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Patterning of brain organoids derived from human pluripotent stem cells

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Abstract

The emerging technology of brain organoids deriving from human pluripotent stem cells provides unprecedented opportunities to study human brain development and associated disorders. Various brain organoid protocols have been developed that can recapitulate some key features of cell type diversity, cytoarchitectural organization, developmental processes, functions, and pathologies of the developing human brain. In this review, we focus on patterning of human stem cell-derived brain organoids. We start with an overview of general procedures to generate brain organoids. We then highlight some recently developed brain organoid protocols and chemical cues involved in modeling development of specific human brain regions, subregions, and multiple regions together. We also discuss limitations and potential future improvements of human brain organoid technology.

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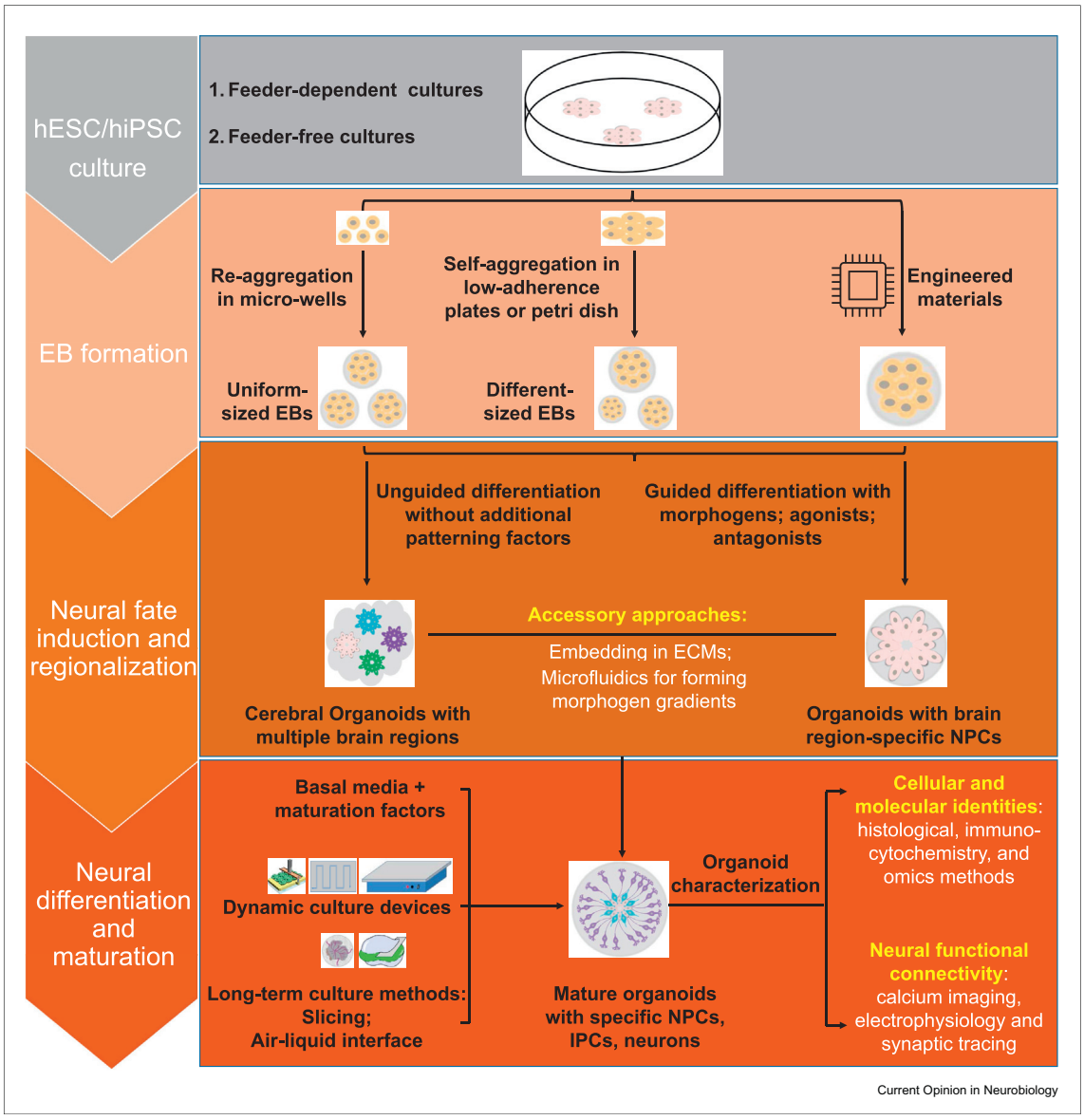
Introduction

