCA hiPS cells carrying this mutation were differentiated into retinal organoids containing photoreceptors within self-organizing 3D optic cups (Nakano et al., 2012; Parfitt et al., 2016). This change in RNA processing suggests that intrinsic RNA processing pathways differ among specialized neuronal cell types such as photoreceptors and contributes to selective vulnerability of cell types to a genetic mutation with widespread expression. When optic cup cultures were treated with an anti-sense oligonucleotide (ASO) designed to correct *CEP290* splicing, *CEP290* mRNA and CEP290 protein increased, the number and length of cilia on photoreceptor progenitors increased, and defective RPGR localization to the cilium was restored (Parfitt et al., 2016). In a subsequent study, *CEP290*-correcting ASOs were initially screened for efficacy in LCA fibroblasts (Dulla et al., 2018). One efficacious ASO, QR-110, was added to 3-month-old LCA retinal organoids and was shown to restore CEP290 protein expression and cilia phenotypes in a dose-dependent manner (Dulla et al., 2018). The safety and efficacy of QR-110 in treating LCA patients with aberrant *CEP290* splicing is currently being assessed in clinical trials.

Different mutations in *CEP290* can also cause Joubert syndrome and related disorders (JSRD), which is more severe than LCA, as it is associated with malformations of the cerebellar vermis and brainstem in addition to retinal abnormalities. While hiPS cell-derived fibroblasts from LCA patients expressed reduced levels of CEP290 protein and had normal cilia, those derived from JSRD patients had complete loss of CEP290 protein and decreased ciliogenesis, suggesting cilia phenotypes are CEP290-dose dependent (Shimada et al., 2017). However, when LCA patients' hiPS cells were differentiated into retinal organoids containing optic cups, photoreceptors exhibited underdeveloped cilia as detected by electron microscopy (Shimada et al., 2017).

Models of Brain Cancer

The understanding of brain tumorigenesis has been significantly advanced by patient-derived tumor xenograft, tumor spheroid, and genetically engineered mouse models (Huszthy et al., 2012). These strategies are limited by donor availability, tumor evolution away from human genetic signatures (Ben-David et al., 2017), and poor clinical translatability (Huszthy et al., 2012), which has motivated the development of alternative models. Brain cancers can arise from several cell types of origin (Zong et al., 2015), and their growth is heavily influenced by a tumor microenvironment containing many cellular lineages including macrophages, endothelia, pericytes, and fibroblasts (Quail and Joyce, 2017) that are not present in current human brain organoid models. Nonetheless, human brain organoids are demonstrating value as models of genetic dysregulation that underlie malignant transformation and tumor growth. In one study, 4-month-old undirected brain organoids were electroporated with a two-plasmid, CRISPR-Cas9-based system in which an HRasG12V oncogene recombined into the genomic *TP53* locus (Ogawa et al., 2018). Electroporated cells took on tumorigenic properties including rapid division rates and invasive growth patterns into the organoid. Transplantation of dissociated human brain organoid-derived tumor cells into the hippocampi of immunodeficient mice caused their death within months. The organoid-derived tumor cells exhibited extensive invasiveness, spread along blood vessels, and were highly angiogenic. Nuclear pleomorphism and necrosis were observed on histopathological analysis. Additionally, patient-derived glioblastoma cells and organoid-derived tumor cells were combined with control human brain organoids in assembloids to model tumor growth pathways (Ogawa et al., 2018).

A second study expanded the range of tumorigenic mutations modeled in undirected brain organoids by combining Sleeping Beauty transposon-mediated oncogene insertion and CRISPR-Cas9-mediated knockouts of tumor suppressors in a nucleofection-based strategy (Bian et al., 2018). Early organoids containing neural stem cells and progenitors that overexpressed *MYC* resulted in tumor growth that transcriptionally and histomorphologically resembled central nervous system primitive neuroectodermal tumors (CNS-PNETs). These organoid-derived tumor cells were phenotypically and transcriptionally distinct from those generated by multiplexed overexpression and knockdown of gene combinations observed across glioblastoma multiforme cases. Neoplastic cerebral organoids (neoCORs) xenografted into the renal subcapsular space of mice proliferated and maintained their subtype identities *in vivo*, in contrast to control organoids that were mostly resorbed. To demonstrate this system's utility in chemotherapeutic drug screening, neoCORs were treated with EGFR inhibitors which only caused reduction in the growth of tumor cells from EGFR-overactivation backgrounds (Bian et al., 2018).

