CHAPTER TWELVE

Building the brain from scratch: Engineering region-specific brain organoids from human stem cells to study neural development and disease

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Abstract

Human brain development is an intricate process that involves precisely timed coordination of cell proliferation, fate specification, neuronal differentiation, migration, and integration of diverse cell types. Understanding of these fundamental processes, however, has been largely constrained by limited access to fetal brain tissue and the inability to prospectively study neurodevelopment in humans at the molecular, cellular and system levels. Although non-human model organisms have provided important insights into mechanisms underlying brain development, these systems do not fully recapitulate many human-specific features that often relate to disease. To address these challenges, human brain organoids, self-assembled three-dimensional neural aggregates, have been engineered from human pluripotent stem cells to model the architecture and cellular diversity of the developing human brain. Recent advancements in neural induction and regional patterning using small molecules and growth factors have yielded protocols for generating brain organoids that recapitulate the structure and neuronal composition of distinct brain regions. Here, we first provide an overview of early mammalian brain development with an emphasis on molecular cues that guide region specification. We then focus on recent efforts in generating human brain organoids that model the development of specific brain regions and highlight endeavors to enhance the cellular complexity to better mimic the *in vivo* developing human brain. We also provide examples of how organoid models have enhanced our understanding of human neurological diseases and conclude by discussing limitations of brain organoids with our perspectives on future advancements to maximize their potential.

1. Introduction

Brain organoids are self-organized three-dimensional (3D) neural aggregates formed from pluripotent stem cells (PSCs) that recapitulate the cytoarchitecture and cellular diversity of the developing brain (Qian, Song, & Ming, 2019). Brain organoids recapitulate key characteristic features of fetal nervous system development, including progenitor zone organization and sequential generation of neurons and glia. Studies in model organisms and *in vitro* combinatorial morphogen screening have informed protocols for differentiating PSC aggregates into organoids that generate

neural progenitors and neurons of various brain regions. We first provide an overview of mammalian brain development from neural tube formation to region specification and neurogenesis, focusing on morphogens involved in specifying regional fates along the dorsal-ventral and rostral-caudal axes, which forms the basis for many region-specific brain organoid protocols. We then delve into human-specific features of brain development and compare advantages and disadvantages of monolayer and three-dimensional *in vitro* cultures. We further review different brain organoids and their applications and finally we discuss limitations and opportunities for future improvements.

1.1 Fundamentals of mammalian brain development

Mammalian brain development is a highly coordinated process that integrates diverse signals across time and space. Brain morphogenesis begins during gastrulation with neural induction of the dorsal ectoderm by signals from the mid-gastrula organizer that secretes Bone Morphogenetic Protein (BMP) inhibitors (Levine & Brivanlou, 2007). Neural fate is often described as the "default fate" as embryonic stem cells (ESCs) readily express neural markers, such as NESTIN, when deprived of any growth factors or morphogens, whereas BMP4 addition promotes epidermal differentiation (Munoz-Sanjuan & Brivanlou, 2002). After gastrulation, the dorsal ectoderm thickens to form the neural plate, which proliferates, invaginates, and separates from the surface ectoderm to form the neural tube in a process known as neurulation (Wilson & Hemmati-Brivanlou, 1997). Following neurulation, the neural tube is patterned along the rostral-caudal axis into three major brain regions, the prosencephalon (forebrain), mesencephalon (midbrain), and rhombencephalon (hindbrain) and the spinal cord. The prosencephalon further segregates into the telencephalon, which forms the cerebral cortex and basal ganglia, and the diencephalon, which forms the retina, thalamus, and hypothalamus. Likewise, the rhombencephalon further segregates into the metencephalon, which forms the pons and cerebellum, and the myelencephalon, which forms the medulla (Fig. 1A).

A widely accepted concept is that the most rostral fate constitutes the primitive identity of the neural tube (Levine & Brivanlou, 2007; Stern, 2001) and after rostral neural fate acquisition, the rostral-caudal and dorsal-ventral axes are defined by positional cues from organizing centers located near to and within the developing neural tube. Regional brain patterning relies on morphogen gradients secreted by secondary organizing centers. Generally, WNT and BMP inhibition enhances rostral neural fate, whereas retinoic acid (RA), WNTs, and FGFs enhance caudal fates in a

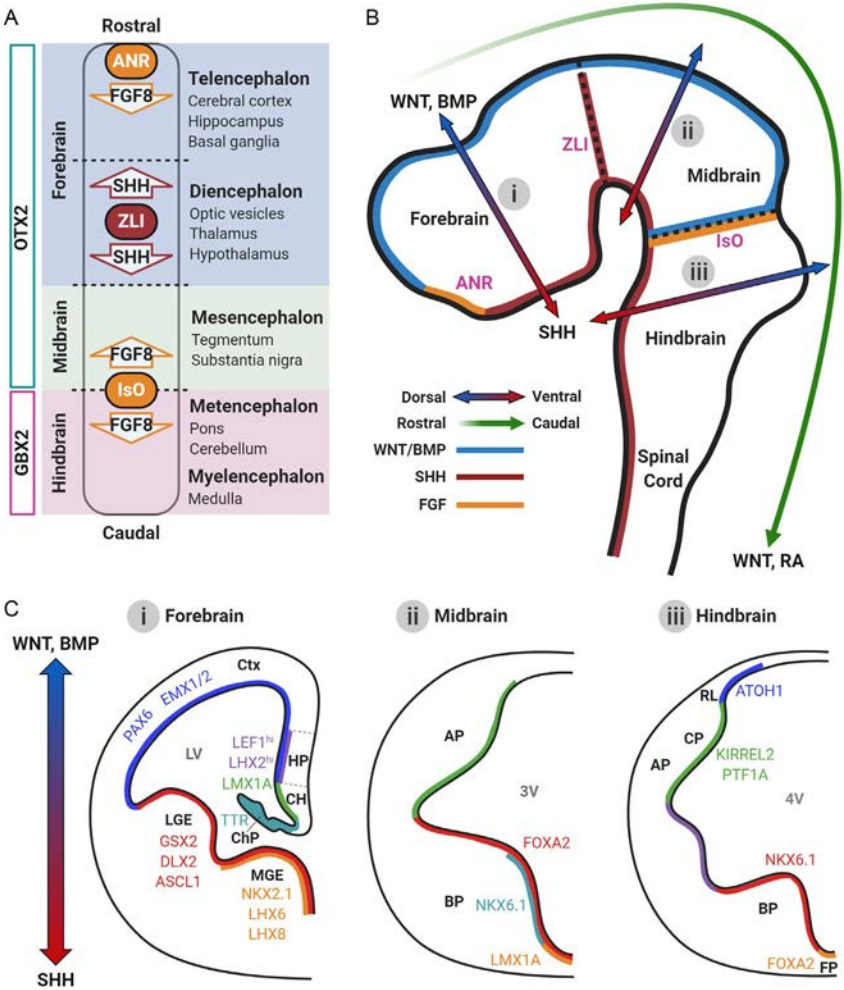

Fig. 1 Regional patterning of the developing neural tube. (A) Simplified diagram of major brain regions and their derivatives along the rostral-caudal axis of the developing neural tube with the location of important secondary organizers and their secreted morphogens. The non-overlapping expression of the transcription factors OTX2 and GBX2 distinguishes the developing forebrain and midbrain from the hindbrain. ANR, anterior neural ridge; ZLI, *zona limitans intrathalamica*; IsO, isthmic organizer. (B) Diagram of the midline sagittal plane of the embryonic mouse brain with the location of important secondary organizers and fate-inducing signals. Increasing WNT and RA specifies fates along the rostral-caudal axis and the opposing actions of WNT/BMP from the roof plate and SHH from the floor plate specifies dorsal and ventral fates. (C) Diagrams of the coronal cross sections of the embryonic mouse forebrain (i), midbrain (ii), and hindbrain (iii) outlining major progenitor zones and their defining transcription factors along the dorsal-ventral axis. Ctx, cortex; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; HP, hippocampus; CH, cortical hem; ChP, choroid plexus; AP, alar plate; BP, basal plate; RL, rhombic lip; CP, cerebellar plate; FP, floor plate; LV, lateral ventricle; 3V, third ventricle; 4V, fourth ventricle.

dose-dependent manner (Wilson & Hemmati-Brivanlou, 1997). Secondary organizers often secrete similar molecules that exert differential downstream effects due to prepatterned cellular competencies. The rostral-caudal axis contains several secondary organizers, including the anterior neural ridge (ANR), the *zona limitans intrathalamica* (ZLI), and the isthmic organizer (IsO) (Fig. 1A). Telencephalic fate induction arises from a discrete group of cells known as the ANR located at the rostral end of the embryo. Secretion of FGF molecules, most notably FGF8, from the ANR induces the adjacent neural ectoderm to express the transcription factor FOXG1, which defines the telencephalon (Rubenstein & Beachy, 1998). The ZLI patterns the diencephalon by secreting sonic hedgehog (SHH), which diffuses locally to specify the pre-thalamus rostrally and the thalamus caudally by activation of the transcription factors DLX2 and GBX2, respectively (Scholpp, Wolf, Brand, & Lumsden, 2006). The IsO which is close to the future midbrain-hindbrain boundary secretes FGF8 and WNT1, which diffuse locally to specify the tectum rostrally and the cerebellum caudally (Chi, Martinez, Wurst, & Martin, 2003).

Neural progenitor fates along the rostral-caudal axis are further specified by signals from the dorsal and ventral poles of the neural tube. Soon after neural induction, BMP is secreted from the lateral ectoderm and diffuses to induce the formation of the roof plate, a temporary glial population that also secretes BMP and WNT to specify dorsal fates (Wilson & Maden, 2005). Conversely, ventral to the neural tube lies the notochord, a transient mesodermal structure that secretes SHH to induce the formation of the floor plate, a temporary glial population that also secretes SHH to specify ventral fates (Wilson & Maden, 2005). Dorsal-ventral patterning of the neural tube is accomplished by the opposing signals of BMP/WNT and SHH, whose combined gradients specify distinct progenitor zones (Fig. 1B). The combination of timed morphogen gradients during brain morphogenesis leads to the restricted expression of distinct transcription factors, which define the identity of early progenitor zones and their competency to generate different neural subtypes (Fig. 1C). For example, the dorsal forebrain, which produces the glutamatergic projection neurons of the cortex and hippocampus, is defined by EMX1/2 and PAX6 expression, whereas the ventral telencephalon, which produces the GABAergic interneurons of the cortex and basal ganglia, is defined by GSX2 expression (Campbell, 2003). Restricted expression of NKX2.1 toward the midline further separates the medial (MGE) from the lateral ganglionic eminence (LGE) that generate different interneuron subtypes (Campbell, 2003).

1.2 Human-specific features

The human brain has undergone a massive increase in relative size over the last few million years of evolution, with major expansion of the surface area and thickness of the neocortex, which is in part responsible for the acquisition of higher cognitive functions in humans (Hill & Walsh, 2005; Lui, Hansen, & Kriegstein, 2011). Comparative studies among the human, non-human primate, and rodent cortex reveal not only an increase in the number, but also the diversity of cortical neurons in humans. The cortical expansion has been attributed to duplications in key neural stem cell regulatory genes, such as NOTCH, and the emergence of an additional neural progenitor population termed outer radial glia cells (oRGCs) situated in the outer subventricular zone (oSVZ), which produce many upper layer neurons (Suzuki et al., 2018). This second wave of neurogenesis from oRGCs is believed to be responsible for the massive increase in cortical size and neuron abundance. For example, several genes that cause congenital microcephaly when mutated, such as abnormal spindle protein (ASPM), were shown to only have minor phenotypes in mice, which lack a distinct oSVZ with oRGCs (Pulvers et al., 2010). In contrast, brain organoids grown from ASPM-mutant human induced pluripotent stem cells (hiPSCs) derived from microcephaly patients showed robust deficits in progenitor proliferation and neuronal generation (Li, Sun, et al., 2017). In addition, human brain development occurs over a much longer timeframe than rodents and disparities in microcephaly phenotypes may relate to differential cell-cycle lengths and division properties that are intrinsic to human cells. Certain mutations may also exert greater effects on oRGC division, adding to the discrepancy. Differences in the size, cellular diversity, and synaptic properties between humans and rodents highlights the need for a cellular model system for human brain development.

