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3D Brain Organoids: Studying Brain Development and Disease Outside the Embryo

Silvia Velasco,[1,2] Bruna Paulsen,[1,2] and Paola Arlotta[1,2]

[1]Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, Massachusetts 02138, USA; email: paola_arlotta@harvard.edu

[2]Stanley Center for Psychiatric Research, Broad Institute of MIT and Harvard, Cambridge, Massachusetts 02142, USA

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Abstract

Scientists have been fascinated by the human brain for centuries, yet knowledge of the cellular and molecular events that build the human brain during embryogenesis and of how abnormalities in this process lead to neurological disease remains very superficial. In particular, the lack of experimental models for a process that largely occurs during human in utero development, and is therefore poorly accessible for study, has hindered progress in mechanistic understanding. Advances in stem cell–derived models of human organogenesis, in the form of three-dimensional organoid cultures, and transformative new analytic technologies have opened new experimental pathways for investigation of aspects of development, evolution, and pathology of the human brain. Here, we consider the biology of brain organoids, compared and contrasted with the endogenous human brain, and highlight experimental strategies to use organoids to pioneer new understanding of human brain pathology.

Contents

THE VALUE AND LIMITATIONS OF ANIMAL EMBRYOS AS MODELS OF THE DEVELOPING HUMAN BRAIN

The essential framework for the cells and tissues of the body is laid down during embryonic development in a process known as organogenesis, ultimately resulting in anatomically defined and functionally integrated organs. This process is highly invariant, producing nearly identical cell types and tissue architecture across individuals. Over a century of work has investigated the mechanisms that build organs and how defective organogenesis leads to developmental disease. However, in the majority of these studies, animals have served as models for understanding human organogenesis. This has led to an impressive body of work on evolutionarily shared biology, but there remain species differences that prevent a full understanding of human development from other model systems; as a result, large gaps in knowledge persist (**Figure 1*a***).

Perhaps no other organ embodies these limitations as uniquely as the brain (Pasca et al. 2014, Quadrato et al. 2016). Building experimental models of human brain development has been challenging. Our brains are different from even our closest primate relatives (Lui et al. 2011, Sousa et al. 2017), and the developing human brain cannot be accessed experimentally (Farahany et al. 2018) (**Figure 1*a***). The availability of embryonic tissue is largely limited to a short and early phase of development, which, given the length of human gestation, can only provide a snapshot of a much more extensive process. Consequently, human brain development, functionality, and disease remain incompletely understood. New experimental models are acutely needed to move forward (**Figure 1*b***).

HUMAN MODELS FOR HUMAN DISEASE: BRAIN DEVELOPMENT IN THE DISH

The need for dynamic cellular models of the developing human brain extends beyond investigation of basic aspects of brain formation and evolution; it also directly affects the study of human diseases originating in neurodevelopmental abnormalities (**Figure 1*b***). For example, nonhuman models are poorly suited to replicating the genetic bases of prominent human neurodevelopmental and neuropsychiatric disorders, such as autism spectrum disorder (ASD) and schizophrenia (SCZ). Sequencing studies have revealed the polygenic architecture of these pathologies: most patients have genetic risk states representing the combined effect of many common variants of

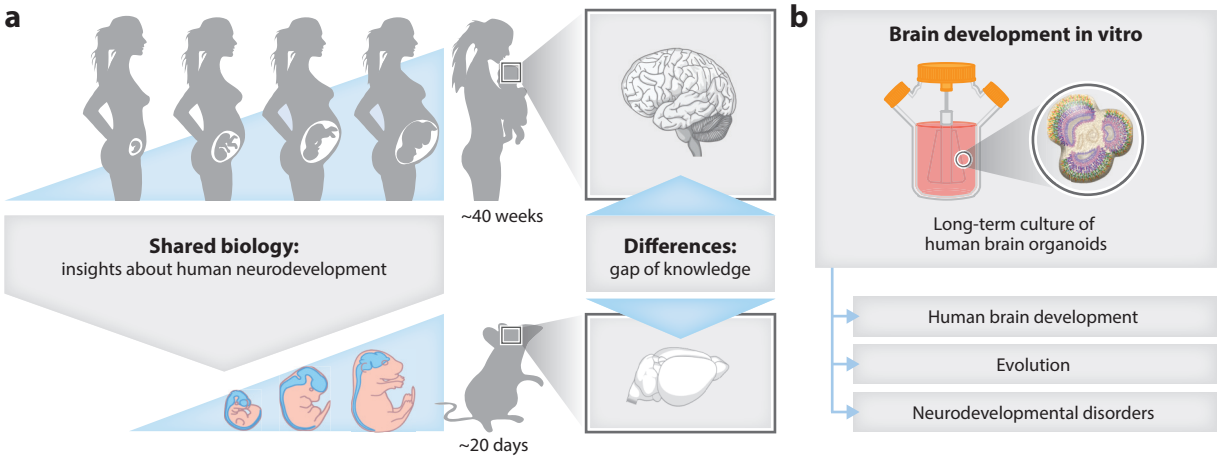

Figure 1

The need for experimental models of the developing human brain. (*a*) The human brain is largely inaccessible during embryonic development, limiting the ability to investigate its development in vivo. Model organisms such as rodents share many biological features with humans and have been very powerful in providing insights about the development of the mammalian brain. However, there are numerous intrinsic interspecies differences that result in large gaps of knowledge about unique aspects of human brain development. (*b*) Brain organoids are a novel tool to observe and perturb selected processes of the developing human brain.