Human brain organoids are neural tissues differentiated from pluripotent stem cells (PSCs) with self-organized three-dimensional (3D) structures that recapitulate some key characteristics of the cell type diversity, cytoarchitectural organization, and developmental trajectories of the embryonic human brain [1,2]. Brain organoid technology overcomes many limitations of conventional approaches, such as 2D neural cultures, and provides platforms for investigation of human brain development and disorders. Human brain organoids have recently been widely used in modeling human neural development (e.g. differentiation [3–5], migration [6] and evolution [7]), neurological and psychiatric disorders (e.g. schizophrenia [8], lissencephaly [9], hypoxia [10], viral infections [4,11–14]), brain cancers [15], drug screening and testing [16,17], AAV capsid selection for gene therapy [18], and have been transplanted into the rodent brain [19–21]. In this review, we first describe common procedures for brain organoid generation, and then focus on patterning of brain region- or subregion-specific organoids. We also highlight emerging technologies for engineering organoids consisting of multiple CNS regions in continuum. Finally, we discuss current limitations and prospects for future improvements.

Generation of brain organoids from human pluripotent stem cells

Generation of brain organoids starts from undifferentiated human embryonic stem cells (hESCs) or induced pluripotent stem cells (hiPSCs) cultured either on mouse-derived feeder cells or under feeder-free conditions (Figure 1). Notably, protocols are usually different between feeder and feeder-free conditions. Second, 3D embryoid bodies (EBs) are formed through the re-aggregation of single-PSC suspensions in microwells, such as Aggrewell [22], V/U-bottom wells, or 3D printed wells [23], or through the self-aggregation of PSCs colonies in low-adherence plates [3,4]. Additionally, engineered materials, such as microfilaments [24] and microfluidic chips [25], have been used. The third step is to differentiate EBs into neural progenitors. Brain organoid protocols are generally divided into unguided and guided categories based on this step. The unguided protocol takes advantage of the property that EBs differentiate preferentially toward neuroectoderm through intrinsic signals simply by the absence of exogenous growth factors in the culture media. The derived cerebral organoids contain tissues resembling multiple brain regions in an unpredictable

Figure 1



Procedures for generation of brain organoids from human pluripotent stem cells. Embryoid bodies (EBs) are formed from hESCs or hiPSCs via re-aggregation, self-aggregation or engineered approaches. EBs can undergo unguided or guided differentiation with specific morphogens or related agonists/antagonists for patterning into organoids with brain region-specific neural progenitors (NPCs). Organoids then go through a long-term culture in basal culture media (such as, neurobasal or DMEM/F-12) with additional factors for promoting maturation (such as, ascorbic acids, cAMP, NT3, BDNF, and GDNF). During this process, dynamic culture devices (e.g. orbital shaker, spinning bioreactor, and microfluidics), and different approaches (e.g. slicing method, or culturing at air-liquid interface) can be used to enhance the diffusion of oxygen, nutrition, and metabolites of organoids and prevent cell death. Organoids are analyzed using different approaches to identify their cellular populations, molecular signatures, neural functions, and network connections. IPC: intermediate progenitor cell.

fashion, including the cerebral cortex, ventral telencephalon, choroid plexus and retina, and sometimes non-neural cells [26]. The guided protocol is based on timed modulation of key morphogen-related signaling pathways, which generates organoids possessing specific brain region components with higher reproducibility [3,4]. For both protocols, EBs are sometimes embedded in the extracellular matrix (ECM), such as Matrigel, to support morphogen gradients and tissue growth, and promote organization in organoids. Brain organoids are further cultured to promote neural differentiation and maturation under

various conditions, such as the slicing method [27], the air–liquid interface method [28,29], and they can be transplanted into animals [19–21]. Organoids of different kinds can also be fused together as "assembloids" to investigate their interactions [6,30]. For organoid characterization, cellular and molecular identities are usually investigated with histological, immunocytochemical, and omics methods [31], including single-cell/bulk RNA-sequencing and ATAC-sequencing [32], whereas physiological functions and synaptic connections can be analyzed by calcium imaging [33], electrophysiology recording, and rabies viral tracing.