Models of Neurodegeneration

Alzheimer's Disease

Alzheimer's disease (AD) is characterized by progressive memory, language, and behavioral dysfunction and is associated with β-amyloid plaques and neurofibrillary tangles in extracellular and intracellular neuronal spaces, respectively. Mouse models lack neurofibrillary tangle pathology, and while monolayer neuronal cultures from AD patients express toxic proteins, they lack both plaques and tangles, which limits their utility as model systems (Drummond and Wisniewski, 2017). In an early non-organoid hES cell-based 3D culture system of neural stem cells in matrigel (Choi et al., 2014), overexpression of *APP* or *PSEN1* gene variants from early-onset familial AD patients induced both amyloid plaque deposition and aggregates of phosphorylated and filamentous tau. Furthermore, inhibition of β- and ɣ-secretase with small molecules decreased the formation of β-amyloid and tau. Culturing hNPCs in 3D for several weeks was sufficient to observe pathological protein aggregates not previously observed in mouse or monolayer culture models (Choi et al., 2014). In follow-up work, neurons and astrocytes derived from hNPCs were co-cultured in a microfluidic-based system with human immortalized SV40 microglia to investigate neuroimmunologic interactions related to AD pathogenesis (Park et al., 2018). Immortalized microglia migrated quicker and caused greater toxicity to neurons and astrocytes transduced with an APP variant carrying multiple FAD mutations versus controls. Furthermore, interferon-ɣ blocking antibodies or knockdown of TLR4 in microglia reduced neuronal toxicity, yielding insights into the cytokine signaling pathways activated in AD (Park et al., 2018).

While many studies have investigated the biogenesis of β-amyloid and tau aggregates seen in AD patient brains, the presence of protein aggregates could also be the result of impaired clearance pathways by glia. Microglia-like cells generated from hiPS cells expressing variants of the APOE protein that confer different susceptibilities to AD were combined with familial AD-derived forebrain organoids to form multi-lineage assembloids (Lin et al., 2018) (Figure 3). Within brain organoids, microglia-like cells from a high susceptibility background (APOE4 variant) had longer processes and poorer uptake of β-amyloid aggregates than controls. Long-term culture for 6 months allowed for the differentiation of neurons and astrocytes within brain organoids, and those generated from the APOE4 background had higher levels of β-amyloid and tau phosphorylation. Furthermore, a genetic change from a high (APOE4) to a low (APOE3) AD gene variant in hiPS cells using CRISPR-Cas9 resulted in organoids with reduced β-amyloid accumulation (Lin et al., 2018).

Frontotemporal Dementia

Identification and investigation of proteins that regulate tau, such as CDK5, could help identify drug targets that might reduce tau pathology seen in AD as well as frontotemporal dementia (FTD). Previous work has shown that CDK5, in association with p25, hyperphosphorylates tau and increases aggregation, while inhibition of CDK5 relieves neurotoxicity and tau pathology in AD models (Piedrahita et al., 2010; Kimura et al., 2014). A subset of FTD patients have genetic mutations in *MAPT*, the human gene encoding tau, and have associated tau pathology (Kimura et al., 2014). Forebrain brain organoids generated from hiPS cells derived from an FTD patient with a MAPT-P301L mutation exhibited increased levels of p25 (Seo et al., 2017). Introducing a CRISPR-Cas9-mediated mutation in p35 that inhibits conversion into p25 resulted in lower levels of total tau and phosphorylated tau in 2-month-old organoids derived from the MAPT-P301L FTD patient. A concomitant increase in synaptophysin was also seen, suggestive of more synapse formation when p25 generation is inhibited (Seo et al., 2017).

