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Brain organoids as models to study human neocortex development and evolution

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Since their recent development, organoids that emulate human brain tissue have allowed *in vitro* neural development studies to go beyond the limits of monolayer culture systems, such as neural rosettes. We present here a review of organoid studies that focuses on cortical wall development, starting with a technical comparison between pre-patterning and self-patterning brain organoid protocols. We then follow neocortex development in space and time and list those aspects where organoids have succeeded in emulating *in vivo* development, as well as those aspects that continue to be pending tasks. Finally, we present a summary of medical and evolutionary insight made possible by organoid technology.

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Introduction

Brain organoids (see Box 1 definitions) have quickly established themselves as invaluable tools for studying mammalian brain development and disease, particularly for species where primary embryonic cerebral tissue is rare and fraught with ethical concerns, as is the case for primates, notably humans. The formation of brain organoids generally relies on principles explained by the neural-default model, where pluripotent stem cells cultured with minimal extrinsic factors preferably undergo neural differentiation, particularly that of the forebrain [1].

In less than two decades, *in vitro* models of human cortical development have led to exciting advances [2,3]. The field effectively began with the generation of two-dimensional (2D) systems, for example, neural rosettes [4], followed by serum-free floating culture of embryoid body-like aggregates (SFEB) [5] and efficient neural rosette generation by dual SMAD inhibition [6]. Progenitors and neurons were then generated in spatiotemporal patterns reminiscent of *in vivo* tissue, starting with the '2.5D' SFEBq culture [7], and culminating with the establishment of more complex and fully free-floating 3D systems five years ago [8,9]. Various protocols have since optimized the established methods, both for cerebral [10••,11••] and forebrain organoids (e.g. [12••,13,14•,15,16••]). Organoids have achieved impressive success in recapitulating key features of human brain development, yet significant differences remain between the developing primary cerebral and the organoid tissue, which set limits for biological and medical applications.

The developing cortical wall (DCW) has become the most widely studied tissue in brain organoids. In this review, we first present a technical comparison of the various brain organoid protocols to understand their different outcomes. We then dissect the spatiotemporal features of cortical development and list those aspects emulated in brain organoids, emphasizing those studies where specific features were first described, as well as the main pending tasks. Finally, we look at new insight into the pathophysiology and evolution of human cerebral cortex development made possible by organoids.

Two major classes of brain organoid protocols

Current brain organoid protocols fall into two major classes, (i) self-patterning (un-directed, entire cerebrum, e.g. [8]) which rely on intrinsic self-organization capabilities, and (ii) pre-patterning (directed, region-specific, e.g. [9]), which drive embryoid bodies (EBs) toward a certain identity using small molecules (Figure 1A, Table 1).

Protocols for self-patterned cerebral organoids

In self-patterning, EBs are embedded in extracellular matrix (ECM, e.g. Matrigel), which supports neuroepithelium self-organization [8,10••,11••,17] (Figure 1A). The first such protocol was reported in 2013 by Lancaster and colleagues [8] and, by not adding instructive signals, the organoids exhibit characteristics of various cerebral

Box 1 Definitions

Organoids are defined as discrete multicellular assemblies that can emulate the main 3D architecture and cell-type composition of a tissue or organ, for at least a defined developmental period. The collective term '*brain organoids*' refers to all organoids that emulate a part or parts of the brain (cerebrum and cerebellum). Organoids that emulate a majority of cerebrum tissue are referred to as '*cerebral organoids*'. Regional organoids that emulate a specific brain tissue are referred to by the tissue name, such as '*forebrain*' organoids. Distinct regional organoids may also be fused, for example dorsal telencephalic organoids together with ventral telencephalic organoids form telencephalon organoids.

A further distinction comes from the protocol used. Brain organoids that develop autonomously, that is, without addition of external signalling molecules, are referred to as '*self-patterned*'. Brain organoids with a development directed by addition of external signalling molecules are referred to as '*pre-patterned*'.

regions [8]. These organoids recapitulate many aspects of both the morphology [8,17] and gene expression [18] of the early developing human neocortex, such as the ventricular zone (VZ). Limitations include notorious cell death at later stages [8,11••,17], which impairs long-term developmental studies. Also, certain progenitor cells show low abundance, and the cytoarchitecture of the basal zones and layers is incomplete (see below) [8,17].

Optimized protocols have therefore used different strategies to overcome such limitations. First, synthetic microfilaments used as scaffolds (Figure 1C), together with a short pulse of a canonical Wnt pathway activator generated more forebrain neuroepithelium and larger ventricle-like structures, while reducing mid- and hindbrain regions [10••]. Furthermore, Matrigel added to the culture medium lead to better maintenance of basal lamina components and to a more faithful cortical plate (CP) cytoarchitecture [10••]. Second, the Arlotta lab [11••], combined (i) fewer starting cells for EB formation, (ii) optimized neural induction and (iii) addition of BDNF to the final differentiation medium, achieving reduced cell death and improved long-term organoid development.