1.3 Comparison of *in vitro* human cell models

Human stem cell-derived models of neural development broadly include monolayer, neurosphere, and brain organoid cultures (Fig. 2). These model systems exist along a spectrum of least to most complex and each offer their own advantages and disadvantages. Monolayer cultures are the simplest systems and consist of adherent neural progenitor cells (NPCs) that undergo neuronal differentiation upon low-density plating and/or the addition of cell-cycle inhibitors (e.g., AraC) or maturation factors, resulting in fairly uniform cultures of adherent neurons. Monolayer cultures can be easily scaled up for large studies or screens by expanding NPCs before neuronal

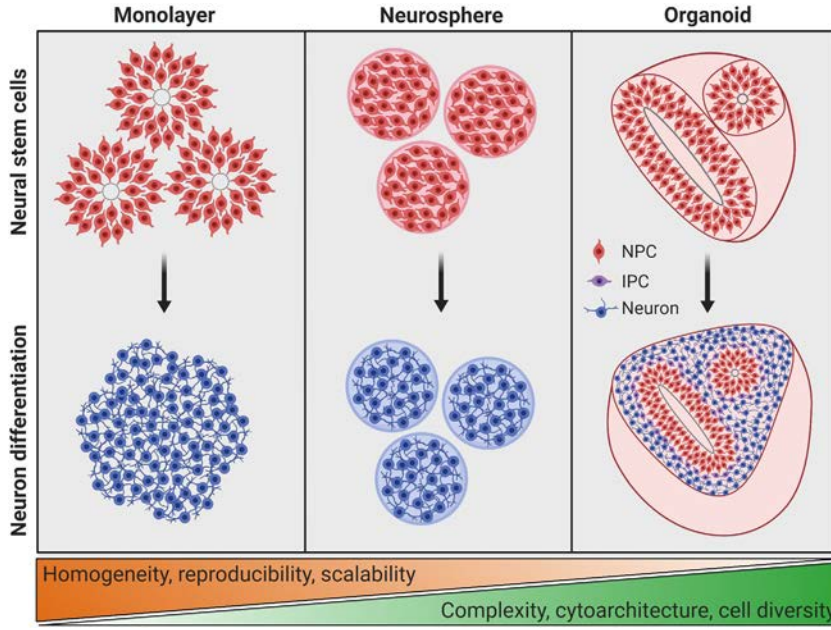

Fig. 2 Comparison of *in vitro* models of human neurodevelopment. Human pluripotent stem cell-derived neural progenitor cells can be cultured as monolayers, neurospheres, or organoids that undergo neuronal differentiation with prolonged culture. Monolayer cultures represent the most homogenous cultures that are reproducible and easily scalable by passaging. Brain organoids represent the most heterogeneous cultures that contain self-organized 3D cytoarchitecture, mixtures of progenitors and neurons, and diverse subpopulations of neural cells. Neurospheres represent an intermediate culture of neural progenitors or neurons in 3D that lack organized structures.

differentiation. Neurospheres are free-floating aggregates of neural progenitors that continuously expand with continued FGF and EGF exposure. Neurospheres permit neurons to interact with one another within a 3D environment, but do not contain progenitor structures and neuronal lamination that resemble the developing brain. Brain organoids exhibit more complex cytoarchitecture over time, consisting of both neural progenitors and developing neurons. Importantly, the structural organization of progenitors into ventricular zones followed by continuous neurogenesis for diverse neuronal subtypes and neuronal migration pattern more accurately resembles human brain development. With this increased complexity comes higher degrees of variability in structure and composition. In contrast to monolayers, 3D aggregates respond less uniformly to culture agents and undergo spontaneous self-organization. This self-organization leads to inter-organoid variability in structure size, shape, and regional identity that

may complicate some analyses. This disadvantage is often outweighed by benefits of more faithfully recapitulating the structural organization and cellular diversity of the fetal brain.

2. Generation of region-specific brain organoids from human stem cells

The foundation for creating human brain organoids begins with undifferentiated cultures of human PSCs, which include both human embryonic stem cells (hESCs) and hiPSCs. Ethical considerations around the use of human embryonic tissue for research has limited the number of hESC lines available for research; however, the discovery that human somatic cells such as fibroblasts and lymphocytes can be reprogrammed into hiPSCs by forced expression of a cocktail of transcription factors has led to a massive expansion of available human pluripotent stem cell lines (Takahashi et al., 2007). hiPSCs have the additional advantage of retaining the genetic background of individuals, making them especially useful for disease modeling. The protocols for generating brain organoids start with 3D hPSC aggregates and fall into two broad categories: unguided and guided differentiation (Fig. 3). We provide an overview of unguided and guided human brain organoid differentiation protocols with an emphasis on studies that define specific morphogen formulations and exposure lengths to specify distinct regional fates (Table 1).

2.1 Unguided differentiation: Cerebral organoids

hPSC aggregates grown in suspension preferentially differentiate toward neural ectoderm in the absence of additional patterning factors. hPSC aggregates are cultured as free-floating embryoid bodies (EBs), embedded into Matrigel, and cultured in serum-free conditions in a spinning bioreactor to enhance nutrient diffusion and support long-term health and tissue growth. These aggregates, termed cerebral organoids, contain neural stem cells that self-organize to form structures with a variety of cell lineages that resemble multiple brain regions, such as the dorsal and ventral forebrain, hippocampus, retina, and choroid plexus (Lancaster & Knoblich, 2014; Lancaster et al., 2013). For example, certain regions within a cerebral organoid recapitulate structural features of the developing cortex, with well demarcated ventricular zones with apical adherens junctions and radially oriented neural progenitor cells. These radial glia cells give rise to a cortical plate with neurons of the various layers of the cortex, including neurons expressing markers such as TBR1, SATB2, CTIP2 and RELN.

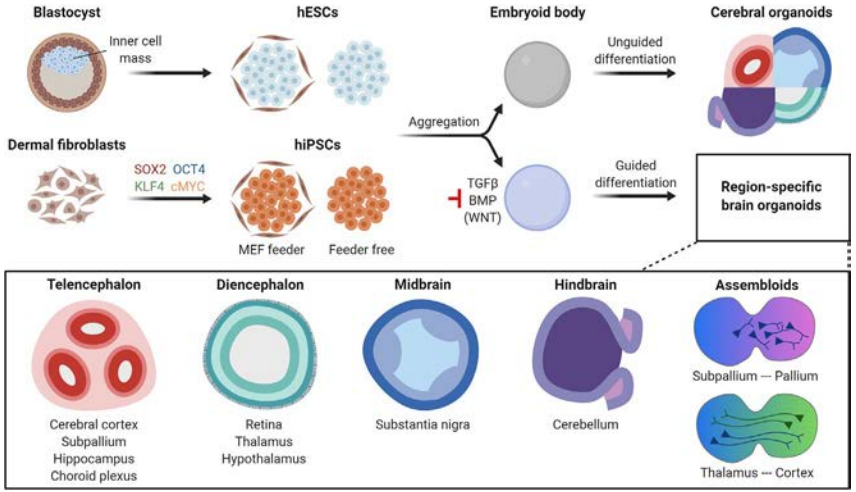

Fig. 3 Generation of brain region-specific organoids from human pluripotent stem cells. Diagram of the major steps for generating cerebral and region-specific brain organoids. Human PSCs, which include hESCs derived from the inner cell mass of human blastocysts and hiPSCs derived from cellular reprogramming of patient-derived somatic cells, such as dermal fibroblasts, are the starting material for generating brain organoids. hPSCs can be cultured on traditional mouse embryonic fibroblast feeder cells or without feeder cells using specialized media formulations. hPSCs are aggregated and cultured in suspension as embryoid bodies that are either placed in medium without morphogens for unguided differentiation toward cerebral organoids or neural induction medium with a series of patterning molecules for guided differentiation toward region-specific organoids. Organoid protocols have been developed for various brain regions of the telencephalon, diencephalon, midbrain, and hindbrain (see Table 1). Fused region-specific brain organoids, termed assembloids, have been developed to study the interactions between neurons of different brain regions.

Table 1 Region-specific brain organoid protocols.

Brain region	Starting cells	Patterning and maturation factors	ECM	Culture apparatus	Reference(s)
Brain (unguided)	ESCs or iPSCs (MEF)	NA (intrinsic)	Matrigel	Spinning bioreactor	Lancaster et al. (2013) and Lancaster and Knoblich (2014)
	iPSCs (MEF)	NA (intrinsic)	Defined hydrogel	Static culture dish	Lindborg et al. (2016)
	iPSCs (FF)	NA (intrinsic)	Matrigel	Spinning bioreactor	Quadrato et al. (2017)
Dorsal forebrain	ESCs (MEF)	SB-431542, IWR1	Matrigel	Static culture dish	Kadoshima et al. (2013)
	iPSCs (MEF or FF)	Dorsomorphin, SB-431542, (XAV-939) FGF2, EGF, BDNF, NT3	NA	Static culture plate	Pasca et al. (2015) and Yoon et al. (2018)
	iPSCs (FF)	Noggin, DKK1, bFGF, EGF, BDNF, GDNF	NA	Static culture plate	Mariani et al. (2015)
	iPSCs (MEF)	A-83, dorsomorphin; CHIR-99021, SB-431542, WNT3A	Matrigel	Miniature spinning bioreactor (SpinΩ)	Qian et al. (2018, 2016)
	ESCs or iPSCs (MEF or FF)	NA (intrinsic) or CycA	Matrigel	Culture plate on orbital shaker	Bagley et al. (2017)
	ESCs or iPSCs (MEF)	CHIR-99021, PLGA microfilament	Matrigel	Culture plate on orbital shaker	Lancaster et al. (2017)
	ESCs or iPSCs (FF)	SB-431542, LDN-193189, XAV-939, BDNF	NA	Culture plate on orbital shaker	Xiang et al. (2019, 2017)
	iPSCs (FF)	SB-431542, IWR1	Matrigel	Spinning bioreactor	Velasco et al. (2019)

Medial pallium	ESCs (MEF)	SB-431542, IWR1e, CHIR-99021, BMP4	NA	Permeable film culture dish	Sakaguchi et al. (2015)
Choroid plexus	ESCs or iPSCs (FF)	CHIR-99021, BMP4	Matrigel	Culture plate on orbital shaker	Pellegrini, Bonfio, et al. (2020)
	iPSCs (FF)	LDN-193189, SB-431542, IWP-2, CHIR-99021, BMP7, BDNF, GDNF	NA	Culture plate on orbital shaker	Jacob, Pather, et al. (2020)
Ventral forebrain	ESCs (MEF)	SB-431542, IWR1e, SAG	Matrigel	Static culture dish	Kadoshima et al. (2013)
	ESCs or iPSCs (MEF or FF)	IWP-2, SAG	Matrigel	Culture plate on orbital shaker	Bagley et al. (2017)
	ESCs or iPSCs (MEF)	Dorsomorphin, SB-431542, IWP-2, SAG, FGF2, EGF, BDNF, NT3	NA	Static culture plate	Birey et al. (2017) and Sloan, Andersen, Pasca, Birey, and Pasca (2018)
	ESCs or iPSCs (FF)	SB-431542, LDN-193189, XAV-939, SHH, purmorphamine, BDNF	NA	Culture plate on orbital shaker	Xiang et al. (2019, 2017)
Optic cup and retina	ESCs (MEF)	IWR1e, SAG, CHIR-99021	Matrigel	Static culture dish	Nakano et al. (2012)
	ESCs (MEF)	BMP4, CHIR-99021, SU5402	NA	Static culture dish	Kuwahara et al. (2015)
	iPSCs (FF)	NA (Intrinsic), RA (photoreceptor maturation)	Matrigel	Static culture dish	Cowan et al. (2020) and Zhong et al. (2014)
Thalamus	iPSCs (FF)	SB-431542, LDN-193189, Insulin, PD0325901, BMP7, BDNF	NA	Culture plate on orbital shaker	Xiang et al. (2019)