Patterning of brain region-specific organoids

Significant progress has been made in generating different brain region-specific organoids by fine turning of distinct morphogens mimicking a similar differentiation program as the developing embryonic central nervous system (CNS) (Figure 2). In general, the "dual-SMAD inhibition" by simultaneously inhibiting the bone morphogenic proteins (BMPs) and TGFβ pathways is used for patterning the neuroectoderm fate. During normal brain development, retinoic acid (RA), WNTs, and FGFs cause early caudalization, while their inhibition promotes rostral differentiation. With appropriate gradients, the neuroectoderm further forms the neural tube and develops along the rostral-caudal axis into the prosencephalon (or forebrain), mesencephalon (or midbrain), rhombencephalon (or hindbrain), and spinal cord. During this process, Sonic hedgehog (SHH) is critical for ventral region patterning, while BMP and WNTs are important for dorsal fate patterning.

Forebrain organoids

The forebrain is further segregated into the telencephalon containing the cerebral cortex and basal ganglia and the diencephalon containing retina, thalamus, and hypothalamus. Several protocols have been developed to pattern brain organoids specifically representing some of these regions (Figure 2).

The cortical organoid is sometimes termed as dorsal forebrain organoid as the cerebral cortex is the major component of the forebrain. The first reported cortical organoid protocol [34] used TGFβ and WNT inhibitors for telencephalic fate patterning and produced tissues with self-organized multilayered ventricular and neuronal zones that expressed markers for radial glia cells and neurons of different cortical layers (CTIP2, TBR1, and SATB2). The Pasca group [3] used dual-SMAD inhibition and then FGF2/EGF for expanding ventricular progenitors without the exogenous ECM. Organoids generated using this approach contain astrocytes and neurons with synapses and appeared to be more consistent from multiple batches and cell lines [22]. The Ming group [4,35] used dual-SMAD inhibition, followed by continuous TGFβ inhibition and WNT activation, then embedding EBs in Matrigel to promote expansion of ventricular structures and cortical-specific progenitors. After long-term culturing with a spinning bioreactor or the slicing method, they observed well-organized six-layer structures identified by expression of REELIN, CUX1, BRN2, SATB2, CTIP2, or TBR1 markers, as well as a prominent progenitor layer containing human enriched outer radial cells. One limitation of all these approaches is the presence of multiple ventricular structures, which could compromise their applications. Recent studies reported methods to generate a single neural tube by geometric constraint [36], or manual dissection of a single neural rosette [37], although it remains challenging to reliably maintain the single-ventricular structure over the long-term.