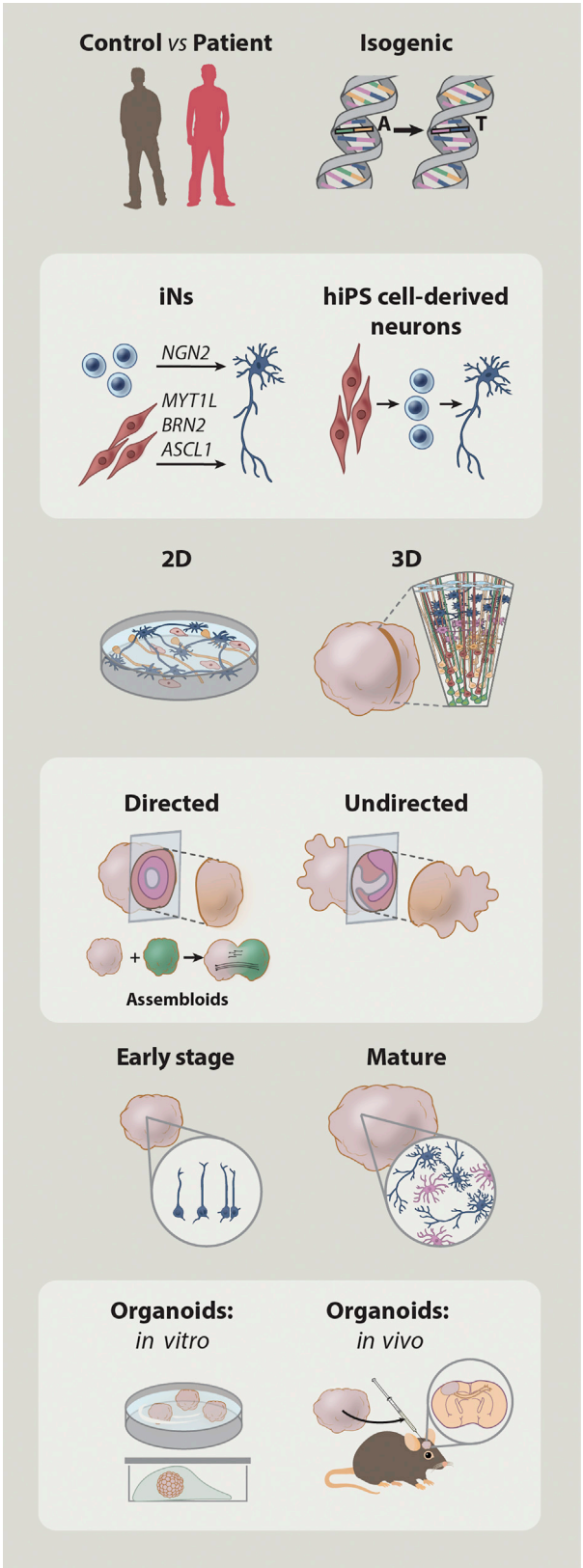

Figure 4. Considerations in Stem Cell-Based Experimental Design
Advances in stem cell technologies in the last decade have provided a multitude of experimental approaches for investigating neuropsychiatric disease. Highlighted here are important decision points to consider when using stem cells to model brain disorders (top to bottom): use of cohorts of patient and controls versus isogenic cell lines modified by gene editing; use of iNs versus hiPS-derived cell types; use of 2D monolayer cultures versus 3D human brain organoids; use of directed organoids combined into assembloids versus undirected organoids that model a diversity of brain regions; use of early-stage organoids versus those cultured for prolonged periods of time; and investigation of organoids *in vitro* versus *in vivo* after xenotransplantation.

Considerations and Limitations of Cellular Models of Brain Disorders

Within the last decade, the diversity of human stem cell-derived models has rapidly expanded. An increasing number of experimental design options are now available (Figure 4), each of which should be carefully considered to effectively address the neurobiological question of interest. For example, the selection of control hiPS cell lines should be aligned with the underlying genetics of the neuropsychiatric disorder being investigated. The use of isogenic hiPS cell lines limits unwanted sources of phenotypic variability caused by differences in genetic background (Chandler et al., 2013; Hoffman et al., 2018; Kilpinen et al., 2017). Therefore, when a specific mutation is suspected to influence a disease process, isogenic lines can be generated using genome engineering to identify and rescue monogenic disease phenotypes. On the other hand, the effects of known and unknown risk-contributing and protective alleles can be captured and compared *in vitro* by generating side-by-side patient and control-derived hiPS cells. This strategy is particularly relevant for investigating highly heritable yet genetically heterogeneous psychiatric conditions, such as bipolar disorder, in which hundreds of low-effect size genetic risk variants may contribute to disease (Birnbaum and Weinberger, 2017; Geschwind and Flint, 2015; Smoller et al., 2018; Sullivan et al., 2012). Unfortunately, deriving large numbers of individual hiPS cell lines from patients and controls is still time consuming and expensive. Another issue that can affect neuronal phenotypes is epigenetic history. Interestingly, direct conversion of somatic cells into iNs (Drouin-Ouellet et al., 2017) has been shown to maintain the age-related epigenetic signature of the patient's fibroblasts (Huh et al., 2016; Mertens et al., 2015a). However, neuronal differentiation of hiPS cells is generally more scalable, can be guided by a wider range of protocols to generate specific cell types, and can be used to model disorders of proliferation and developmentally programmed fate specification.