Continued

Table 1 Region-specific brain organoid protocols.—cont'd

Brain region	Starting cells	Patterning and maturation factors	ECM	Culture apparatus	Reference(s)
Hypothalamus	iPSCs (MEF)	LDN-193189, SB-431542, WNT3A, SHH, purmorphamine, FGF2, CNTF	NA	Miniature spinning bioreactor (SpinΩ)	Qian et al. (2018, 2016)
Midbrain	iPSCs (MEF)	LDN-193189, SB-431542, SHH, FGF8, purmorphamine, CHIR-99021, BDNF, GDNF	NA	Miniature spinning bioreactor (SpinΩ)	Qian et al, (2016, 2018)
	ESCs (FF)	SB-431542, Noggin, CHIR-99021, SHH, FGF8, BDNF, GDNF, cAMP	Matrigel	Culture plate on orbital shaker	Jo et al. (2016)
	NESCs	SB-431542, dorsomorphin, CHIR-99021, purmorphamine, BDNF, GDNF, cAMP, TGFβ	Matrigel	Culture plate on orbital shaker	Monzel et al. (2017)
Cerebellum	ESCs (MEF)	SB-431542, FGF2, Insulin, FGF19, SDF1	NA	Static culture dish	Muguruma et al. (2015)

Summary of protocols for generating region-specific brain organoids, including the starting cells, patterning and maturation factors, extracellular matrix (ECM), and special culture conditions used in each procedure. Selected references are provided for further reading. MEF, mouse embryonic fibroblast; FF, feeder-free.

While unguided differentiation of cerebral organoids generates remarkable cell diversity of the developing brain, it has two main drawbacks: (1) unguided differentiation leads to unavoidable contamination of non-neural cell lineages, such as mesoderm and endoderm; and (2) the conglomerate of many brain regions is non-uniform, with high degrees of structural and cell type variability among organoids. One effort to improve forebrain specificity and the quality of ventricular structures was to embed hPSC aggregates with engineered microfilaments, which improved the size and reproducibility of forebrain ventricular structures within cerebral organoids, although structural and cell fate variability still remains (Lancaster et al., 2017).

2.2 Guided differentiation: Region-specific brain organoids

To enhance region specificity and organoid reproducibility for developmental studies and disease modeling, several groups have devised protocols for generating regionally patterned brain organoids by influencing the fate of neuroepithelial cells. Guided differentiation protocols utilize small molecules and recombinant growth factors with precise timing of exposure to instruct hPSC aggregates to differentiate into structures and cell types of specific brain regions. The majority of protocols involves specification of major brain regions, such as the forebrain, midbrain, and hindbrain, but many protocols have been further optimized to enrich for subregions of the brain including the cerebral cortex, medial pallium, thalamus, hypothalamus, substantia nigra and cerebellum (Fig. 3). The first step toward generating regionally patterned brain organoids is to form an EB. This can be accomplished using stem cell cultures that rely on mouse embryonic feeder (MEF) cells or medium formulations that allow stem cell expansion under feeder-free conditions (Fig. 3). Stem cell colonies are enzymatically detached or dissociated and reaggregated and transferred to suspension culture to form EBs, which are guided towards neuroectoderm fate by inhibiting the BMP and TGFβ pathways, commonly referred to as "dual-SMAD inhibition." This inhibition mimics the effects of the mid-gastrula organizer during development and is generally accomplished by using a combination of small molecule inhibitors of signaling pathways of BMP (LDN-193189 or Dorsomorphin) and TGFβ (SB-431542 or A-83). Small molecules are generally preferred over recombinant growth factors due to their improved stability and lower cost. Many protocols also include simultaneous WNT inhibition with small molecules (XAV-939 and IWP-1/2), which enhance neural induction and help to specify rostral fate. Efficient neural ectoderm specification provides a blank state for further patterning to specific regions of the brain.

2.2.1 Cortical organoids

Cortical organoids are the most abundant and perhaps most well-studied group of brain organoids due to their relatively simplistic patterning procedures. Cortical fate is often observed as the default fate of neuroectodermal EBs and several protocols have emerged that apply additional small molecules, growth factors, or extracellular matrix to improve region specification, ventricular zone expansion, and neuronal layering (Qian et al., 2019). In general, cortical organoid protocols involve enrichment for neural ectoderm at the EB stage followed by a ventricular zone expansion phase using small molecules or Matrigel, and then a neurogenesis phase with basal medium containing nutrients important for neural differentiation and survival. The Sasai group first reported the generation of cortical organoids containing cortex-like structures after embedding EBs patterned to neural fate with the TGFβ and WNT antagonists SB-431542 and IWR1 into Matrigel (Kadoshima et al., 2013). The resulting organoids contain several ventricular structures, each with a defined ventricular zone enriched for $FOXG1^+$ forebrain progenitors. Most of the ventricular structures contain $PAX6^+$ dorsal forebrain radial glia cells (RGCs) that give rise to a neural plate containing neurons expressing CTIP2, TBR1, and SATB2. Interestingly, certain regions within these organoids express GSH2, a marker for ventral forebrain progenitors, and GAD65, a marker for GABAergic interneurons, contributing to increased forebrain neuronal diversity at the cost of regional purity. Ventricular RGCs within these organoids undergo stereotypical interkinetic nuclear migration as occurs in the developing mammalian cortex. The Ming group found that continued TGFβ inhibition combined with exposure to the WNT agonist CHIR99021 led to marked expansion of neural progenitors within Matrigel and the formation of large ventricular structures that are highly enriched for dorsal forebrain progenitors (Qian et al., 2018, 2016). After about 50 days in culture, these forebrain organoids contain a particularly large and well-defined oSVZ containing $HOPX^+$ oRGCs that resembles the developing human cortex. This protocol involves the additional use of a spinning bioreactor which improves nutrient and oxygen diffusion for long-term maintenance. In contrast, the protocol developed by the Pasca group uses FGF and EGF to expand ventricular progenitors in the absence of any added extracellular matrix (Pasca et al., 2015). Given the role of FGF and EGF in astroglial fate specification, these human cortical spheroids (hCSs) contain not only cortical neurons expressing TBR1, CTIP2 and SATB2, but are also enriched with astrocytes.

Although most protocols for cortical organoids exhibit poor lamination and layer distinction, some have shown the sequential generation of neurons expressing layer-specific markers in the correct order, such as RELN, TBR1, CTIP2, FOXP1, SATB2, BRN2, RORB, and CUX1 (Qian et al., 2016, 2020). These organoids also exhibit similar transcriptome and epitranscriptome as the developing human brain (Yoon, Ringeling, et al., 2017). A major limitation of cortical organoids, which extends to other regional organoids, is the presence of many ventricular structures in one organoid. This complicates analysis because the ventricular structures come in varying shapes and sizes and neurons produced by each can mix, blurring any potential layering. Reducing the number of ventricular structures and improving cortical layer formation and specification will be important for the next generation of cortical organoids. Another challenge in dorsal forebrain patterning is arealization—specifying the different neocortical subregions, such as the frontal, temporal, and occipital lobes; however, the factors that determine or promote subregion identity are not well understood (Cadwell, Bhaduri, Mostajo-Radji, Keefe, & Nowakowski, 2019). Accompanying this challenge is a lack of specific markers to adequately access the identity of subregions, as distinctions between these regions are not discrete and often depend on differences in layering and subcortical projections, which are not adequately modeled in organoids.

2.2.2 Choroid plexus and hippocampal organoids

The choroid plexus of the lateral ventricles arises from the dorsal telencephalon and plays a critical role in the production of cerebrospinal fluid (CSF) and formation of the blood-CSF barrier, which restricts free passage of solutes between the blood and CSF (Lun, Monuki, & Lehtinen, 2015). The most dorsal structures of the forebrain are patterned by high WNT and BMP signaling from the roof plate, with early *in vitro* studies demonstrating the sufficiency of BMP4 exposure to induce choroid plexus from neuroepithelial cells (Fig. 1C) (Watanabe et al., 2012). The Sasai group developed a protocol to produce self-organized choroid plexus epithelium from hESCs by prolonged exposure of EBs to high levels of the WNT agonist CHIR99021 and BMP4 (Sakaguchi et al., 2015). The resulting structures contained cuboidal epithelium expressing the choroid plexus-defining markers TTR and AQP1. The Lancaster group established a modified protocol which relies on exposing EBs to the same patterning molecules combined with Matrigel embedding to generate choroid plexus organoids (CPOs) that produce

CSF-containing structures (Pellegrini, Bonfio, et al., 2020). Transcriptomic analysis showed recapitulation of the diversity of human choroid plexus epithelial cells and proteomic analysis confirmed the presence of key human CSF components. These CSF-producing CPOs have an intact barrier function which recapitulated the *in vivo* CNS permeability to small molecules. More recently, the Ming group established an optimized protocol by exposing neural patterned EBs to high levels of CHIR99021 and BMP7 to generate CPOs that were highly enriched for cells expressing TTR, AQP1, OTX2 and ion channels and molecular transporters important for CSF generation (Jacob, Pather, et al., 2020). CPOs generated by this protocol form epithelial extensions resembling the choroid plexus without the addition of Matrigel, but do not form CSF-containing inclusions. While CPOs provide useful model systems to study human choroid plexus epithelial cell function, an important drawback, as in other types of brain organoids, is the lack of integration with functional vasculature and pericytes which form the complete blood-CSF barrier. Therefore, reconstructing a complete, physiological system remains a future challenge.

The hippocampus, a conserved structure among vertebrates that plays a critical role in learning and memory, arises from the medial pallium of the developing telencephalon which lies adjacent to the more midline cortical hem and choroid plexus structures (Fig. 1C) (Grove & Tole, 1999). The cortical hem, an important secondary organizer in the telencephalon, secretes WNTs, which act in combination with BMP signals from the choroid plexus to induce the medial pallium (Subramanian, Remedios, Shetty, & Tole, 2009). The neuroepithelium closest to the cortical hem receives the highest levels of WNT and becomes the dentate neuroepithelium, which produces neurons, astrocytes and adult neural stem cells of the dentate gyrus (Berg et al., 2019). The remaining portion of the medial pallium receives lower WNT and segregates into the *cornu ammonis* (CA) neuroepithelium, which produces the pyramidal neurons of CA1–3. The Sasai group discovered that medial pallium fate could be induced by exposing EBs to high levels of WNT and BMP signals, but only for a brief period of time (Sakaguchi et al., 2015). This exposure induces cortical hem tissue, which in turn induces adjacent medial pallium neural progenitor fate defined by the combined expression of FOXG1, LEF1, LHX2, and PAX6 (Fig. 1C). However, hippocampal neurons are not produced even with prolonged culturing time. Instead, dissociation of 3D medial pallium tissue and long-term maintenance as monolayer cultures produced electrically active neurons expressing the hippocampal marker ZBTB20 and more specific

dentate granule neuron marker PROX1 and CA3 pyramidal neuron marker KA1. Future improvements are necessary to produce hippocampal-specific organoids not only with the full diversity of hippocampal neurons, including pyramidal neurons of CA1 and CA2, hilar mossy neurons, and GABAergic interneurons, but also to recapitulate the organized structure and typified circuitry of the mature hippocampus.