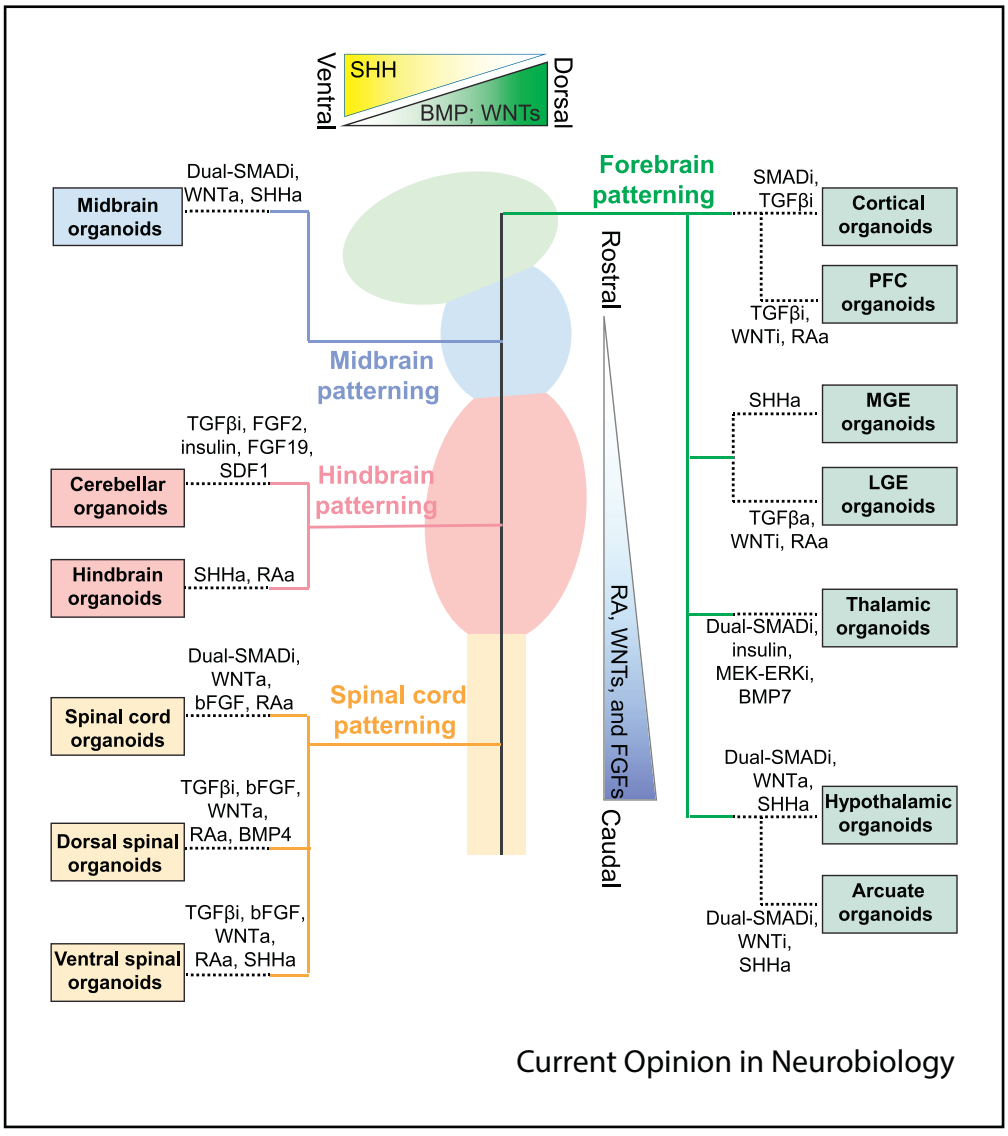
The ventral forebrain comprises the medial (MGE) and lateral (LGE) ganglionic eminence, which is patterned by high SHH and low WNT activity, respectively. Several protocols generated MGE organoids by activating the SHH pathway [6,34,38,39]. RNA-sequencing and immunostaining identified the presence of MGE-specific neural progenitors and diverse interneurons. These organoids were fused with cortical organoids to model interneuron migration, cross–region interactions, and brain disorders, such as Timothy syndrome. The striatum mainly originates from the LGE. Striatal organoids were patterned with TGFβ activation (Activin A), WNT inhibition (IWP-2), and RA activation (SR11237), leading to expression of LGE-specific and striatal medium spiny neuron markers [40]. Fused cortico-striatal assembloids exhibited unidirectional synaptic connections from excitatory cortical neurons to striatal GABAergic medium spiny neurons and were used to model Phelan-McDermid syndrome.

Thalamus and hypothalamus are developed from caudal and rostral diencephalon, respectively. Thalamic organoids were generated using dual-SMAD inhibitors and insulin for early caudal neural fate induction, then a MEK-ERK inhibitor (PD0325901) for antagonizing excessive caudalization and BMP7 for thalamic fate patterning [41]. Thalamic identities, including specific neural and progenitor cell populations, were validated by scRNA-sequencing. Furthermore, fused thalamic-cortical organoids exhibited reciprocal axonal projections. Hypothalamic organoids were generated by exposing hiPSC-derived neuroectoderm to high SHH and WNT signaling [4], leading to expression of hypothalamic neural progenitor and peptidergic neuronal markers.