Protocols for pre-patterned brain organoids

In contrast to self-patterning protocols, pre-patterning protocols use small molecules to drive the organoids to a certain regional specificity (Figure 1A). The Sasai lab was the first to obtain pre-patterned 3D-like forebrain organoids, by combining the SFEB system with free-floating and coated-dish culture (SFEBq) [7]. A decisive step forward was published in 2013 [9], using only free-floating SFEBq culture and adding Wnt and SMAD inhibition to induce forebrain identity in the EBs. In contrast to self-patterning cerebral organoid protocols, these EBs are not embedded in Matrigel (Figure 1A), but Matrigel is added to the culture medium. Similar to the original Lancaster cerebral organoids, the Kadoshima forebrain organoids recapitulate many aspects of the early developing human neocortex [9] and show similar shortcomings [8,9,10••].

These limitations also encouraged the improved protocols, most of which maintain SMAD inhibition [12••,13,14•,15] and lack Matrigel embedding [13,14•,15,16••] (Figure 1A). Two of them deserve special comment. First, in contrast to the other pre-patterning protocols, Qian and colleagues [12••,19] include a step of Matrigel embedding after dual SMAD inhibition. This supported neuroepithelium induction by canonical Wnt pathway activation and further SMAD inhibition. After neuroepithelium formation, the embedding Matrigel is removed and only at later stages added to the culture medium. These steps resulted in a more discrete oSVZ-like zone, exhibiting a greater relative abundance of basal radial glia (bRG), and in more distinguishable layer-like neuron arrangements in the CP. Also, by using miniaturized spinning bioreactors, a more reproducible and scaled-up organoid production was achieved [12••,19]. Second, Watanabe and colleagues [16••], removed the SMAD inhibition and Wnt signalling modulation, but added LIF at later stages. This increased the abundance of bRG, promoted basal lamina formation, improved an upper layer-like neuron arrangement in the CP, and promoted astrocyte production [16••].

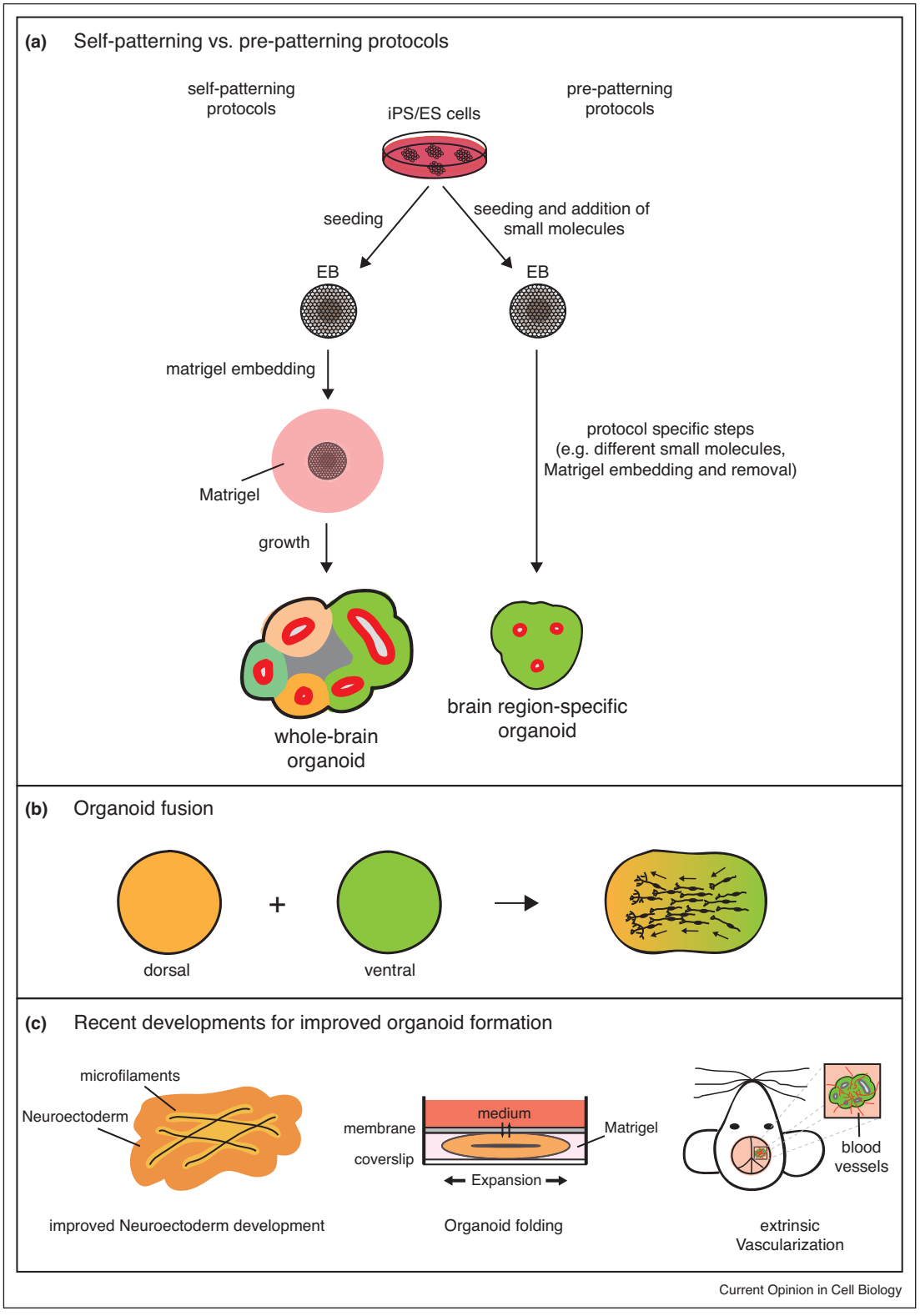
Four recent protocols have also aimed at further directing forebrain identity, that is, dorsal vs. ventral telencephalon. Organoids are directed toward a ventral identity by activation of the Shh pathway [14•,15,16••,20•], as first described in 2011 by Danjo and colleagues [21]. Of note, the protocol by Bagley and colleagues involves Matrigel embedding, as in the original self-patterning Lancaster protocol, but uses small molecules to either inhibit Wnt signaling and activate the Shh pathway to induce ventral identity, or inhibit the Shh pathway to induce dorsal identity [20•]. All four protocols resulted in very pure forebrain organoids of either dorsal or ventral identity [14•,15,16••,20•].