2.2.3 Subpallial organoids

The subpallium is divided into MGE, LGE, and CGE that generate different interneuron subtypes (Kepecs & Fishell, 2014). SHH from the floor plate is critical in specifying ventral progenitor fate and has been used to differentiate hPSCs into interneurons in monolayer cultures (Germain, Banda, Becker, Naegele, & Grabel, 2013; Liu et al., 2013; Maroof et al., 2013; Nicholas et al., 2013). Several protocols have emerged for generating brain organoids expressing markers for subpallium neural progenitors and the inhibitory neurons they produce (Bagley, Reumann, Bian, Levi-Strauss, & Knoblich, 2017; Birey et al., 2017; Kadoshima et al., 2013; Xiang et al., 2017). Different exposure lengths and concentrations of SHH and/or the SHH agonist purmorphamine among these protocols may be responsible for the induction of broad ventral vs MGE-specific identity and the differences in interneuron diversity. Most of the neural progenitors in these protocols express NKX2.1, a marker for the MGE, and produce inhibitory neurons characterized by the expression of GAD67 and the production of GABA. A limitation of these protocols is the production of mainly SST, CR, and/or RELN interneurons with low abundance of PV, NPY, or VIP interneurons. Refining these protocols to optimize the production of interneurons with correct subtype proportions remains a major challenge. Additionally, subpallium organoids do not contain any structures resembling the organization of the caudate or putamen nuclei, limiting studies of their development.

2.2.4 Optic cup and retinal organoids

The optic cup and neural retina arise from optic vesicles in the rostral diencephalon. The retina is a highly organized structure with distinct layers composed of defined cell types, including rod and cone photoreceptors. Generation of optic cup-like structures was first demonstrated by the Sasai group by embedding EBs into Matrigel and exposing them to WNT signaling *via* the GSK3β antagonist CHIR99021 (Nakano et al., 2012). Early retinal structures contain $CHX10^{+}$ progenitors that self-organize and produce a multilayered neural retina containing cells expressing the photoreceptor

markers CRX and Recoverin. Many of these photoreceptors expressed the rod marker NRL or the early cone marker RXR-γ. Several protocols for retinal organoids have since emerged, each with modifications to either improve retinal cell purity, cellular organization, or function (Kuwahara et al., 2015; Zhong et al., 2014). Recently, the Roska group optimized a protocol to generate light-sensitive, multilayered human retinal organoids with functional synapses (Cowan et al., 2020). Despite these efforts, no protocol has been able to generate all layers of the retina with definitive structural and synaptic organization. A major challenge remains in reproducing the highly laminar organization of the neural retina. Single-cell transcriptomic studies comparing the mammalian retina to retinal organoids have shown recapitulation of the diverse subtypes of retinal neurons and human cell type-specific (e.g., NRL, rods; ARR3, cones) and disease-associated genes, and will provide a valuable resource to improve existing protocols (Cowan et al., 2020).

2.2.5 Thalamic and hypothalamic organoids

The thalamus and hypothalamus arise from the caudal diencephalon and play important roles in circuit integration and regulation of homeostasis. Unlike the highly laminated structure of the cortex and retina, neurons within the thalamus and hypothalamus organize into discrete nuclei. The Park group utilized caudalizing effects of early insulin exposure followed by BMP7 treatment to pattern EBs toward thalamic fate (Xiang et al., 2019). The MEK-ERK inhibitor PD0325901 was used during BMP7 treatment to prevent excess insulin-induced caudalization toward midbrain fate. This patterning combination induced robust expression of TCF7L2, a marker for the developing thalamus. By scRNA-seq they identified populations of thalamic progenitors, glutamatergic neurons, and GABAergic neurons of the thalamus, emphasizing the neuronal diversity of their system.

Specification of the hypothalamus is defined by the transcription factor RAX1, which is specific to only the hypothalamus and retina during development, which can be distinguished from each other by structural features. Hypothalamic neurons are defined by the expression of many neural peptides, including POMC, NPY and TRH that mark specific subsets of neurons. Several reports have described protocols for generating hypothalamic neurons *via* monolayer culture (Merkle et al., 2015; Rajamani et al., 2018; Wang, Egli, & Leibel, 2016), but only one study describes hypothalamic organoids specifically. The Ming group used a combination of WNT and SHH agonists, including CHIR99021, purmorphamine, and

SHH, followed by FGF2 and CNTF exposure to pattern hypothalamic organoids (Qian et al., 2018, 2016). Neural progenitors in early organoids expressed general hypothalamic marker RAX1 and subtype-specific markers NKX2.1 and FOXA2. Mature organoids contained a diverse collection of neurons expressing the neuropeptides NPY and POMC and the dopaminergic marker TH. Whether these organoids can generate neurons that represent the entire spectrum of neuropeptide secretion remains uncertain and may require protocol modifications to enrich for neuronal subtypes of interest. With both thalamic and hypothalamic organoids, the challenge remains in reconstructing organized nuclei structures and the cellular diversity seen *in vivo*.

2.2.6 Midbrain organoids

The midbrain, which includes the tegmentum and substantia nigra, arises from the developing mesencephalon and plays important functions in information relay between the forebrain and spinal cord, controlling movement and sensory processing. Several protocols for generating midbrain organoids involve the exposure to FGF8 and SHH (Fig. 1) (Jo et al., 2016; Monzel et al., 2017; Qian et al., 2018, 2016). The derivation of midbrain progenitors is defined by expression of FOXA2. Most protocols focus on generating dopaminergic neurons of the substantia nigra, which is of special interest due to their role in the pathogenesis of Parkinson's disease and other related movement disorders. Dopaminergic neurons can be distinguished by the expression of PITX3, tyrosine hydroxylase (TH), the enzyme require to make dopamine, and DAT, the dopamine transporter. Although these protocols all generate TH^+ neurons, whether they belong to the substantia nigra remains debatable. One study from the Ng group uses the presence of pigment, a defining feature of dopaminergic neurons in the substantia nigra, as a criteria to claim that their dopaminergic neurons are truly of the substantia nigra identity (Jo et al., 2016). Further studies that optimize dorsal-ventral patterning will hopefully lead to more defined protocols for generating other midbrain regions.

2.2.7 Hindbrain organoids

The hindbrain arises from the developing rhombencephalon and consists of the cerebellum, pons, and medulla, which play important roles in movement augmentation and information relay to the spinal cord (Fig. 1). Insights into the mechanisms instructing cerebellar differentiation have led to several protocols for generating cerebellar neurons from mESCs and hESCs

(Erceg et al., 2010; Muguruma et al., 2010; Salero & Hatten, 2007; Su et al., 2006; Tao et al., 2010). The Sasai group first reported a protocol to generate self-organized cerebellar tissue that includes regions of cerebellar plate neuroepithelium giving rise to Purkinje cells and interneurons, and rhombic lip neuroepithelium giving rise to granule cells and neurons of the deep cerebellar nuclei (Muguruma, Nishiyama, Kawakami, Hashimoto, & Sasai, 2015). They showed that treatment of hESC aggregates with FGF2, SB431542, and insulin suppressed forebrain fate and induced expression of caudal marker GBX2 and isthmus organizer genes FGF8 and WNT1. Continued culture resulted in neuroepithelium expressing the cerebellar plate markers KIRREL2, PTF1A, and EN2, which generated four different cerebellar neuron precursors, including Purkinje cells, Golgi cells, granule cells, and DCN projection neurons. The addition of FGF19 induced formation of neural tube-like structures that recapitulate the dorsal-ventral polarity of the developing hindbrain. On the other hand, sequential addition of FGF19 and SDF1 induced the formation of a continuous neuroepithelium that differentiates into neurons that organize into a multilayered structure seen in cerebellar development. The cerebellum organoids described by the Sasai group represent an important advancement in recapitulating the major structural organization, progenitor subtypes, and neuronal composition of the cerebellum. However, there was poor survival of long-term cultures, which limits the structural maturation beyond the first trimester of development and necessitates organoid dissociation and 2D culture to observe maturation of cerebellar neuron subtypes. Additionally, the formation of neural networks and appearance of characteristic lobular morphogenesis of the mature cerebellum is missing. Further refinement of patterning methods may lead to more specific protocols for the pons and medulla, other regions in the hindbrain.

3. Advancements in cellular complexity of brain organoids

Although improved neural ectoderm induction and regional patterning have greatly enhanced the fate specificity and reproducibility of brain organoid protocols, these improvements have the caveat of producing predominantly excitatory or inhibitory neurons, which does not recapitulate *in vivo* neuronal diversity. Efficient neural induction also restricts the production of non-neuroectodermal cells, such as microglial, endothelial, and meningeal cells that are critical to the development and function of

the human brain. Additionally, despite long-term culture, brain organoids typically contain few glial cells, such as astrocytes and oligodendrocytes, and display only rudimentary synaptic circuitry and electrical activity, limiting their applicability for studying the complex physiology of the human brain and the mechanisms that go awry during disease. There are significant recent advancements in improving the cellular complexity and maturity of brain organoids to create a model system that more faithfully recapitulates the *in vivo* condition. These innovations include protocol modifications to model interactions between brain regions, enhance glial cell production, reconstitute resident immune cells and vasculature, improve healthy organoid longevity and maturity, and study long-range neuronal projections of human neurons (Fig. 4).

3.1 Fusion of region-specific brain organoids

Organoids patterned to specific brain regions contain a more homogenous population of progenitors and neurons with reduced variability among organoids, but this improvement also limits the potential for studying interactions between different brain regions. Several groups have developed methods to overcome this limitation by fusing region-specific organoids, termed "assembloids" (Fig. 3). This methodology offers the benefits of regional purity with the ability to study neuronal interactions. For example, to reproduce the mixed glutamatergic and GABAergic neuronal diversity of the cortex, the first studies fused dorsal forebrain or cortical organoids with subpallial organoids and observed the selective migration of interneurons, mimicking the process that occurs in normal forebrain development (Bagley et al., 2017; Birey et al., 2017; Xiang et al., 2017). In these assembloids, migrating interneurons undergo stepwise cell movements through the process of nucleokinesis, form synapses with glutamatergic neurons, and display increased morphological complexity (Marton & Pasca, 2020). Another study investigated the connections between the thalamus and the cortex by fusing cortical organoids with thalamic organoids and observed reciprocal axonal projections, mimicking the connections that occur in the normal brain (Xiang et al., 2019). After fusion, thalamic neurons form functional synapses with cortical neurons and exhibit higher-frequency firing, suggesting improved maturity following circuitry integration. These studies represent a step forward in improving neuronal diversity and modeling inter-regional connections in brain organoids but require more in-depth characterization of cell-type specific projections and consequences of circuitry formation on