Three-dimensional cultures are alluring for their novelty; however, monolayer (2D) cultures retain certain advantages such as their homogeneity and scalability, which can make them more suitable for high-throughput genetic and pharmacologic screens. Cells in monolayer can more uniformly access nutrients, small molecules, peptides, and dissolved gases in culture media, while the center of large organoids may be starved due to limitations in diffusion. On the other hand, brain organoids model more physiologically relevant cellular interactions and can recapitulate structural and organizational features of the brain that may not be present in monolayer cultures. When using brain organoids, it is important to keep in mind the suitability of various approaches available for effective experimental design. Human brain organoids generated

by directed differentiation resemble specific brain regions that might be relevant to a specific neurobiological or disease process. By modulating culture conditions, protocols have been developed to generate human brain organoids resembling the cerebral cortex (Paşca et al., 2015), the ventral forebrain (Birey et al., 2017), the cerebellum (Muguruma et al., 2015), the midbrain containing functional dopaminergic neurons relevant to Parkinson's disease (Jo et al., 2016), and the spinal cord relevant to motor neuron diseases (Ogura et al., 2018). Directed brain organoids can be fused to generate assembloids that model cell-cell interactions and neural circuit formation and could be used to model region-specific disease processes such as hippocampal loss in Alzheimer's disease. Alternatively, undirected brain organoids contain a large diversity of cell types, including non-ectodermal lineages, and could be used to map the expression patterns of spatially restricted genes relevant to disease. However, the stochastic nature of this differentiation and the intrinsic differentiation propensities of individual hiPS cell lines result in high inter-organoid variability. An additional consideration is organoid culture duration and cellular maturation. One specific advantage of organoids over monolayer cultures is the ability to maintain 3D cultures for very long periods of time *in vitro* to achieve maturation milestones. For example, initial studies indicate that astrocytes in cortical organoids start resembling postnatal primary human astrocytes after 9–10 months in culture (Sloan et al., 2017). The conservation of developmental timing *in vitro* will be critical for investigating late-stage processes, such as gliogenesis and myelination, which have been previously difficult to study in humans. Moreover, this feature could be leveraged to understand and potentially control neuronal and glial maturation and ultimately develop more chronologically relevant human cellular models of age-associated neurodegeneration. Finally, the use of human brain organoids should be considered within the larger framework of investigative approaches for studying nervous system disorders. Findings in human brain organoids may reproduce those identified in other systems; however, phenotypes may unsurprisingly differ because of inherent species-specific differences. For example, microcephaly-related gene mutations in mice and human brain organoids have different effects on neural proliferation (Johnson et al., 2018; Li et al., 2017a; Pulvers et al., 2010), and mutations in *PTEN* gene cause neuroepithelial overgrowth in human but not mouse organoids (Li et al., 2017b).

Advancing Organoid Models of Brain Disorders

To further advance the application of brain organoid technologies to neuropsychiatric disorders, challenges in several domains will need to be addressed. First, investigations have demonstrated high levels of inter-organoid variability reflecting the stochastic nature of undirected differentiation protocols (Camp et al., 2015; Quadrato et al., 2017). Next-generation organoids should increase reproducibility, scalability, and internal spatial predictability of individual cell types and macrostructural features contained. Use of directed brain organoids generated with protocols that constrain developmental programs and defined starting numbers of stem cells may decrease inter-organoid variability. Further improvements in these areas may be achieved by incorporating bioprinting, nanotechnology, microfluidics, and biomaterials into differentiation, long-term culture, and experimental protocols. Additionally, methods are being investigated to vascularize organoids *in vitro* and *in vivo* (Mansour et al., 2018; Pham et al., 2018). Further protocol development in this area may improve nutrient diffusion to enhance organoid growth potential and long-term viability and might even enable better modeling of the blood-brain barrier, relevant to many disease processes and pharmacokinetics. The programs for deriving a greater diversity of brain regions and assembling them into mature functional circuits must be identified to expand the applicability of organoids to more brain disorders. For example, generating assembloids containing an organoid resembling the ventral tegmental area could enable the investigation of dopaminergic mesocortical pathways relevant to psychiatric disorders. Similarly, developing human brain organoids resembling the thalamus would enable the study of sensory inputs' effect upon cortical organoids in the context of disease. Recently, human brain organoids grafted into mouse brains were shown to integrate into the host neural networks (Mansour et al., 2018). Future studies achieving wiring of human brain tissue to sensory modalities in the animal cortex may provide another opportunity to model the impact of sensory inputs on human brain tissue. Ultimately, experimental models of complex psychiatric disorders will only be as useful as their ability to capture advanced human brain processes. As human brain organoids will recapitulate more sophisticated aspects of brain function, discussing the ethical implications will be essential (reviewed in Bredenoord et al., 2017; Farahany et al., 2018; Lavazza and Massimini, 2018).

Neuropsychiatric diseases cause considerable morbidity and mortality (The Lancet Neurology, 2017; Vigo et al., 2016), and progress in developing safe and efficacious treatments has been limited by inherent difficulty investigating neurobiological processes occurring in the human brain. Research on human brain organoids is contributing to our understanding of a wide variety of neuropsychiatric conditions and may ultimately facilitate innovation that leads to desperately needed treatments and improvements in patient outcomes.

DECLARATION OF INTERESTS

Stanford University has filed patent applications that covers the generation and assembly of region-specific neural spheroids.

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