Midbrain and hindbrain organoids

The substantia nigra, a dopaminergic neuron-enriched region in the midbrain, plays key roles in Parkinson's

Figure 2



Patterning of brain region-specific organoids. The *ex vivo* brain organoids are patterned following similar differentiation programs of distinct brain regions as *in vivo*. The developing neural tube is generally patterned into rostral-caudal regions by low to high gradients of RA, WNTs, and FGFs, and into dorsal-ventral regions by the opposite gradients of BMP/WNTs and SHH. Shown are examples of patterning of different brain region specific organoids by manipulating specific signaling pathways. SMADi: SMAD inhibitors including BMPi or TGFβi; BMPi: BMP inhibitors including Noggin, LDN-193189, or Dorsomorphin; TGFβi: TGFβ inhibitors including SB-431542 or A-83; WNTi: WNT inhibitors including IWR-1, IWP-2, or XAV-939; Notchi: Notch inhibitor DAPT; MEK-ERKi: MEK-ERK inhibitor PD0325901; WNTa: WNT activators including WNT3A or CHIR 99021; SHHa: SHH activators including recombined SHH, purmorphamine, or SAG; TGFβa: TGFβ activator Activin A; RAa: RA activators including RA, SR11237 or Vitamin A.

disease. At least seven midbrain organoid protocols were developed by six research groups [4,42–47] (Figure 2). Neuroectodermal EBs were first induced by dual-SMAD inhibition together with WNT activation. Some protocols embed EBs in Matrigel to promote tissue growth and structural organization [43–45,47]. To pattern EBs toward the midbrain floor plate, SHH activation and FGF8 treatment were commonly used [4,42,43,47], although some protocols showed that FGF8 is not essential [44–46]. They all reported the presence of dopaminergic neurons with TH or DAT markers and dopamine synthesis, and some with dopamine receptors. Further refinement of patterning methods will hopefully model other midbrain regions, such as the ventral tegmental area.

The hindbrain comprises the medulla, pons, and cerebellum. Cerebellar organoids have been generated using a TGFβ inhibitor, FGF2 and insulin for early cerebellar neuroepithelium induction [48] (Figure 2). Sequential

addition of FGF19 and SDF1 produces a continuous polarized neural-tube-like structure that can be further developed into laminated cerebellar cytoarchitectures with rhombic lip-like zones. Cerebellar precursors for Purkinje cells, Golgi cells, granule cells, and DCN projection neurons were validated by immunostaining. However, it remains challenging to establish long-term cultures to generate well-organized neural networks with lobular morphogenesis. Some other hindbrain organoids were recently generated by activation of SHH and RA without pre-SMAD inhibition [49]. Immunostaining and qPCR identified several hindbrain markers (GBX2, 5-HT, CHAT and HB9) in a tubular structure at day 30. Functionality was demonstrated by increased 5-HT synthesis and TPH2 expression in response to specific metabolites of gut microbiota at day 85. However, maintenance of the structural organization and reproducibility of organoids for long-term cultures remain challenging, since variability of the 5-HT neuron proportion increased, and the tubular structure was missing at day 60. These organoid methods recapitulate some features of early-stage hindbrain and can be used for modeling diseases, such as Dandy–Walker syndrome. Further efforts are needed to generate well-organized tissues during long-term culturing as well as to model some other hindbrain regions, such as medulla and pons.

Spinal cord organoids

The spinal cord bridges the brain and body and is essential for sensory input and motor output. Its dorsal and ventral neural development is guided by high BMP/WNT and SHH signaling, respectively, while the rostro–caudal fate is patterned by high to low RA gradients. The Takahashi group [50] used TGFβ inhibition combined with bFGF and WNT activation, followed by continuous RA and BMP4 treatment at the late stage to generate dorsalized spinal cord-like organoids with enriched dorsal spinal progenitors and four types of dorsal spinal cord interneurons (Figure 2). They further generated intermediate and ventral spinal organoids by removing BMP4 and adding low or high concentrations of an SHH agonist, respectively. Another study further demonstrated that the dosage, timing and duration of BMP4 treatment modulate cell types and organization in dorsal spinal cord organoids [51]. However, these methods did not produce morphologically or functionally mature neurons. A recent study generated spinal cord organoids by guiding hiPSCs to a caudal fate with dual-SAMD inhibitors and a WNT activator in 2D, making EBs with bFGF, and then replacing bFGF with RA for neural differentiation and maturation [52]. These organoids recapitulated many aspects of spinal cord development, and produced neurons exhibiting mature markers, dendritic spines, and spontaneous and evoked neural activity with short-term plasticity.