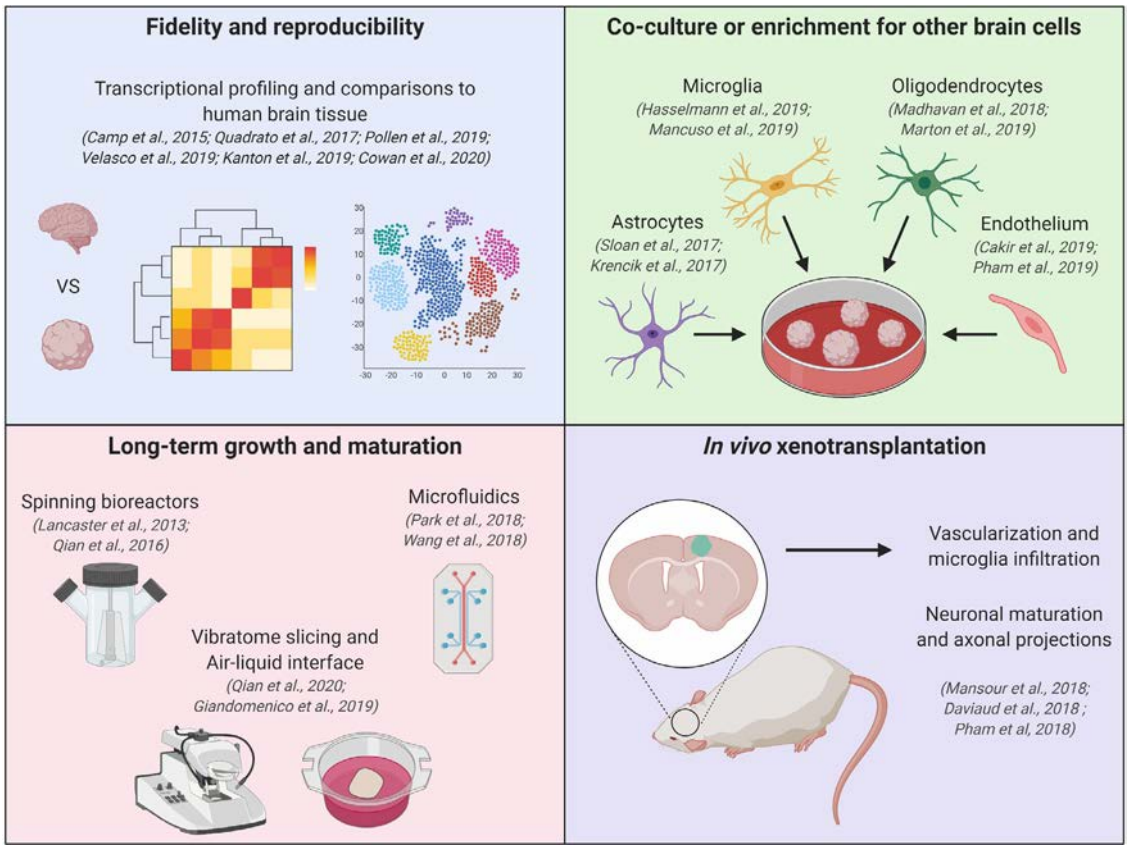

Fig. 4 Improvements and extensions of brain organoid technology. Diagram illustrating major improvements and extensions of human brain organoid technologies, including high-throughput genomics and transcriptomics to compare organoids to human brain tissue and improve reproducibility, co-culture with additional cell types to more fully reconstitute the cellular composition of the human brain, modifications to improve long-term growth and maturation, and *in vivo* xenotransplantation to improve maturation and study axonal projections.

neuronal function. The development of more region-specific protocols will allow modeling of additional regional interactions, such as cortical-striatal circuits, with the added possibility of multi-regional interactions, such as the cortex-hippocampus-amygdala fear and memory circuitry and the cortex-thalamus-spinal cord motor circuitry.

3.2 Enhancing glial cell production and maturation

In the human brain, astrocytes outnumber neurons and play critical roles in maintaining brain homeostasis, such as regulating synapse development and function, modulating blood flow and solute penetrance through the blood-brain barrier, and responding to injury (Sofroniew & Vinters, 2010). Oligodendrocytes support signal transduction by forming insulating myelin sheaths and providing metabolic support to axons (Simons & Nave, 2015). Several brain organoid protocols report the emergence of astrocytes and oligodendrocytes after long-term culture, but their appearance is variable and with low abundance. Given that gliogenesis follows neurogenesis, one approach to improving the abundance and maturation of astrocytes in organoids was to maintain cortical organoids in culture for greater than 100 days (Sloan et al., 2017). The number of astrocytes increased over time and transcriptional comparison to primary human astrocytes revealed striking similarities with the expression of mature astrocyte genes increasing with organoid age. Despite the improvement in astrocyte abundance and maturity, this approach is limited by the need to maintain cultures for very long periods of time accompanied with reduced neuronal abundance and the complete abolishment of ventricular zones and neuronal lamination. The ability of brain organoids to recapitulate the remarkable astrocyte diversity of the human brain, including fibrous and protoplasmic subtypes has just begun to be explored (Qian et al., 2020).

Several brain organoid protocols report the late emergence of oligodendrocyte precursor cells and oligodendrocytes with even some reports of myelination of neuronal axons (Pamies et al., 2017; Qian et al., 2020). In one study, ventral forebrain organoids were found to produce more oligodendrocytes at earlier timepoints than dorsal counterparts, representing the different waves of oligodendrogliogenesis in human development (Kim, Xu, et al., 2019). A recent study described a modified protocol to generate oligo-cortical spheroids that reproducibly make oligodendrocytes and myelin (Madhavan et al., 2018). After cortical patterning, treatment with platelet-derived growth factor AA (PDGFR-AA) and insulin-like growth

factor 1 (IGF-1) drives the expansion of OPC populations and further treatment with thyroid hormone (T3) induces oligodendrocyte differentiation and myelination by 10 weeks in culture. Robust myelination was observed by electron microscopy. Exposure of oligo-cortical organoids to the pro-myelination drugs T3, clemastine, and ketoconazole increased myelination and expression of MYRF (myelin regulatory factor). This approach, however, produces oligodendrocytes at the expense of neurons and lacks obvious structural organization of neurons and axons.

3.3 Reconstitution of resident immune cells and vasculature

Because of improved neuroectoderm specification, brain region-specific organoids are relatively homogeneous for neural precursors, which consequently do not produce other cell types of the brain, including microglia, endothelial, and meningeal cells. Reconstituting these cell populations remains an important endeavor to more accurately mimic *in vivo* cellular diversity (Fig. 4). Microglia are the brain's resident macrophages that perform essential functions in maintaining homeostasis, modulating neuronal circuits, and surveying the environment (Hammond, Robinton, & Stevens, 2018; Hickman, Izzy, Sen, Morsett, & El Khoury, 2018). Microglia are also the primary responders to brain injury and infection and are implicated in the pathogenesis of many neurological disorders, such as Alzheimer's disease (Li & Barres, 2018). Cerebral organoids generated from unguided differentiation protocols were reported to innately contain $IBA1^+$ microglia-like cells, likely derived from mesodermal progenitor cells (Ormel et al., 2018; Quadrato et al., 2017). Although these cells were shown to resemble human microglia morphologically and transcriptionally, it is unknown whether these cells correspond to yolk sack-derived microglia or other tissue macrophages (Bennett & Bennett, 2020). Additionally, the variable and uncontrolled appearance of microglia-like cells in cerebral organoids limits their utility for developmental and disease modeling. The recent emergence of protocols to derive human microglia-like cells *in vitro* (Abud et al., 2017; Douvaras et al., 2017; Haenseler et al., 2017; McQuade et al., 2018; Muffat et al., 2016; Pandya et al., 2017) has created the possibility of adding pure cultures of microglia-like cells to brain organoids (Jacob & Bennett, 2020). Microglia-like cells co-cultured with cerebral organoids readily invade and integrate, expressing microglia-specific markers and displaying ramified-like morphologies (Abud et al., 2017) and microglia co-cultured with forebrain

organoids show improved maturation and responses to inflammatory stimuli (Song et al., 2019). Although microglia co-culture methodologies are promising, more work is needed to improve the reproducibility and microglia function for disease modeling.

Endothelial cells lining major blood vessels and capillaries supply the brain with nutrients and play important, yet incompletely understood roles in neural development (Paredes, Himmels, & Ruiz de Almodovar, 2018). The innate presence of endothelial cells has been reported in brain organoids (Bhaduri et al., 2020), but like microglia-like cells, their presence is highly unreliable and with the caveat of a lack of organization into vessel-like structures. To vascularize brain organoids, studies have taken one of several approaches: co-culture brain organoids with endothelial cells, induce differentiation of endothelial cell precursors within organoids, or xenotransplant organoids into mouse brains and allow host vasculature to infiltrate. A pair of studies co-cultured either iPSC-derived endothelial cells or umbilical vein endothelial cells with brain organoids and observed robust engraftment with the formation of capillary-like structures (Pham et al., 2018; Shi et al., 2020). An alternative approach used hESCs engineered to ectopically express human ETV2 to produce endothelial cells within cortical organoids that formed vascular network-like structures with reported improvement to neuron maturity (Cakir et al., 2019). Transplantation of these vascularized organoids into mice showed integration with host vasculature with functional perfusion (Cakir et al., 2019). Despite the presence of endothelial cells that form structures resembling capillaries, there is a lack of concrete evidence that these cells are functional and influence the maturation or function of neurons within brain organoids. Importantly, serum-free medium formulations used commonly in brain organoid cultures may not be sufficient to promote endothelial cell maturation and function, and critically, media is unlikely to flow naturally through these vessels *in vitro*. Flow could theoretically be enhanced by integrating vascular structures with mechanical pumps, a possibility not yet explored.

3.4 Technical modifications for long-term culture

Inevitable hypoxic conditions at the center of large organoids hinders long-term brain organoid health and prevents neurons, astrocytes, and/or oligodendrocytes within brain organoids from reaching full maturity. Spinning bioreactors have been widely used to help increase nutrient and

oxygen diffusion within organoids by agitating and better circulating the medium (Fig. 4). In the original cerebral organoid protocol, the Lancaster group made use of large magnetic spinning flasks to improve diffusion (Lancaster et al., 2013). These flasks have allowed organoids to maintain growth long-term but are limited by taking up extensive incubator space and are associated with a high cost. To address this constraint, the Ming group developed a miniature spinning bioreactor which operates on a standard 12-well plate (Qian et al., 2016). This innovation drastically reduces incubator space but is limited by needing to 3D print parts and maintain motors. Although spinning bioreactors have permitted organoids to grow to large sizes, the growth remains constrained by physical limits of oxygen diffusion into the organoid core. Two groups have devised methodologies to circumvent the hypoxia problem by culturing organoids as thick slices, similar to methods used for brain slice electrophysiology. Although both studies employ the same general methodology, they have distinct applications. The Lancaster group cultured their cerebral organoid slices on an air-liquid interface, which improved oxygen availability (Giandomenico et al., 2019). They witnessed improved axonal projections, with deep layer neurons having horizontal projections and upper-layer neurons projecting vertically. The Ming group maintained cortical organoid slices immersed in culture media on an orbital shaker to preserve the health of the ventricular zone that often dies due to its location at the hypoxic center of organoids (Qian et al., 2020). By preserving the progenitor zones and laminar neuronal structure within organoids, they develop more distinct layer separation and neuron identity, allowing better modeling of diseases that may have defects in layer organization or neuron identity specification. Also worth mentioning are microfluidic technologies that introduce constant medium flow through brain organoids that replenishes nutrients (Fig. 4). Using a microfluidic organoid-on-a-chip platform, the Qin group describes a system that not only increases nutrient perfusion into brain organoids, but permits the constant replacement of disease-related chemicals, such as nicotine, avoiding issues with chemical degradation during long-term culture (Wang, Wang, Zhu, & Qin, 2018). The advent of technologies to increase nutrient and oxygen diffusion into brain organoids has drastically improved cellular health and has permitted labs to culture organoids long-term to further maturation. Future improvements to bioreactor and microfluidic technologies may allow organoids to better recapitulate late gestational and early post-natal development.

3.5 *In vivo* orthotopic xenotransplantation

Neurons within the brain form immediate- and long-distance connections with neurons throughout the brain. While assembloids have been developed to investigate aspects of the synaptic connectivity between two distinct brain regions, all current organoid models fail to recapitulate the intricate network connectivity of the human brain, a major limitation that cannot be solved by current technology. As an alternative strategy, several groups have devised methods for direct orthotopic xenotransplantation of whole brain organoids into mouse brains (Daviaud, Friedel, & Zou, 2018; Dong et al., 2020; Mansour et al., 2018). In these studies, portions of the mouse brain are removed and replaced with cerebral organoids. These grafts were shown to mature and send projections throughout the mouse brain. With time, host vasculature invades organoid grafts and supplies nutrients to core regions. Additionally, host microglia migrate into grafts and disperse similarly to what is seen in the surrounding mouse brain. The xenotransplantation has led to improved organoid maturation and the integration of endothelial cells and microglia often not present in *in vitro* cultures. It has also permitted the study of human neuron synaptic targeting and electrophysiology in an *in vivo* system. Despite these advancements, xenotransplantation is limited by requiring immune-deficient animals and the different timing of neurodevelopment in humans and mouse, which results in the early depletion of progenitors and loss of human specific features.