small effect, compared to a much smaller proportion of cases associated with monogenic changes, i.e., rare loss-of-function mutations in specific genes (Geschwind & Flint 2015, Gratten et al. 2014). Knowledge of the genetic underpinnings of these disorders can be used experimentally as a handle to identify the neurobiological substrates of pathology: the cell types and circuits affected, the developmental events that go awry, and the molecular mechanisms involved. Yet, this type of polygenic contribution cannot be easily studied in animal models; while much knowledge has emerged from analysis of single-gene loss-of-function mutations, it is experimentally infeasible to create transgenic models bearing variable combinations of precise edits across many genes. Human experimental models of the developing brain are therefore essential platforms to complement work in animals.

This goal of generating models of the developing human brain raises the question of which, if any, aspects of brain development can be recapitulated outside of in vivo embryogenesis, and of what parts of such a precisely orchestrated process can be experimentally induced and controlled in vitro. To pursue this aim, over decades the field has developed ever-improving cell culture models of brain development; the recent emergence of human brain organoids represents a major advance in modeling the cellular complexity and structural features of the embryonic human brain in vitro.

The ability to induce differentiation of pluripotent stem cells (PSCs) along specific developmental pathways has been used to derive a wide variety of cell types of many tissues (Murry & Keller 2008). Classically, these experiments have primarily relied on externally patterned models in which sequential exposure to exogenously provided instructive cues such as growth factors directs development toward a preselected cell fate (Wichterle et al. 2002). However, such externally patterned models generally produce relatively homogenous cultures consisting mostly of a single cell type or a few closely related cell types; in addition, they have typically employed two-dimensional (2D) monolayer culture systems, which limit the degree of architectural complexity and cell–cell interaction that can develop.

Over the past several years, new models have emerged that take advantage of the remarkable robustness of cell-intrinsic developmental programs to produce more complex in vitro tissues. In these models, collectively termed organoids, minimally patterned cells grown in three-dimensional (3D) culture spontaneously develop some of the cellular diversity and architectural features of the endogenous organ through a process of self-organization (Sasai et al. 2012, Simunovic & Brivanlou 2017, Turner et al. 2016). Organoid models have been produced for a variety of tissues, suggesting that the ability for multilineage differentiation and spatial organization to emerge under permissive conditions without direct instruction is broadly shared across organs (Clevers 2016). These self-organizing organoid systems represent a significant advance in in vitro models; compared to externally patterned models, they ultimately produce tissues that more closely replicate the cellular and architectural complexity of the endogenous tissue and allow study of the developmental processes leading to this complexity without the constraints imposed by extensive external instruction.

In the case of the brain, it was determined some time ago that stem cell–derived neural progenitor cells (NPCs) in 2D culture can exhibit largely self-guided organization into structures resembling the neural tube (i.e., rosette-like structures where a lumen is surrounded by NPCs) (Elkabetz & Studer 2008). The later demonstration that stem cell–derived cultures were similarly able to give rise to 3D polarized structures replicating more complex aspects of the nervous system (Sasai 2013) affords new opportunities to use PSCs to model early steps of brain development in the dish.

The seminal work of Yoshiki Sasai and colleagues (Eiraku et al. 2011, Nakano et al. 2012) showed the remarkable ability of stem cells to generate an optic cup–like structure through cell-intrinsic programs alone. In these experiments, dissociated PSCs cultured in the presence of minimal factors could spontaneously give rise to the primordium of an optic cup, in which neural retinal tissue develops surrounded by retinal pigmented epithelium. Remarkably, this cellular system produced some of the distinctive cell types of the retina and could organize into layers resembling the endogenous early retina. Sasai and colleagues' work indicated the presence of an intrinsic self-organizing program in retinal progenitor cells, which emerged in vitro under permissive conditions, without the influence of neighboring tissues such as the surface ectoderm and lens. Within the nervous system, it is clear that this not only is a property of retinal tissue but also extends to other brain regions, including the cerebral cortex (Kadoshima et al. 2013, Lancaster et al. 2013, Mariani et al. 2012, Pasca et al. 2015, Qian et al. 2016, Quadrato et al. 2017, Sloan et al. 2017, Velasco et al. 2019), midbrain (Jo et al. 2016, Monzel et al. 2017, Qian et al. 2016), hippocampus (Sakaguchi et al. 2015), hypothalamus (Qian et al. 2016), and cerebellum (Muguruma et al. 2015).