A major achievement using these specific forebrain organoids is the fusion of two organoids with different identity (Figure 1B). Fused organoids of dorsal and ventral telencephalic identity have been generated either by incubating them in the same tube [14•] or tissue culture well [15], or by embedding the differently pre-patterned EBs in the same Matrigel droplet [20•]. This allows the study of tissue interactions, for example, the migration of interneurons from the ventral to the dorsal forebrain organoid.

Cerebral cortex development in organoids vs. *in vivo* – achievements vs. pending tasks

We now discuss which aspects of human cortical development have been emulated, and which differences remain between brain organoid and *in vivo* neocortical development. We also outline what in our opinion are the

Figure 1



Overview of current brain organoid developments. **(a)** Diagram depicting the generation of brain organoids in self-patterning vs. pre-patterning protocols. **(b)** Diagram depicting the fusion of brain region-specific organoids (in this example dorsal and ventral forebrain) to study interactions between brain regions (e.g. migration of interneurons). **(c)** Cartoon depicting recent developments for improved organoid formation, that is, microfilaments for improved neuroectoderm formation, microfabricated chip for organoid folding and transplantation into the mouse brain for extrinsic vascularization.

Table 1

Comparison of the two major classes of brain organoid protocols.

	Self-patterned organoids	Pre-patterned organoids
Generation	Self-organization in Matrigel	Directed development by addition of small molecules
Specificity	Various brain regions	Brain (sub)region-specific
Advantages	Highly complex, different identities in one organoid	Highly pure organoids of one identity, reproducibility
Disadvantages	Low reproducibility, batch-to-batch variability	Interactions between brain regions only possible by fusion
Usage	E.g. model of microcephaly [[11••]], model of Zika virus infection [44,45•,48], analysis of neuronal networks [11••], interspecies (human vs. chimpanzee vs. orangutan) comparison of brain development [28••], study of physics of brain folding [41].	E.g. model of microcephaly [50], model of Zika virus infection [12••,16••,46,47,49], model of Miller-Dieker syndrome [51,52], interspecies (human vs. chimpanzee vs. macaque) comparison of brain development [31•], study of interactions between brain regions [14•,15,20•], study of autism spectrum disorder [66].

main pending tasks to improve organoid modelling of human corticogenesis. First, we compare the origin of the cortical stem cells and general aspects of cortex formation in organoids vs. *in vivo*. Then, to the extent to which we understand corticogenesis [22–27], we dissect the features of brain organoids relative to fetal human cerebral neocortex development in a systematic apical-to-basal manner.

Stem cell origin

The cells of origin and their developmental timeline are a first difference between primary brain tissue and organoids. Embryonic stem cell (ESC) lines have been the canonical source for generating brain organoids [7–9]. Despite their success, it remains risky to assume that cultured ESCs are equivalent to ESCs *in vivo*. Even more different are induced pluripotent stem cells (iPSCs), which are of somatic origin. Nevertheless, broad success has been achieved using iPSCs, especially for medical insight using patient-derived cell lines (see below).