4. Modeling neurological diseases using brain organoids

Studying and understanding the mechanistic and biological basis of brain disorders has remained a challenge due to the lack of access to human brain tissue for research, the complexity and heterogeneity of disease presentation among individuals, and the inability of current model systems to accurately recapitulate human disease phenotypes (Amin & Paşca, 2018; Wen, Christian, Song, & Ming, 2016). Although analysis of post-mortem human brain tissue has provided insight into the histological components of brain disorders, such analysis has not been fruitful toward studying disease mechanisms. Although animal models have contributed greatly in establishing a link between cellular and systems-level pathology in an intact physiological system, there remain species-specific differences that limit their ability to

accurately model human disorders (Schnoll et al., 2019). Human, especially patient iPSC-based monolayer cultures have provided ways to study mechanisms of nervous system disorders in specific brain cell types with the advantage of retaining patients' genetic backgrounds, but lacking the complexity of the brain (Amin & Paşca, 2018). Brain organoids more accurately recapitulate the development, structural organization, and cellular heterogeneity of the human brain (Camp et al., 2015; Lancaster et al., 2013; ; Luo et al., 2016), therefore offering a more promising *in vitro* human model. Broadly, brain organoids permit modeling of neurological disorders of both extrinsic and intrinsic etiologies (Adams, Cugola, & Muotri, 2019; Zhang, Song, & Ming, 2020). Extrinsic etiologies include brain environmental stimuli such as chemicals, pathogens, and neoplastic cells which can be modeled by adding external agents or cells to organoid cultures (Fig. 5).

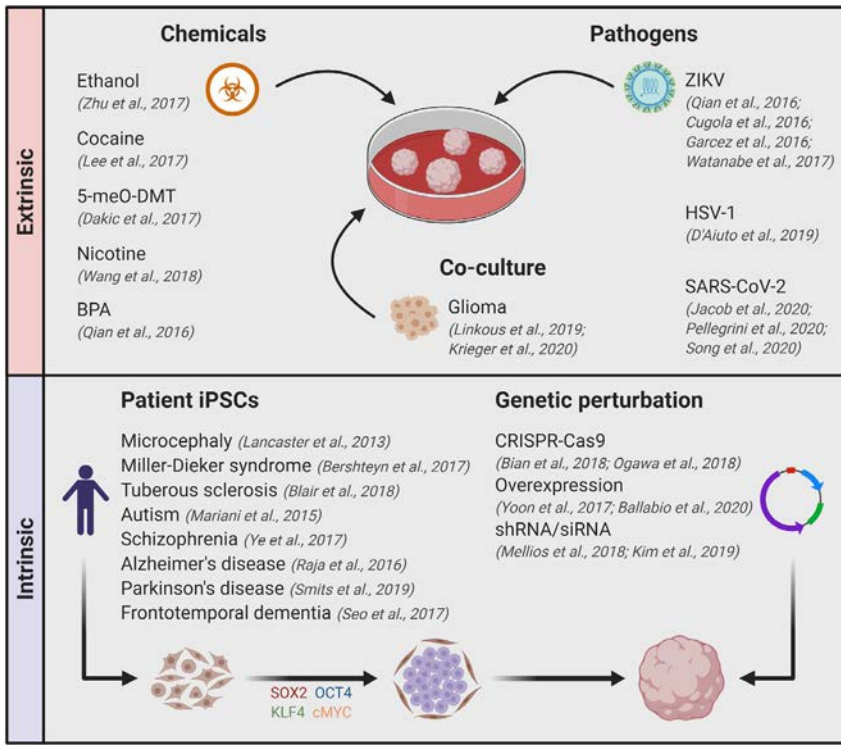

Fig. 5 Modeling neurological diseases of extrinsic and intrinsic etiology using brain organoids. Brain organoids have been used to model neurological diseases resulting from extrinsic factors (exogenous chemicals, pathogens, or neoplastic cells) and intrinsic factors (patient hiPSCs harboring disease-causing mutations or genetic perturbations that introduce aberrant gene expression into otherwise healthy PSCs or organoids). See Table 2 for a more comprehensive list of neurological diseases modeled in brain organoids.

Intrinsic etiologies include genetic abnormalities such as large chromosomal aberrations and mutations in protein coding or gene regulatory sequences, which can be modeled with organoids from patient-derived iPSCs or iPSCs modified by genomic editing or introduction of recombinant DNA (Fig. 5). We highlight examples of how brain organoids have been applied to study a wide range of both extrinsic and intrinsic nervous system pathologies, including neurodevelopmental and neuropsychiatric disorders, neurodegenerative and infectious diseases, and brain cancers (Table 2).

4.1 Neurodevelopmental and neuropsychiatric disorders

The phenotypes and mechanisms underlying many neurodevelopmental and neuropsychiatric disorders have been studied using brain organoids generated from patient iPSCs or hPSCs edited to harbor disease-associated mutations. Cephalic disorders are a group of congenital conditions characterized by gross abnormal development of the central nervous system that are often a result of genetic abnormalities, which cause severe defects in the structure and function of the brain. Brain organoids have been applied for studying a range of cephalic disorders, including microcephaly (Gabriel et al., 2016; Lancaster, 2013; Li, Sun, et al., 2017), macrocephaly (Li, Muffat, et al., 2017), and lissencephaly (Bershteyn et al., 2017; Iefremova et al., 2017). Microcephaly is characterized by a reduced head size compared to those of the same age and gender groups and may derive from several conditions or chromosomal abnormalities that lead to abnormal brain growth. Two such forms of microcephaly, autosomal recessive primary microcephaly (MCPH) (Lancaster, 2013; Li, Sun, et al., 2017) and Seckel syndrome (Gabriel et al., 2016), have been modeled in brain organoid cultures. MCPH patient-derived cerebral organoids are smaller and exhibit reduced progenitor zones, decreased neurogenesis and impaired neuronal activity compared to controls (Lancaster, 2013; Li, Sun, et al., 2017). Seckel syndrome patient-derived organoids have a similar phenotype in addition to increased length of cilia on apical neural progenitors (Gabriel et al., 2016). Conversely, macrocephaly is associated with an increased head size compared to those of the same age and gender groups, and loss-of-function mutations in *PTEN* have been shown to contribute to this phenotype (Butler et al., 2005). Organoids derived from *PTEN*-knockout hESCs exhibit increased proliferation, surface area, and organoid volume-a clinical feature of macrocephaly (Li, Muffat, et al., 2017). Lissencephaly is a cephalic disorder characterized by reduced or absent gyration in the brain, which may result from

Table 2 Neurological disease modeling using brain organoids.

Disease/Disorder	Mutation	Organoid model	Reference(s)
Neurodevelopmental			
Macrocephaly	PTEN KO iPSCs	Cerebral organoids	Li, Muffat, et al. (2017)
Microcephaly	Mutant ASPM patient iPSCs	Cortex-like organoids	Li, Sun, et al. (2017)
	WRD62 KO and mutant CDK5RAP2 patient iPSCs	Cerebral organoids	Lancaster et al. (2013)
	Mutant CPAP patient iPSCs	Cerebral organoids	Gabriel et al. (2016)
Lissencephaly	Miller-Dieker syndrome patient iPSCs	Cerebral and forebrain organoids	Bershteyn et al. (2017) and Iefremova et al. (2017)
	LIS1 heterozygous KO ESCs	Brain organoids	Karzbrun, Kshirsagar, Cohen, Hanna, and Reiner (2018)
Neuronal heterotopia	PLEKHG6 OE and knockdown iPSCs	Cerebral organoids	O'Neill et al. (2018)
	Mutant DCHS1 and FAT4 patient iPSCs	Cerebral organoids	Klaus et al. (2019)
Rett Syndrome	MECP2 mutant patient iPSCs	Cerebral organoids	Mellios et al. (2018)
Sandhoff disease	HEXB mutant patient iPSCs	Cerebral organoids	Allende et al. (2018)
Angelman syndrome	UBE3A KO and AS patient iPSCs	Cortical spheroids	Sun et al. (2019)
Leber congenital amaurosis	CEP290 mutant patient iPSCs	Retinal organoids	Parfitt et al. (2016)

Hypoxia	Control ESCs and iPSCs	Cerebral and brain organoids and cortical spheroids	Boisvert, Means, Michaud, Madri, and Katz (2019), Daviaud, Chevalier, Friedel, and Zou (2019) and Paşca et al. (2019)
Neuropsychiatric			
Autism	Idiopathic autism patient iPSCs	Cerebral organoids	Mariani et al. (2015)
	Heterozygous CHD8 KO iPSCs	Cerebral organoids	Wang et al. (2017)
	Timothy syndrome patient iPSCs	Subcortical spheroids	Birey et al. (2017)
Schizophrenia	Mutant DISC1 patient iPSCs	Forebrain and sliced cortical organoids	Qian et al. (2020) and Ye et al. (2017)
	Mutant DISC1 iPSCs	Cerebral organoids	Srikanth et al. (2018)
	16p13.11 microduplication patient iPSCs	Cerebral organoids	Johnstone et al. (2018)
	Schizophrenia patient iPSCs	Cerebral organoids	Stachowiak et al. (2017)
Neurodegenerative			
Alzheimer's disease	Mutated APP or PSEN1 AD patient iPSCs	Brain organoids	Raja et al. (2016)
Frontotemporal dementia	Mutated Tau P301L FTD patient iPSCs	Cerebral organoids	Seo et al. (2017)
Parkinson's disease	Mutant LRRK2 PD patient iPSCs	Midbrain organoids	Smits et al. (2019)
	Mutant LRRK2 iPSCs	Midbrain organoids	Kim, Park, et al. (2019)

Continued

Table 2 Neurological disease modeling using brain organoids.—cont'd

Disease/Disorder	Mutation	Organoid model	Reference(s)
Huntington's disease	Expanded CAG repeat iPSCs	Cortical organoids	Conforti et al. (2018)
Retinitis pigmentosa	RP patient iPSCs	Retinal organoids	Deng et al. (2018)
Infectious			
Zika virus	Control iPSCs	Cerebral and forebrain organoids	Cugola et al. (2016), Garcez et al. (2016), and Qian et al. (2016)
Herpes simplex virus	Control iPSCs	Brain organoids	D'Aiuto et al. (2019)
Cytomegalovirus	Control iPSCs	Brain and cortical organoids	Sison et al. (2019) and Sun et al. (2020)
Japanese encephalitis virus	Control iPSCs	Cortical organoids	Zhang et al. (2018)
SARS-CoV-2	Control iPSCs	Cerebral, cortical, hippocampus, hypothalamus, midbrain, and/or choroid plexus organoids	Jacob, Pather, et al. (2020), Pellegrini, Albecka, et al. (2020), Ramani et al. (2020), and Song et al. (2020)
Chemical exposure			
Bisphenol A	Control iPSCs	Forebrain organoids	Qian et al. (2016)
Ethanol	Control iPSCs	Brain and cerebral organoids	Arzua et al. (2020) and Zhu et al. (2017)
Cocaine	Control iPSCs	Neocortical organoids	Lee et al. (2017)

5-MeO-DMT	Control iPSCs	Cerebral organoids	Dakic et al. (2017)
Nicotine	Control iPSCs	Brain organoid-on-a-chip	Wang et al. (2018)
Cannabinoid	Control iPSCs	Microfluidic assembled cerebral organoids	Ao et al. (2020)
Neoplastic			
Tuberous Sclerosis	TSC1 and TSC2 KO iPSCs	Cortical spheroids	Blair et al. (2018)
Glioma	HRAS OE and TP53 KO	Cerebral organoids	Ogawa et al. (2018)
	MYC, EGFR, and EGFRvIII OE; CDKN2A/B, NF1, PTEN, and TP53 KO	Cerebral organoids	Bian et al. (2018)
	Control iPSCs and GSCs	Cerebral organoids	Linkous et al. (2019)
	Control iPSCs and GSCs	Cerebral organoids	Krieger et al. (2020)
Medulloblastoma	Control iPSCs	Cerebellar organoids	Ballabio et al. (2020)

Summary of neurodevelopmental and neuropsychiatric disorders, neurodegenerative and infectious diseases, chemical exposures, and neoplasms modeled using brain organoids. Selected references are provided for further reading. KO, knock-out; OE, over-expression.

defective neuronal proliferation and radial migration, leading to the appearance of a smooth cerebral cortex (Icenogle & Kaplan, 1981). Forebrain organoids generated from patients with Miller-Diekler syndrome (MDS), a form of lissencephaly, were found to be smaller with reduced proliferation in the ventricular zone-like layer (Iefremova et al., 2017). Additionally, oRGCs in organoids appeared to spend longer periods of time in mitosis, while neurons exhibited decreased ability to sustain saltatory migration, traveling less linearly at slower speeds (Bershteyn et al., 2017). Lastly, neuronal heterotopias involve the abnormal presence of neurons in areas such as the white matter, which is believed to be the consequence of abnormal neural migration during development. Modulation of PLEKHG6 primate isoform expression in cerebral organoids reproduced features of periventricular heterotopia, with defects in neural progenitor differentiation and RhoA dependent neuronal migration (O'Neill et al., 2018). Similarly, cerebral organoids derived from patient iPSCs with mutations in *DCHS1* and *FAT4* exhibit changes in the morphology of neural progenitor cells and result in defective neuronal migration dynamics (Klaus et al., 2019).