DIFFERENT STRATEGIES FOR INDUCING BRAIN TISSUE DEVELOPMENT IN VITRO

Brain organoids are complex 3D structures, derived from in vitro stem cell cultures that are able to replicate some of the cell type– and tissue-specific features of the brain (**Figure 2*a***). Within this broad definition, brain organoids have been produced following many different protocols, which generally contain sequential phases of culture conditions designed to promote neural induction, regionalization, and long-term growth. Early examples include human 3D structures resembling the dorsal forebrain and containing distinct neuronal and progenitor zones organized in the same apicobasal order as in the human fetal cortex, which were described by Yoshiki Sasai's group (Eiraku et al. 2008, Kadoshima et al. 2013). The emergence of brain structures from PSCs in vitro was concomitantly reported by the group of Jurgen Knoblich (Lancaster et al. 2013), which showed that by combining PSC-derived embryoid bodies with artificial extracellular matrix (Matrigel), large 3D structures containing multiple distinct brain regions could be formed even in

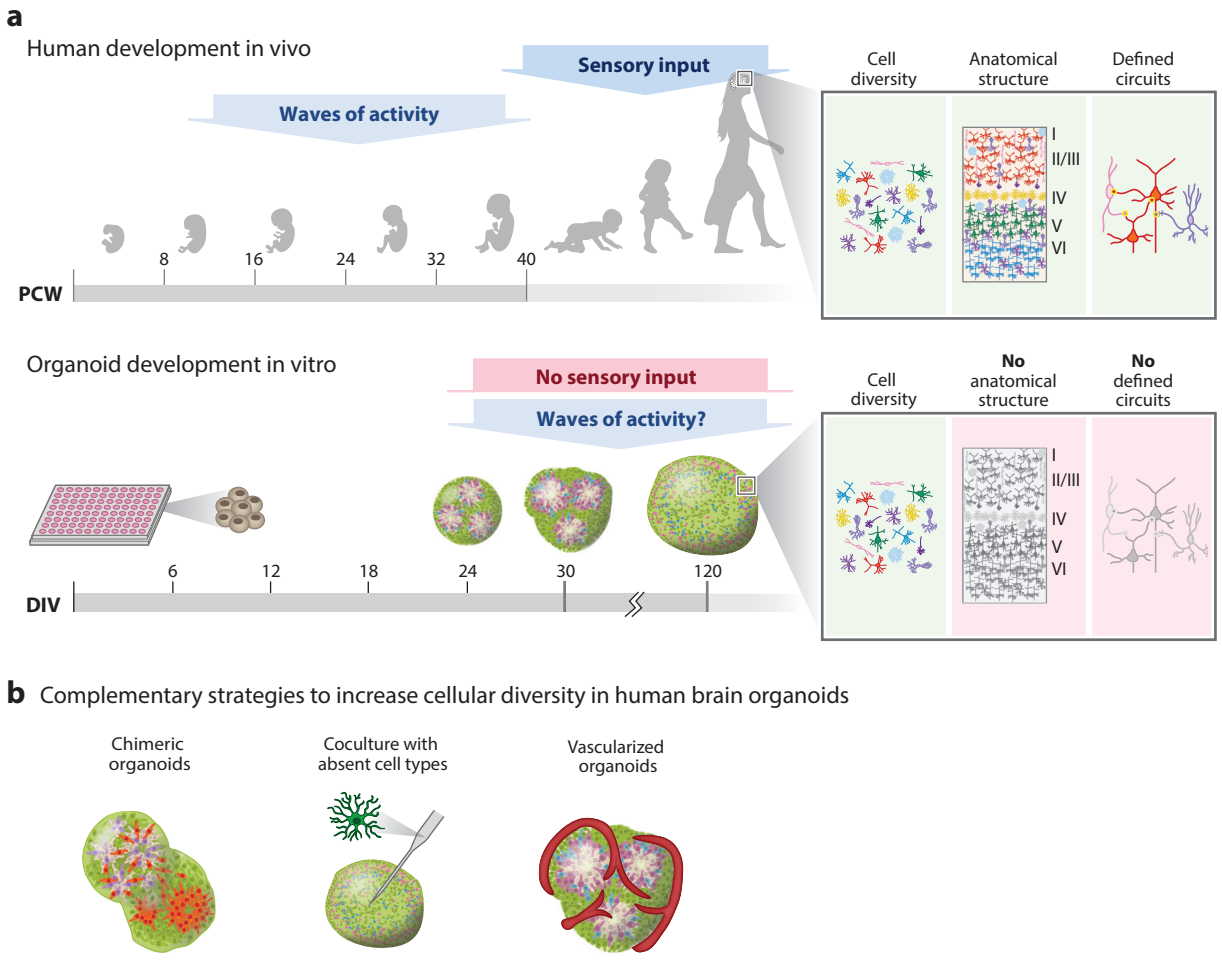

Figure 2

Challenges and current limitations of human brain organoids. (*a*) Human brain organoids can generate most of the cell types present in the developing cerebral cortex with potentially high reproducibility; however, anatomical structures are not fully recapitulated in brain organoids, and well-defined circuits are also not formed. Differences in the maturation of circuits may be influenced by the lack of experience-dependent stimulation of developing networks, which is an important influence in vivo but absent in organoids. (*b*) Some of the cell types of the brain do not spontaneously develop in brain organoids. Current approaches to integrate missing cell types include generation of chimeric organoids (by combining organoids patterned to form different brain regions), coculture with absent cell types (such as microglia), and provision of exogenous vasculature. Abbreviations: DIV, day in vitro; PCW, postconception week. Organoids in figure adapted with permission from Quadrato et al. (2016).