Together, tremendous progress has been made in generating various brain region-specific organoids and we expect many more to come in the near future. Most approaches focus on generating organoids with desired cell types and diversity, which need to be better benchmarked with endogenous human cell types not only at the transcriptome level, but also epitranscriptomic [53] and epigenetic levels [54]. More attention also needs to be devoted to proper cytoarchitectural organization and long-term maintenance.

Patterning of organoids modeling subregions of the brain

Many brain subregions play critical roles in specific functions and disorders and organoids with subregion specificity can lead to a better understanding of their development, functions and pathologies. The Ming group showed the first example of organoids (ARCO) modelling the human arcuate nucleus (hARC) of the hypothalamus [55], which is essential for transmitting signals of hunger. The protocol uses dual-SMAD inhibition followed by extended WNT inhibition and triple SHH activation (SHH, SAG, and purmorphamine) (Figure 2). Immunostaining showed hypothalamic progenitor markers at day 15 and hARC markers, including OTP, DLX, TBX3, and POMC, at day 40. scRNA-seq analysis aided by machine learning showed highly similar signatures between the native neonatal hARC and hiPSC-derived ARCOs. Furthermore, Prader–Willi syndrome patient-derived hARC organoids exhibited molecular, cellular and functional deficiencies.

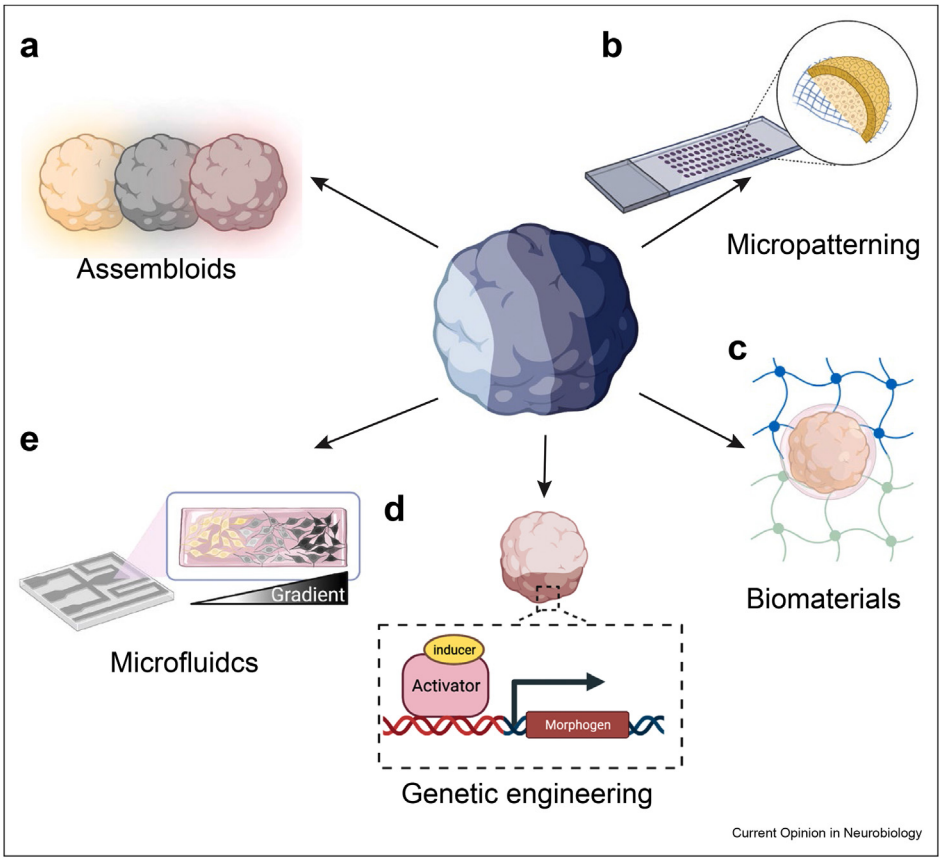
Two groups recently reported that upregulated RA signaling is related to mid-fetal stage human prefrontal cortex (PFC) development [56,57]. Aided by this knowledge, the Nowakowski group generated cortical organoids based on a previous protocol [34] and then added vitamin A starting from day 35. scRNA-sequencing identified a higher proportion of PFC-like excitatory neurons in RA-treated organoids, which was confirmed by histology of SATB2, CTIP2 and AUTS2 co-expression [56]. However, whether RA treatment could also produce cells of other brain regions, such as striatal neurons as reported in another study [37], has not been examined.