Brain organoids have also been used to model other neurodevelopmental diseases, such as Sandhoff disease, Rett syndrome, and Angelman syndrome. Sandhoff disease is an autosomal recessive lysosomal storage disorder characterized by the accumulation of lysosomal GM2 ganglioside, which results in developmental regression, neurodegeneration, and macrocephaly (Bley et al., 2011; Tavasoli et al., 2018). Patient iPSC-derived cerebral organoids showed an excess accumulation of GM2 ganglioside, along with increases in cellular proliferation and organoid size, features similar to what is observed clinically (Allende et al., 2018). Rett syndrome is a neurodevelopmental disorder caused by X-linked mutations in *MECP2*, and is characterized by developmental regression and the onset of gait abnormalities in female infants (Ip, Mellios, & Sur, 2018; Neul et al., 2010) and congenital encephalopathy and early death in male infants (Schüle, Armstrong, Vogel, Oviedo, & Francke, 2008). Rett syndrome patient-derived cerebral organoids showed an upregulation of two micro-RNAs, miR-199 and miR-214, which are involved in ERK and AKT signaling and important for neurogenesis and neural differentiation. The organoids also displayed enlarged ventricular zones, increased neural progenitor proliferation, and impaired neurogenesis (Mellios et al., 2018). Angelman syndrome is a disorder characterized by microcephaly, severe intellectual deficit, paroxysms of laughter, and hyperactivity that result from loss of function of the imprinted *UBE3A* gene (Buiting, Williams, & Horsthemke, 2016). Cortical spheroids derived from patient iPSCs or

UBE3A knock-out iPSCs showed augmented potassium channel activity, leading to increased intrinsic excitability in neurons and aberrant network synchronization (Sun et al., 2019).

Psychiatric diseases comprise a heterogeneous set of disorders with both idiopathic and known genetic causes. Schizophrenia is chronic psychiatric disorder that influences early brain development, and manifests as a combination of psychotic symptoms including hallucinations, delusions, and cognitive dysfunctions (Kahn et al., 2015). Specifically, rare mutations in *DISC1* have been associated with schizophrenia, and forebrain organoids derived from patients with a 4 base pair frameshift mutation in *DISC1* exhibited a delay in cell cycle progression related to disrupted DISC1 and NED1/NDEL1 protein interactions (Ye et al., 2017). In a separate study, forebrain organoids also displayed deficits in cortical neuron subtype and layer specification related to disrupted WNT/β-catenin signaling (Qian et al., 2020). Additionally, brain organoids derived from *DISC1*-mutated iPSCs were found to lack ventricular-like structures and had decreased progenitor proliferation (Srikanth et al., 2018).

Autism Spectrum Disorder (ASD) represents a group of heterogeneous developmental conditions that are characterized by impairments in social and emotional interactions and speech, with some patients exhibiting macrocephaly (Geschwind, 2008). Patient cortical organoids with macrocephalic features (larger size) were shown to have differential gene expression in pathways related to cell fate and proliferation, cytoskeletal regulation of dynamic cellular growth, guidance, and maintenance, synaptic assembly and channel functioning, and upregulated GABAergic enzyme synthesis (Mariani et al., 2015) . Consistent with this, cerebral organoids derived from iPSCs with heterozygous knockout of *CHD8*, a major risk factor for ASD, showed dysregulation of *DLX* genes that play a critical role in GABAergic interneuron differentiation (Wang et al., 2017). Timothy Syndrome, which is an autosomal-dominant disorder caused by a missense mutation in a gene encoding the calcium channel *CACNA1C*, is characterized by ASD. Neurons in patient-derived cortical organoids showed calcium signaling defects and delayed GABAergic interneuron migration (Birey et al., 2017).

Together, these studies demonstrate the validity of brain organoids for capturing molecular, cellular and structural phenotypes of neurodevelopmental and neuropsychiatric disorders, and provide the opportunity for investigating the underlying mechanisms. Brain organoids were first applied to model disorders with large structural deficits, as the phenotypes are usually obvious enough to detect reliably, despite variability among organoids.

In contrast, neuropsychiatric disease models are mostly limited to those with known genetic causes, which permits the use of isogenic lines with corrected mutations to confirm causality. As brain organoid technologies improve in regard to inter- and intra-organoid variability, diseases with more subtle phenotypes may be modeled with more confidence.

4.2 Neurodegenerative diseases

Neurodegenerative diseases are a diverse group of disorders that are characterized by the progressive deterioration of the nervous system resulting from neural and glial cell death or impaired neuronal function. Brain organoids generated from patient iPSCs or hPSCs edited to contain disease-causing mutations have been used to model several neurodegenerative diseases including Alzheimer's disease (AD), frontotemporal dementia (FTD), and Parkinson's disease (PD) (Grenier, Kao, & Diamandis, 2020).

AD, the most common neurodegenerative disease, is characterized by the progressive loss of memory and language with associated behavioral dysfunction. AD patients have β-amyloid plaques in the extracellular brain parenchyma and neurofibrillary tangles comprised of hyperphosphorylated tau in the intracellular neuronal spaces (Kumar, Singh, & Ekavali, 2015). Mouse models of familial AD (fAD) exhibit synaptic and memory deficits with β-amyloid pathology, but do not recapitulate other key aspects, such as neurofibrillary tangles (Duff, 2001), while AD-hiPSC-derived neurons exhibit elevated β-amyloid and phosphorylated tau, but do not develop β-amyloid plaques or neurofibrillary tangles (Israel et al., 2012; Kondo et al., 2013; Muratore et al., 2014; Yagi et al., 2011). Thick-aggregate cultures of human stem cell-derived neurons with fAD mutations provided the first evidence that 3D cultures could recapitulate both plaque and tangle pathology (Choi et al., 2014). Later, patient-derived brain organoids harboring APP duplications or PSEN1 mutations were shown to develop age-dependent extracellular β-amyloid aggregation, hyperphosphorylated tau protein and endosome abnormalities (Raja et al., 2016). Treatment with β- and γ-secretase inhibitors reduced amyloid and tau pathology.

FTD is a neurodegenerative disease characterized by atrophy in the frontal and temporal brain lobes and an increase in intracellular protein aggregates. Patient-derived cerebral organoids carrying the Tau P301L mutation showed increased levels of p25, which hyperphosphorylates tau and increases aggregation (Seo et al., 2017). Pharmacological inhibition of p25 generation reduced levels of phosphorylated tau and increased expression of synaptophysin in organoids.

PD is a neurodegenerative disease associated with abnormalities in movement, balance, and coordination (Fearnley & Lees, 1991). The most common cause of autosomal-dominant PD is a gain-of-function G2019S missense mutation in leucine-rich repeat kinase 2 (LRRK2), which has been shown to be associated with mitochondrial dysfunction (Hsieh et al., 2016; Langston, Ballard, Tetrud, & Irwin, 1983; Park, Davis, & Sue, 2018), impaired dopamine signaling leading to the degeneration and progressive loss of dopamine neurons (Fearnley & Lees, 1991), and the accumulation and aggregation of a-synuclein in the neuronal cytosol, known as Lewy bodies, causing damage to other cellular components (Kim, Park, et al., 2019; Spillantini, Crowther, Jakes, Hasegawa, & Goedert, 1998; Spillantini et al., 1997; Stefanis, 2012). Indeed, midbrain organoids generated from iPSCs with the *LRRK2* G2019S mutation exhibited similar pathological features of patient brains, including increased accumulation and aggregation of a-synuclein (Kim, Park, et al., 2019) and decreased dopaminergic neurons (Smits et al., 2019).

4.3 Chemical exposures

Brain organoids, mostly cortical organoids, have been used to study the effects of chemical exposures on fetal development and neuronal function by directly adding toxic molecules or drugs of abuse to the culture medium. Exposure to bisphenol A, a chemical found in many household plastics, reduced neural progenitor cell proliferation and ventricular zone thickness in forebrain organoids (Qian et al., 2016). Ethanol exposure induced premature neural progenitor differentiation, attenuated neuronal maturation and neurite outgrowth, and led to increased cell death in brain organoids in a concentration-dependent manner (Arzua et al., 2020; Zhu et al., 2017). Cocaine exposure led to a CYP3A5-induced generation of reactive oxygen species, reduced neural progenitor cell proliferation, and premature neuronal differentiation in cortical organoids (Lee et al., 2017). 5-MeO-DMT exposure led to altered expression of proteins associated with anti-inflammation, long-term potentiation, and dendritic spine formation (Dakic et al., 2017). Nicotine exposure induced premature neuronal differentiation, disrupted progenitor regionalization, and abnormal neurite outgrowth in a brain organoid-on-a-chip model (Wang et al., 2018). Cannabinoid exposure reduced neuronal maturation, impaired neurite outgrowth, and decreased spontaneous firing in microfluidic assembled cerebral organoids (Ao et al., 2020). Although these early studies helped in gaining understanding of the effects of many chemicals on brain development, the results must

be interpreted with caution due to important limitations. Because brain organoids are isolated from other functioning organ systems, models of chemical exposure do not accurately account for metabolism and bioavailability, despite best attempts to mimic the bioactive compound and physiological concentrations. Second, brain organoids do not have an intact blood-brain barrier which functions to limit diffusion of chemicals into the brain parenchyma and cerebrospinal fluid. Third, it has been increasingly appreciated that individual genetic predispositions may affect response to certain chemicals. Most of these studies utilize a modest number of stem cell lines, limiting the broad generalizability to the general population. Improvement of organoid technology combined with high throughput screening methods that employ many iPSC lines from individuals of diverse genetic backgrounds may better inform disease mechanisms in the future.