the absence of regionalizing factors. Histological analysis revealed a complex heterogeneous tissue containing regions resembling the cerebral cortex, choroid plexus, and retina. Importantly, these studies indicated that species-specific features could be replicated in culture. For example, both of these organoid types formed outer radial glia (oRG) progenitors, a cell type that is virtually absent in rodents but characteristic of the developing human cortex (Hansen et al. 2010) and potentially involved in its evolutionary expansion (Fish et al. 2008, Lui et al. 2011, Pollen et al. 2015). This finding suggests that brain organoids may become a valuable model for studying species-specific developmental events in experimentally intractable species.

Since then, countless other brain organoid protocols have been established to address specific needs for more extensive development, reproducibility, and scalability (Arlotta & Pasca 2019, Di Lullo & Kriegstein 2017, Kelava & Lancaster 2016, Pasca 2018, Quadrato et al. 2016). Some of these methods rely largely on principles of self-assembly and self-patterning (Lancaster et al. 2013, Quadrato et al. 2017). These models are characterized by the presence of broad regional identities and are therefore termed whole-brain organoids. Other systems rely on the use of signaling molecules to direct the formation of specific brain regions, and can be referred to as patterned brain organoids (Kadoshima et al. 2013, Mariani et al. 2012, Pasca et al. 2015, Qian et al. 2016, Sloan et al. 2017, Velasco et al. 2019).

Whole-brain organoids (pioneered in Lancaster et al. 2013) are produced largely by self-organization and with minimal external patterning signals, offering a unique platform to investigate the endogenous capacity of neural stem cells to generate appropriate cell types and geometry and how subsequent steps of brain development emerge from these earlier structures. Simplistically, one can think of these organoids as less artificially induced models and thus possibly more reflective of endogenous principles of brain development. However, one of the main limitations of this type of organoid system is the high degree of variability in cell type distribution and overall morphology both between individual organoids and across experimental batches. This lack of reproducibility poses major challenges to the use of organoids as experimental systems for the investigation of biological and pathological processes.

Patterned brain organoids can produce select parts of the brain through the use of exogenous signals to direct formation of specific regional identities (e.g., cortex or basal ganglia) and show promise in many respects, including organoid-to-organoid reproducibility. Organoids containing tissue representing multiple brain regions, which would be difficult to produce within the same 3D system, can instead be assembled via fusion of separate organoids patterned to different regions (Bagley et al. 2017, Birey et al. 2017, Xiang et al. 2017) (**Figure 2*b***). A similar approach could potentially be used to incorporate cell types that would not otherwise self-emerge within a single organoid system (Pasca 2019) (**Figure 2*b***).

3D BRAIN ORGANOIDS: WHAT IS MADE AND WHAT IS MISSING

In recent work, organoid cultures have been optimized to grow for extended time periods, which has provided an opportunity to examine how far development of the brain can progress outside the embryo and to investigate later stages of brain formation, including cellular diversification and maturation (Quadrato et al. 2017, Trevino et al. 2020). Many studies have shown that over time organoids can progressively generate a variety of cell types appropriate for endogenous brain regions (Eiraku et al. 2008, Kadoshima et al. 2013, Lancaster et al. 2013, Quadrato et al. 2017, Velasco et al. 2019). Some studies in particular have used single-cell RNA sequencing (scRNAseq) to profile large numbers of cells from long-term cultures of both whole-brain organoids (Camp et al. 2015, Quadrato et al. 2017) and patterned organoids (Velasco et al. 2019). More recently, scATACseq (single-cell assay for transposase-accessible chromatin followed by high-throughput sequencing) of organoids has also provided highly resolved maps of DNA accessibility and primed the field for understanding the gene regulatory networks that govern organoid development (Trevino et al. 2020). The results of these studies jointly support three main conclusions.

First, both whole-brain and patterned organoids can generate many of the major cell types of a complex brain region, the cerebral cortex; in whole-brain organoids, most cell types of the retina are also generated (Quadrato et al. 2017).