Generation of brain subregion-specific organoids is the next frontier for brain organoid technology and overcoming limitations of insufficient knowledge on developmental mechanisms for promoting subregion identities in humans will be essential for future progress [56,57].

Engineering multi-region CNS organoids

Another frontier of brain organoid technology is to generate organoids modeling multiple CNS regions together. Current methods include unguided cerebral

Figure 3



Engineering of multi-region organoids. (**a**) Region-specific organoids can be combined to form assembloids, which can exhibit interneuron migration as well as functional long-range neural connections. (**b**) Micropatterning on glass slides results in geometric restriction of hiPSC colony growth, which can result in the formation of complex 3D tissues when combined with media containing matrigel or suspension culture. (**c**) By controlling the mechanical properties of the cellular environment, biomaterials can regulate the structural development of organoids. (**d**) Genetic engineering of cells offers a means of creating synthetic organizers within organoids that direct cell differentiation through the release of different morphogens. (**e**) Microfluidic devices can deliver spatial gradients of cellular morphogens or small molecules, thereby regionally patterning cells toward different fates.

organoids [26] and assembloids from fusion of individually patterned organoids of different CNS regions [30] (Figure 3a). However, the unguided nature of cerebral organoid generation leads to significant variability, whereas the lack of smooth continuums and structural intermediaries in assembloids can potentially yield inconsistent or spurious neural connections between different regions. To address these shortcomings, engineering solutions leveraging recent work in the fields of biomaterials, microfluidics and synthetic biology are increasingly being utilized [58–60].

Bioengineering approaches are uniquely suited for mimicking the complex array of biophysical cues [61,62] and morphogens [63,64] involved in regulating the developing CNS. A recent study used a combination of glass micropatterning of hiPSC cultures to spatially confine their growth with a 4% Matrigel environment to enable the formation of 3D cellular structures (Figure 3b). By adding BMP4 to the media, Karzbrun et al. [36] were able to induce folding of the basal tissue into a 3D structure reminiscent of the neural tube, containing $PAX6^{+}$ neural tissue organized around a single lumen and surrounded by an outer layer of ectodermal tissue. This microengineered system could generate a variety of tissue types, including 3D structures containing a dorsal-ventral (D-V) axis. In another approach, D-V patterning can emerge in a subset of both murine [65] and human [66] neural-tube organoids by precisely tuning the stiffness of hydrogel matrices for encapsulation (Figure 3c). Furthermore, Cederquist et al. [67] demonstrated a genetic engineering approach for D-V patterning by using small aggregates of stem cells with inducible SHH expression positioned at a single pole of their forebrain organoids to create a spatial gradient of SHH signaling, allowing ventral forebrain fates to emerge close to the induced signaling center and more dorsal forebrain regions able to develop toward to

the opposite pole [67] (Figure 3d). Finally, microfluidic devices can be used for D-V patterning by delivering opposing spatial gradients of SHH and BMP4/7 for mouse ESCs embedded in Matrigel/Geltrex [68], akin to what occurs *in vivo* (Figure 3e).

Microfluidic devices have also been developed to recapitulate patterning of the neural tube and spinal cord along the anterior-posterior (A-P) axis (Figure 3e). Rifes et al. [69] developed a device that exposes a 2D layer of hESCs to a linear gradient of glycogen synthase kinase 3 inhibitor (GSK3i) to pattern an A-P axis [69]. This cellular monolayer expands to become around 100 μm in thickness over 2 weeks as cells differentiate into continuously distributed forebrain, midbrain or hindbrain progenitors as a function of their position along the GSK3i gradient [69]. Critically, the tissue contains an isthmic organizer between the midbrain and hindbrain boundaries, a group of cells that produce soluble factors that further instruct regional brain development in the neural tube [69–72]. The lack of isthmic organizers in single-cell transcriptomic data from cerebral organoids [69] highlights the strength of their microfluidic system to deterministically and spatially pattern cells along a smooth continuum. An analogous microfluidic gradient approach also successfully patterned hiPSCs along the A-P axis of the spinal cord to generate diverse types of motor neurons [73].