4.4 Infectious diseases

Like chemical exposures, brain organoids have been used to study the effects of infectious diseases on neural development by directly adding pathogens to the culture medium. Following the World Health Organization's declaration of a public health emergency based on mounting epidemiological evidence associating Zika virus (ZIKV) infection in pregnant mothers with babies born with microcephaly, several groups used brain organoids to model ZIKV infection. Brain organoid cultures exposed to ZIKV exhibited selective infection of neural progenitor cells and astrocytes, resulting in cell death, reduced proliferative zones, and disrupted cortical layer organization, corroborating the microcephalic phenotype observed in babies with vertical transmission of ZIKV (Cugola et al., 2016; Dang et al., 2016; Garcez et al., 2016; Ming, Tang, & Song, 2016; Qian et al., 2016; Watanabe et al., 2017). Because of this strong and clinically relevant phenotype, brain organoids were further used to screen and test for compounds that could reduce infection (Watanabe et al., 2017; Xu et al., 2016; Zhou et al., 2017) and to better understand the mechanisms responsible for ZIKV-induced microcephaly (Dang et al., 2016; Wen, Song, & Ming, 2017; Yoon, Song, et al., 2017). 05wHerpesvirus infection, leading to a disease that infects epidermal cells and establishes latent reservoirs in neurons, has also been modeled in brain organoids and exhibited multiple changes, including viral spreading, morphological changes and cellular fusion of infected neurons (D'Aiuto et al., 2019). More recently, several studies have used various region-specific brain organoid protocols to study neurotropism of the SARS-CoV-2 virus

and its potential consequences on brain cell function. Using cortical, hippocampus, hypothalamus, midbrain, and choroid plexus organoids, the Ming group revealed a specific SARS-CoV-2 tropism for choroid plexus epithelial cells that leads to productive infection, cell death, increased inflammatory cytokine transcription, and decreased transcription of genes important for cerebrospinal fluid production and barrier formation (Jacob, Pather, et al., 2020). This tropism was replicated by the Lancaster group using CSF-producing choroid organoids (Pellegrini, Albecka, et al., 2020). These studies highlight the applicability of brain organoids to model infectious disease, identify tropism and potential phenotypes, to understand pathogen-host interactions, and to serve as a platform for drug screening and developing therapeutic strategies.

4.5 Brain cancers

Malignant brain tumors are among the most devastating and lethal neoplasms affecting children and adults (Aldape et al., 2019). Although our understanding of brain tumor biology has been advanced by *in vitro* tumor stem cell lines, these model systems are limited by drifting away from human genetic signatures and poor clinical translatability (Ledur, Onzi, Zong, & Lenz, 2017; Lee et al., 2006). Brain organoids have been used to study tumorigenesis in Tuberous Sclerosis, adult glioblastoma, and pediatric medulloblastoma. Tuberous Sclerosis Complex (TSC) is a developmental disorder with heterozygous germline mutations in *TSC1* or *TSC2*, characterized by epilepsy, ASD, intellectual disabilities, and psychiatric disorders (Curatolo, Moavero, & de Vries, 2015). Patients with TSC commonly show focal regions of disorganized and dysmorphic neurons and glia, called cortical tubers, that result from haploinsufficiency or loss of heterozygosity of TSC (Martin et al., 2017). Cortical organoids derived from cells with homozygous knockout of *TSC1* and *TSC2* showed reduced and delayed expression of neuronal markers, increased glial cells, and dysmorphic neurons and glia, similar to cellular phenotypes seen in patients' tubers (Blair, Hockemeyer, & Bateup, 2018).

To model glioblastoma, one study employed a two-pronged approach to study both intrinsic tumorigenesis and extrinsic tumor cell migration using cerebral organoids (Ogawa, Pao, Shokhirev, & Verma, 2018). Combined HRasG12V overexpression and TP53 knock-out was achieved in cerebral organoids by electroporating vectors encoding Cas9 nuclease with guide RNAs. Cells with successful integration became transformed and exhibited

increased proliferation and rapid invasion which destroyed surrounding organoid structures. Orthotopic xenotransplantation of dissociated cultures recapitulated key glioma phenotypes, such as extensive invasion into the surrounding brain parenchyma, migration along blood vessels, and stimulation of angiogenesis. Furthermore, when fusing glioma stem cell tumorspheres with cerebral organoids, extensive tumor cell invasion and proliferation were found. Some of these results were replicated in other studies by co-culturing glioma stem cells with cerebral organoids (Krieger et al., 2020; Linkous et al., 2019). Similar to this strategy, combined transposon-mediated oncogene insertions and CRISPR/Cas9-mediated tumor suppressor knockouts were applied to cerebral organoids to generate neoplastic cerebral organoids (Bian et al., 2018). For example, overexpression of MYC resulted in formation of tumors that resembled central nervous system primitive neuroectodermal tumors. These organoids are useful for chemotherapeutic drug screening, with EGFR inhibitors reducing the growth of tumor cells with EGFR overactivation. On the other hand, combined overexpression of OTX2 and c-MYC in cerebellar organoids induced medulloblastoma-like tissue, and EZH2 inhibition could inhibit the growth and induce apoptosis of the tumor-like tissue (Ballabio et al., 2020). These studies demonstrate the utility of brain organoids to model tumor initiation and tumor cell invasion and to test therapeutic strategies. However, the observed changes in this unique model should also be further validated because of brain organoid limitations (see below). Furthermore, modifications of a single or a few oncogenes in brain organoids do not necessarily recapitulate the entire genetic and/or epigenetic landscape of human tumors. Recent development of glioblastoma organoid models derived directly from surgically resected tumor tissue have been shown to better maintain the cellular, transcriptional, and genetic heterogeneity of patients' tumors (Hubert et al., 2016; Jacob, Ming, & Song, 2020; Jacob, Salinas, et al., 2020).

5. Limitations and perspectives

Cerebral organoids and region-specific brain organoids have expanded our toolset for studying human brain development and mechanisms underlying many neurological diseases, but they remain imperfect model systems with several important caveats, including limited protocol reproducibility among different hPSC lines, heterogeneity among organoids, lack of definitive methods and markers to accurately assess cell

identity, and absence of structures that fully recapitulate the organization of the human brain. We discuss these limitations and offer some insights on minimizing their effect and improving brain organoid technology.

5.1 Variability among stem cells and heterogeneity between organoids

Inherent variability among hPSC lines limits the reproducibility of differentiation protocols and the ability to accurately compare brain organoids derived from control and disease lines. This variability arises from several sources, including starting cell type (e.g., hESC or hiPSC), reprogramming method (e.g., episomal vectors, Sendai virus), culture substrate (e.g., MEF feeder cells or Matrigel), expansion medium (e.g., knock-out serum replacement, mTeSR1, E8), and genetic heterogeneity among individuals. Pluripotency is a complex cellular state regulated by gene expression controlled by transcription factors, chromatin modifiers, and non-coding RNAs and the state of the hPSCs can have profound effects on differentiation capacity (Enver, Pera, Peterson, & Andrews, 2009; Young, 2011). Additionally, there are noticeable differences in the gene expression, epigenetic landscape, and mutational load between hESCs and hiPSCs that can impact their differentiation potential (Bilic & Izpisua Belmonte, 2012). These differences often manifest as differential responses to signaling molecules and a propensity to differentiate to certain lineages based on the identity of cells that were originally reprogrammed, the age of the individual from which they were harvested, and the reprogramming methodology (Malik & Rao, 2013). Addressing hPSC variability, thorough characterization of differentiation capacity and efficiency using multiple hESC and hiPSC lines is needed when reporting on a new brain organoid methodology. Additionally, each study should include sufficiently detailed methodology so others can reproduce culture conditions. Furthermore, when comparing brain organoids derived from both control and disease lines to report a phenotype, it is imperative that the lines are derived from the same source (e.g., dermal fibroblasts or B lymphocytes) and generated using the same reprogramming method in parallel to limit variability. Multiple control and disease lines should be used when available with more than one clone per line to rule out clone-specific effects. When possible, in-family and/or isogenic controls should be used to control for differences in genetic background. CRISPR-edited lines should be screened for off-target effects in major regulatory and protein coding regions of the genome. Comparisons between unrelated controls and disease lines should

be avoided when possible and the use of lines with mutations that accurately reflect the patient condition over full gene knockouts or overexpression are preferred. Lastly, it is important to perform regular quality control on hPSCs to ensure they do not accumulate harmful mutations or microorganisms, such as mycoplasma, which can affect differentiation and disease phenotypes.

3D differentiation lends itself to much more variation in structure, size, and cell composition than 2D monolayer cultures since it relies on self-organization of stem cells and progeny as they are patterned. There is no definitive way to eliminate heterogeneity among organoids, but the effect of this heterogeneity can be limited in mechanistic and disease studies by using proper controls and devising consistent methods for quantification and analysis. Using defined medium compositions which do not rely on components with substantial batch-to-batch variations (e.g., animal serum and Matrigel) will likely improve organoid reproducibility as serum-free medium supplements and defined hydrogels have become more common. Similarly, recombinant growth factors can have large batch-to-batch variability and poor stability in culture, therefore stabilized growth factors and small molecule agonists and antagonists should be used whenever possible. Methods to control starting cell number within hPSC aggregates (e.g., 96-well self-aggregation and Aggrewell plates) should also be used when possible to reduce size variability.

5.2 Accurately accessing cell identity

Commonly used markers for characterizing neural progenitor regional identity and neuronal subtype specification in human brain organoids rely mostly on knowledge from studies in model organisms. While many of these markers are conserved, it is increasingly appreciated that there are critical differences due to human-specific features. An important advance has been the development of single cell or single nucleus RNA sequencing technologies that have enabled researchers to study the transcriptomes of individual cells of the human brain. These efforts have improved reference databases for cell type assignment, but have not solved the issue entirely. For example, most datasets are generated from careful dissections of specific brain regions, allowing researchers to classify cell identities based on preconceived knowledge of the specific brain region. Although organoids often contain ventricular structures that resemble the primitive brain, the structures alone are inadequate to assign brain region identity. Second, although there exist good marker genes for several cell populations, these markers are often only

specific when looking within small subdivisions of the brain. Throughout the entire brain, different neuron types may share marker expression. Therefore, both positive and negative markers should be used when possible. For example, when assessing dorsal forebrain progenitor fate, a combination of positive markers (e.g., PAX6, FOXG1, EMX1) and negative markers (e.g.,o NKX2.1, GSX2, FOXA2) should be used. While the single cell transcriptome has been widely used for cell type classification, it should be noted that mRNA expression does not always directly translate to protein expression. Lastly, cells within brain organoids represent fetal development and are usually more immature than those found in the adult human brain, and often lack the specific signatures of human brain cells (Bhaduri et al., 2020). Because neurons in organoid culture do not reach full maturity, different neuronal subtypes may often blend in single cell analyses, with few clear distinctions for two main subtypes of glutamatergic neurons and GABAergic neurons. Therefore, it is essential to use a combination of techniques, such as immunohistochemistry, RNA sequencing, and electrophysiology for the classification of cell and regional identities. More thorough characterization of cell identity in brain organoids will provide a better framework for understanding human brain development and disorders.

5.3 Structural organization

Despite rapid advancements in patterning, most region-specific brain organoids do not fully recapitulate the intricate structural organization of neurons and cell type complexity of brain regions. Perhaps the best studied is the cerebral cortex with its highly organized laminar neuronal organization into distinct layers. Brain organoid protocols for cortical development recapitulate progenitor zone layers and rudimentary neuronal layering but have yet to reproduce the full neuronal organization of the cerebral cortex. Likewise, although medial pallium organoids generate diverse neurons of the hippocampus, they fail to recapitulate the structural organization of the CA and dentate gyrus subfields. Similarly, hypothalamic and thalamic organoids fail to recapitulate the formation of discrete nuclei. Improving structural organization will likely rely on advancements in bioengineered scaffolds or the addition of important guidance cells or molecules.

6. Summary

Recent advancements in region-specific brain organoids have contributed greatly to our understanding of the morphogens that guide neural progenitor fate specification and the processes of human neuron generation.

By utilizing precise timing and combinations of signaling molecules, researchers have guided hPSC aggregates to form primitive structures resembling the organization and neuronal diversity of specific brain regions. Although brain organoid models show great promise for developmental and disease studies, limitations in reproducibility and maturity restrict their utility. Improvements in protocol reproducibility and inter-organoid variability combined with techniques to improve cellular diversity and maturity will allow researchers to continue studying the human brain in ways that were previously impossible. Ultimately, brain organoids provide a complementary system, that in combination with other *in vitro* culture methodologies and animal models will help us better understand human brain development neurological diseases.

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Conflict of interest

None.

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