Second, the cell types produced in these different organoid models have transcriptional identities that are correlated to those of the corresponding cell types in the endogenous brain (Camp

et al. 2015, Quadrato et al. 2017, Velasco et al. 2019). This is true despite the fact that molecular signatures of metabolic stress can emerge in culture (Bhaduri et al. 2020). These studies also show that cell types appropriate for a given species (e.g., oRG cells that are characteristic of the developing human cortex) can spontaneously emerge in vitro, offering an opportunity to study such species-specific cell types in an experimentally tractable system (Kadoshima et al. 2013, Lancaster et al. 2013, Pollen et al. 2019, Quadrato et al. 2017, Sloan et al. 2017, Velasco et al. 2019).

Third, across many cell classes, the temporal sequence in which cell types appear mimics the endogenous order (e.g., radial glia are made before corticofugal neurons, which in turn precede callosal neurons and astrocytes). This is in agreement with a body of prior work in other systems showing that cortical neural progenitors cell-intrinsically control the temporal sequence of generation of their cellular progeny, even when cultured outside the cortex (Qian et al. 2000).

This is not to say that cells made in vitro are fully identical to the ones made in vivo, nor that the same degree of similarity will be observed across different analytic modalities (e.g., morphology, physiology). However, the data to date suggest that the process by which cell types are generated may be mechanistically so highly constrained that it is relatively insensitive to the growth environment (e.g., a variety of organoid models and the endogenous brain). This in turn suggests that genetically encoded instructions are sufficient, at least for the cerebral cortex, to drive progenitor cells to generate a certain compendia of cell types without the context of the embryo.

Likewise, certain organizational features of the developing nervous system spontaneously emerge in a reliable, highly stereotyped manner. Neural tube–like structures (i.e., rosettes) can emerge from unspecified stem cells with minimal instruction. Regionally patterned germinal zones can emerge from unspecified sheets of neuroepithelia and subsequently produce regionally and species-appropriate cell types (e.g., oRG), following a predefined temporal sequence. These properties are therefore likely built into the system, and offer a unique opportunity for mechanistic investigation of aspects of human brain development that would not otherwise be accessible.

One of the most impressive properties of embryonic development is the robustness with which each step of organogenesis proceeds, ensuring that each embryo generates the same compendium of cell types, organized in similar anatomical and functional structures (Silbereis et al. 2016). Such robustness does not necessarily emerge in organoids, and one of the greatest limitations in the field has been organoid-to-organoid and batch-to-batch variability. Several groups have investigated the reproducibility of organoid models, initially using bulk transcriptional profiles or measurements of morphological features (Mariani et al. 2015, Qian et al. 2016, Watanabe et al. 2017). In a recent study, Sergiu Pasca's group (Yoon et al. 2019), by comparing single-cell transcriptional profiles of cortical organoids across multiple human PSC lines and experiments, showed that organoids could show high reliability of differentiation, independently of the stem cell line of origin. Other studies also employed scRNAseq to investigate organoid cellular composition in pooled organoids (Bershteyn et al. 2017, Sloan et al. 2017, Xiang et al. 2017). Two other studies profiled individual organoids via scRNAseq, allowing the first quantification of organoid-to-organoid and batch-to-batch variability. The first showed that, in whole-brain organoids, cells of the forebrain were only produced by a subset of organoids and that even these successful organoids presented variability in the specific forebrain cell types generated (Quadrato et al. 2017). However, a more recent study showed that under specific growth conditions, cellular diversification of a complex brain region like the cerebral cortex can occur in organoids with extremely high levels of organoid-to-organoid, line-to-line, and batch-to-batch reproducibility, comparable to the variability observed between individual brains in vivo (Velasco et al. 2019). Moreover, cell types in individual organoids are generated following highly reproducible developmental trajectories, resembling the endogenous temporal order, across lines and batches (Velasco et al. 2019).

While the formation of cell types from patterned neuroepithelia can spontaneously occur in organoids, it is becoming clear that other features of the endogenous brain do not self-emerge. Noticeable omissions include the organization of cells into defined anatomical structures (e.g., cortical lamina, thalamic nuclei), regional patterning, and the formation of cell type–specific long-distance and local connectivity (e.g., the formation of specific commissures) (**Figure 2*a***).

The in vivo processes that lead from patterning of germinal zones to the formation of higher-order anatomy are complex. The formation of cortical layers, for instance, is correlated to both the birth order of the neurons and the presence of a radial glia scaffold; early-born cells occupy the inside layers (apical), while each wave of later-born neurons migrates along radial glial cells to increasingly basal positions, thus leading to an inside-out arrangement (Angevine & Sidman 1961, Gaspard & Vanderhaeghen 2011, Greig et al. 2013, Molyneaux et al. 2009). In 2008, Eiraku et al. (2008) demonstrated for the first time the self-formation of a stratified cortical tissue within organoids. Generation of embryoid bodies under low–growth factor conditions allowed the formation of a remarkable apicobasally polarized tissue, with the presence of four distinct zones (ventricular, early and late cortical plate, and Cajal-Retzius) never observed before in neurosphere cultures. However, in all of the cortical organoid models to date, cell layering is limited to the formation of separate germinal zones and a distinct cortical plate. The cortical plate has never been reported to achieve a clear morphological separation of the six cortical layers formed in vivo (Eiraku et al. 2008, Kadoshima et al. 2013, Lancaster et al. 2013, Quadrato et al. 2017, Velasco et al. 2019) (**Figure 2*a***). This suggests that the proper formation of cortical lamina may require additional factors that are missing in current organoid models.