Moving forward, one challenge for these engineering approaches is to extend the time over which they can be applied, as brain organoid development often occurs over the course of many months. Seo et al. [74] combined initial micropatterning of stem cell cultures with subsequent suspension culture in order to form elongated spinal cord organoids with D-V-like features. In addition, methods for dynamically altering the composition or release of soluble factors from biomaterials [59] as well as synthetic biology strategies for creating improved signaling centers via gene circuits that autonomously regulate cellular aggregation [75] could prove to be invaluable for long-term organoid culture. These and future methods could potentially yield forebrain organoids containing both prefrontal and motor cortex regions, whole-brain organoids with forebrain, midbrain and hindbrain, and hypothalamic organoids with various nuclei.

Limitations and future directions

The last decade has witnessed the tremendous progress of brain organoid technologies. Starting from hESCs or hiPSCs, researchers could now model the development or disorders of diverse brain regions and subregions. Many key features of the developmental human brain, including the molecular signatures, cellular composition and functions, structural organization, and cross–region interaction have been recapitulated by organoids. Despite significant advances, it should be emphasized that organoid technology is still an emerging discipline and has various limitations. For example, lack of arealization and limited tissue organization hampered the study of cross–region interactions and functions in organoids. High variability complicates preclinical studies and interpretation of phenotypes. Besides, organoids only model a limited time window of early development stages and produce limited cell types and atypical physiology [76,77]. All these features have compromised certain applications of brain organoids.

With a clear understanding of these limitations, improvements are needed to fully realize the potential of brain organoid technologies. On a micro scale, orchestrating types, timing and gradients of morphogens can help generate more specific subregion organoids. On a macro scale, brain organoids with appropriately organized multiple regions and cell populations can be created by controlling spatial gradients of patterning factors to model cross–region interactions and network genesis. Most organoid protocols were unable to maintain well-organized 3D architectures over the long-term. This could also be improved via maintaining proper and dynamic gradients of morphogens and proper extracellular matrix during long-term cultivation. The reproducibility of organoids in regards to the size, structures, and cellular composition remains challenging. Standardizing controllable culture conditions, like starting cell number, organoid density, and media change frequency, will help to reduce batch-to-batch variabilities. Cultivation with defined hydrogel or ECM-free approaches could also reduce the variability caused by batch effects of the Matrigel. Robust protocols require validation with multiple cell lines and batches across laboratories. Current brain organoids mostly lack several cell types of the developing brain, such as microglia and endothelial cells, which can be reconstructed by co-culture. Lastly, current brain organoids mainly recapitulate early embryonic stages and future developments are needed to extend this model into later developmental stages with formation of functional neuronal circuits and columns as seen in the human brain. Within the next few years, we will have an increased array of brain organoids for the field to investigate basic biology of human brain development, to model various developmental brain disorders, and to test therapeutic treatments. Along with such rapid advances, ethical issues need to be considered and frequently updated [78].

Conflict of interest statement

Nothing declared.

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using artwork adapted from the template "CREB Signaling Pathway", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates. The research in the authors' laboratories were supported by grants from the National Institutes of Health (R35NS116843 to H.S, and R35NS097370, RF1MH123979, R01MH125528 and U19AI131130 to G-l.M.) and the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation (to G-l.M.)

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- •• of outstanding interest

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This study used human cortical, hippocampus, hypothalamus, midbrain, and choroid plexus organoids to investigate the neurotropism of SARS-CoV-2 infection. The study revealed a selective SARS-CoV-2 tropism for choroid plexus epithelial cells and showed that SARS-CoV-2 infection leads to increased cell death and transcriptional dysregulation. Together with #14, this highlights the application of human brain organoids in responding to a global pandemic.

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Similar as #11, this study used human cerebral organoids and choroid plexus organoids to test SARS-CoV-2 neurotropism. The study showed that SARS-CoV-2 receptor ACE2 is enriched in mature choroid plexus cells rather than in neurons. SARS-CoV-2 productively infects choroid plexus cells and damages the choroid plexus epithelium.

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