Regional patterning of organoids has to date largely focused on protocols that confer a specific regional identity upon the whole culture, although organoids containing tissue representing multiple brain regions have been successfully created by fusion of individual differently patterned organoids (Bagley et al. 2017, Birey et al. 2017, Xiang et al. 2017) (**Figure 2*b***). During endogenous brain development, the division of neurogenic regions into functional domains is regulated by gradients of morphogens and gene expression (Sansom & Livesey 2009); in the cortex, organizing centers are one of the main sources of such regionalizing morphogens (Tiberi et al. 2012). Recently, Lorenz Studer's lab (Cederquist et al. 2019) developed a strategy to introduce an artificial organizing center into forebrain organoids in order to enable self-organization along the dorsoventral and anteroposterior positional axes: By embedding a cluster of cells expressing inducible Sonic Hedgehog (SHH) at one pole of the organoid, the authors created a SHH protein gradient that was capable of inducing consistent spatial separation of tissue expressing regional markers.

Finally, although various brain organoid models can generate a broad compendia of cell types after extended periods in culture, not all of the cell types found in the human cortex are present in brain organoids. In particular, cell types that normally originate from other germ layers, such as endothelium, pericytes, and microglia, do not emerge in most organoid protocols. Yet, there is increasing evidence that these cell types influence important aspects of cortical development, from morphogenesis to circuit refinement, and there are ongoing efforts to incorporate them into brain organoid systems (Abud et al. 2017, Song et al. 2019) (**Figure 2*b***).

For the future, recapitulating morphogenesis of complex organ structures such as the cerebral cortex will likely require modulating both molecular signals and physical context. Microfabricated culture environments may provide control over factors such as morphogen gradients, cell attachment or migration (such as replicating the pial basement membrane to allow attachment and radial orientation of radial glia), or dynamic mechanical signals (such as tension or pressure) that may contribute to morphological organization.

DEVELOPMENT AND MATURATION OF NEURAL CIRCUITS IN BRAINS AND ORGANOIDS

Although many organoid models have shown varying degrees of neuronal activity and synaptic connectivity, they have not yet demonstrated the ability to replicate the well-defined local and long-distance connectivity and circuit architecture of the endogenous brain (**Figure 2*a***). In one of the first mouse brain organoid models, Eiraku et al. (2008) showed that oscillatory Ca^{2+} waves synchronously activated neurons over long distances, as observed in the early postnatal mouse cortex. More recently, using a human brain organoid model that supports the differentiation of photoreceptor-like cells in addition to forebrain neuron subtypes, Quadrato et al. (2017) showed that after 8 months of differentiation, 4 out of 10 organoids had light-responsive units displaying attenuated firing rates and upregulation of cFos (an activity-dependent gene) after light exposure. To improve neuronal survival and axon outgrowth, Lancaster and colleagues (Giandomenico et al. 2019) developed a new strategy to artificially model the formation of circuits with functional neuronal output by culturing cerebral organoids in an air–liquid interface system. The authors were able to show active neuronal networks and extensive axon outgrowths reminiscent of nerve tracts, which were able to innervate spinal cord tissue and induce coordinated muscle contractions in co-cultured mouse spinal cord–muscle explants (Giandomenico et al. 2019). Another study reported that while the majority of cerebral organoids in their model showed some spontaneous neural activity, robust synchronized network activity only emerged after organoid-derived neurons were dissociated and replated in adherent culture (Sakaguchi et al. 2019). Collectively, these findings demonstrate that while brain organoids have the ability to establish spontaneously active neuronal networks, which may serve as useful systems to observe and modulate aspects of circuit formation, connectivity pattern, and cellular maturation, none of the described models present robust and reproducible circuit organization.

It is likely that stereotypical circuit formation does not occur naturally in the current models in part due to the lack of defined tissue architecture, the absence of relevant cell types, and the relative immaturity of neurons and neural circuits, even upon long periods of growth and maturation in vitro. In addition, in vivo, coordinated neuronal activity across networks (spontaneous waves of synchronized neuronal activity during prenatal development and temporally synchronized activity induced by sensory stimuli after birth) is involved in both neuronal maturation and the development and refinement of synaptic connectivity (Kirkby et al. 2013) (**Figure 2*a***). To date, no organoid model has incorporated the ability to induce this type of coordinated activity, although it is possible to imagine designs that apply optogenetic techniques to enable synchronized stimulation of neurons or subsets of neurons to foster maturation and circuit refinement.

ORGANOIDS AS EXPERIMENTAL SYSTEMS OF HUMAN NEUROLOGICAL DISEASE

While today brain organoids remain primitive replicas of the endogenous brain, they provide a unique opportunity to investigate mechanisms underlying human neurological diseases. Despite the great medical and societal impact of neurodevelopmental and neuropsychiatric pathologies such as ASD, SCZ, and bipolar disorder, many basic questions remain unanswered. We currently have only partial knowledge of the cell types and neural circuits that develop abnormally and of the cell types and circuits that risk-associated genes affect. These issues are vital to understanding the etiology of these disorders and to identifying cellular and molecular targets for therapeutic progress.

Many neurodevelopmental and neuropsychiatric disorders have been shown to have a strong genetic basis, which could be leveraged to define their neurobiological substrates by probing the

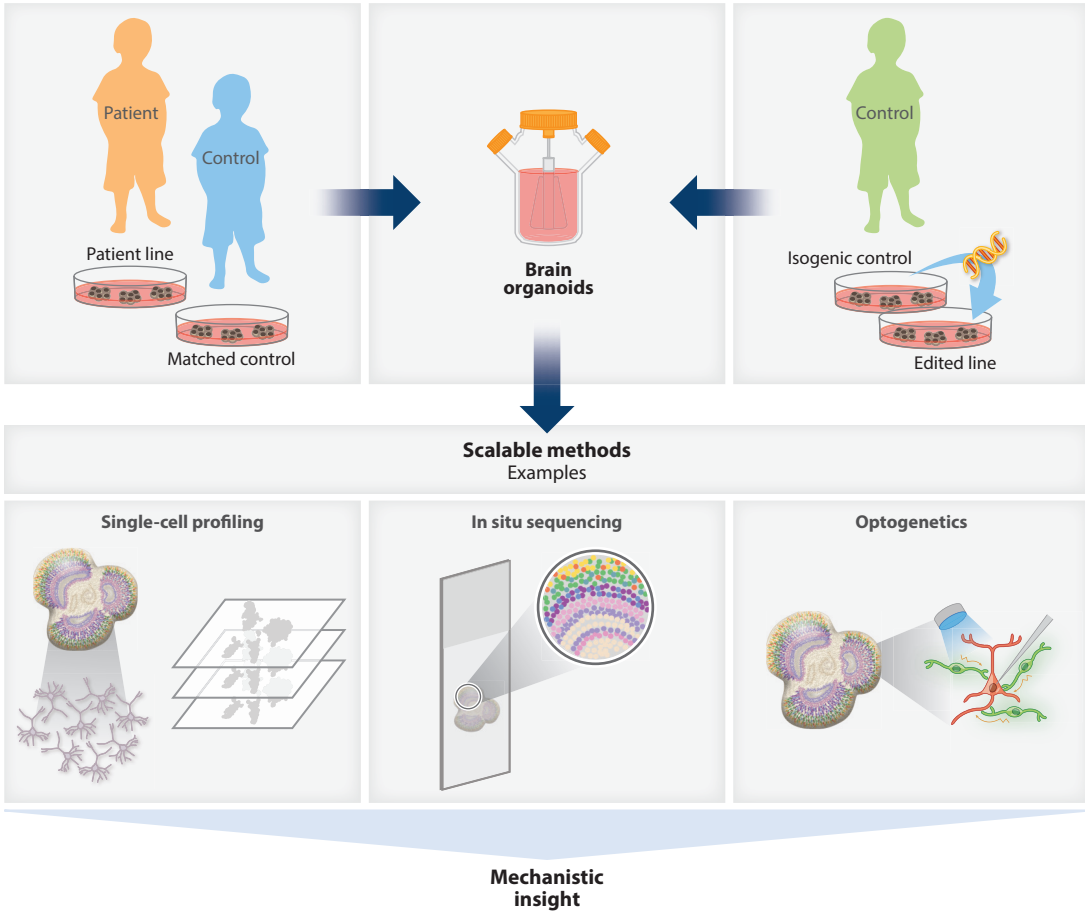

Figure 3

Use of organoids for mechanistic understanding of neurodevelopmental disorders. Pluripotent stem cell lines derived from patients and matched controls (to model polygenic states) or lines with engineered mutations and isogenic controls (to study specific disease-associated genes) can be used to generate brain organoids. The combinatorial use of scalable methods, such as multimodal single-cell profiling, in situ sequencing, and optogenetics, can be applied to organoids to potentially define the neurobiological substrates (i.e., cell types, biological processes, and molecular mechanisms) of complex neurodevelopmental diseases.

role of disease risk–associated genes in human brain development. However, these pathologies are genetically complex, and the majority of patients present polygenic states characterized by large numbers of variants of individually small effect (Geschwind & Flint 2015, Gratten et al. 2014). These types of genetic effects cannot feasibly be studied in animal models; however, human brain organoids grown from patient-derived PSC lines enable modeling such genetic states (**Figure 3**). Given the importance of genetic background for the phenotypic manifestation of even rare de novo mutations, organoids can also serve as unique models for the effect of risk-associated alleles in the context of human genetic variation. Furthermore, gene engineering can be used to correct mutated alleles in patient-derived lines, or to introduce targeted mutations into control lines, providing otherwise genetically identical pairs of experimental and control organoid cultures (**Figure 3**).

Although the field is still developing progressively more sophisticated organoid model systems, some early examples of using organoids to identify neurobiological mechanisms of disease have

been published. For example, organoids have been used to identify mitotic defects in oRG derived from patients with a form of lissencephaly called Miller-Dieker syndrome (Bershteyn et al. 2017, Iefremova et al. 2017). A pioneering study investigated early events of ASD using brain organoids generated from inducible PSC lines derived from patients with idiopathic ASD with macrocephaly, demonstrating overproliferation of neural progenitors and expansion of GABAergic interneurons in patient-derived organoids (Mariani et al. 2015). Brain organoid systems also allow study of dynamic processes that are difficult to capture in static tissue samples; for instance, a chimeric system fusing dorsal and ventral forebrain organoids was used to reveal defects in interneuron migration kinetics associated with Timothy syndrome, a monogenic neurodevelopmental disease characterized by ASD and epilepsy (Birey et al. 2017). Other studies have used brain organoids to investigate brain pathologies, including *CDK5RAP2*-dependent microcephaly (Lancaster et al. 2013), tuberous sclerosis (Blair & Bateup 2020), and Zika virus infection (Ming et al. 2016). These types of studies are only bound to grow as organoid models improve in sophistication and robustness.

The potential utility of brain organoids to understand human neurological disease is also being enabled by transformative advancements in analytic technology, in particular new tools for unbiased molecular assays, across multiple modalities (e.g., transcription, epigenetics, genetics), with single-cell resolution, and with capacity for scalability. These technologies have been critical for the study of organs that are highly cellularly diverse such as the brain, where bulk tissue analysis fails to be informative because of the large number of different cell types and states that are present.

In organoids, high-throughput, single-cell molecular profiling is particularly powerful, since, in addition to containing a variety of cell types and states, they carry the additional challenge of lacking anatomical reference points and of potentially high organoid-to-organoid variability (**Figure 3**). For example, high-throughput scRNAseq approaches have been applied to investigating the cellular composition of brain organoids with unprecedented molecular detail (Birey et al. 2017, Camp et al. 2015, Giandomenico et al. 2019, Pollen et al. 2019, Quadrato et al. 2017, Sloan et al. 2017, Velasco et al. 2019, Yoon et al. 2019). The arsenal of single-cell molecular tools has grown exponentially in the past years, and multimodal integration of transcriptomic, genomic, epigenomic and proteomic data collected from the same single cell will soon provide a comprehensive picture of cell identity and state, allowing disease comparisons on a more finely resolved level. Moreover, the spatial information provided by techniques for transcriptomic profiling of sectioned tissue, such as seqFISH (Shah et al. 2016), MERFISH (Chen et al. 2015, Moffitt et al. 2016), or Slide-seq (Rodriques et al. 2019), will link single-cell molecular profiles to high-resolution spatial mapping of the local cellular context, leading from the analysis of cell-intrinsic effects of disease states on cells to the effects of cellular neighborhood and the local tissue environment.

The use of single-cell biology approaches in 3D brain organoid systems has great potential to shed light on the molecular mechanisms that underpin complex neurodevelopmental disorders, for instance, by identifying the cell types, cell states, or developmental trajectories that are affected by risk-associated genes. Techniques that combine scRNAseq with pool-screening techniques for introducing gene edits, such as Perturb-seq (Adamson et al. 2016, Dixit et al. 2016, Jaitin et al. 2016), can become particularly powerful in organoids as a means to assay the functional effect of larger panels of risk genes in the same genetic and experimental background.

CONCLUSIONS

For centuries, scientists have been fascinated by the human brain and how development generates its myriad cell types, complex connectivity patterns, and refined higher-order structures to ultimately enable function. Yet, most of what we know today about the development of the human brain comes from the study of animal models, and thus emphasizes shared biology. However, the

human brain is different from that of rodents, for example, and many aspects of its development reflect species-specific events that cannot be fully investigated in other organisms. As a result, the science of human brain development has remained relatively descriptive and fragmented.

The advent of transformative technologies in high-resolution genomic and imaging analyses, combined with progress in stem cell biology, opens the door to the experimental exploration of the human brain in both healthy and pathological states. For as primitive as brain organoids still are, there is growing understanding of their properties, and more concrete approaches are being evolved to improve anatomical organization, the formation of defined circuits, and the acquisition of more mature properties of neurons and networks. The application of these systems reaches beyond the study of human brain development and evolution into broader science and medicine. Rapid progress in the field is advancing organoid systems toward more robust and reproducible cellular models, which may soon enable applications in industry for therapeutic screening and in the clinic as diagnostic tools for better patient classification and treatment. These approaches hold great promise, justifying growing investments into the study of organoid biology. Organoid models represent one of the best current opportunities to investigate aspects of the biology and pathobiology of the human developing brain that were never before accessible to science and medicine.

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