In the developing embryo, neuroectodermal cells generate neuroepithelial cells, the primary neural stem cells that generate – directly or indirectly – all other progenitor cells, which in turn produce all neurons and glial cells of the developing brain. These events are recapitulated to some extent during brain organoid development, albeit usually in weeks rather than the months necessary *in vivo*. Despite being an intrinsic difference, this shorter duration of organoid development does save experimental time. In any case, this *in vitro* environment and accelerated time frame, as well as the differences listed below, should all be kept in mind when interpreting organoid studies. Ideally also, results from organoids should be directly validated against primary tissue, and with data from several independent cell lines, to discriminate between line-specific and species-specific features [8,18,28••].

Tissue formation

In embryos, ventricle formation starts by neurulation, when the neural plate folds upon itself to form the neural tube that fills with cerebrospinal fluid (CSF). The rostral side of the neural tube is where the cerebral ventricles form. In brain organoids, luminal structures do not seem to arise by neurulation. They either self-organize into hollow neuroectodermal spheres [5], or arise directly as 3D rosettes, likely by apicobasal polarity establishment, apical constriction and lumen formation [4,5,7,29,30]. Sheets of neuroepithelial-like tissue may also forgo lumen formation and instead expand laterally with inward-curving sides [3,7–9]. In some cases of sustained growth, a side of predominant dorsocaudal identity can join a distal static side without this identity. Although no neural tube-like structure is formed, this seems to create a lumen with a dorsoventral arrangement reminiscent of aspects of the cerebral ventricles [9]. Reproducibility in the size, number and 3D arrangement of the ventricle-like structures remains also an issue, despite numerous recent advances [10••,11••,12••].

The neuroepithelium

The neuroepithelium is the foundational tissue of the central nervous system. Many features of a neuroepithelium-like 3D tissue have been successfully generated *in vitro* since the first forebrain organoid study [7].

I) Cell identity of organoid neuroectoderm-like tissue was established by immunohistofluorescence (IF) for Sox1, and a subsequent cortical neuroepithelium-like identity was demonstrated by IF for N-cadherin, FoxG1/Bf1, Pax6, Sox2 and Six3 [7]. Single-cell RNA sequencing (scRNA-seq) and/or IF also corroborated the identity of apical progenitors (APs, the collective term for neuroepithelial cells and apical radial glia (aRG)) in human [11••,18] and chimpanzee [28••,31•] cerebral organoids. Furthermore, the epigenetic landscape of human cerebral organoid cells was shown to resemble that of the fetal cortex [32].

II) Pseudostratification has been shown, demonstrating a single true layer of neuroepithelial cells that elongate apicobasally and undergo interkinetic nuclear migration [7].

III) Apical cell polarity has been shown by the presence of apical tight and adherens junctions, apical primary cilia, and apical markers at or near the organoid ventricular surface such, as prominin-1 and aPKC, and mostly symmetric proliferative divisions at the apical ventricular surface [7].

IV) Lumina filled with fluid are present in brain organoids as one of their key defining architectural characteristics [7,8,9]. Nevertheless, it is unknown how similar the composition of this fluid is to physiological CSF.

V) A basal lamina containing laminin at the organoid outer border has been documented [7]. Improvements in basal lamina composition and persistence have been achieved when organoids were grown with Matrigel, as embedding [8] or as culture medium component [9]. Mesenchymal cells that secrete ECM components, such as type IV collagen, have also been found by scRNA-seq and electron microscopy of human cerebral organoids [18].

Pending task I). The meninges and most of their molecular and structural input remain absent from brain organoids. Protocols partially compensate by including some factors they secrete, like retinoic acid [7,8].

The ventricular zone

With the onset of neurogenesis, several distinct zones are formed in the DCW. Many of the original neuroepithelium features such as pseudostratification, apical cell polarity and luminal contact are maintained in the VZ.

I) Cell identity of dorsal cortex in brain organoid APs has been established by IF for EMX1 [7]. Regional areas have also been identified, for example, by AUTS2 (prefrontal) and TSHZ2 (occipital) [8]. In addition, scRNA-seq has contributed to document AP identity in primate cerebral organoids [11••,18,28••].It remains unclear to what extent organoid APs replicate the switch from neuroepithelial cells to aRG, a hallmark of the *in vivo* VZ [33,34].

II) Symmetric vs. asymmetric cell division. As is the case *in vivo*, most AP divisions at early stages of human brain organoid development occur with a cleavage perpendicular to the apical surface [7]. Non-vertical divisions that generate more basal progenitors (BPs) tend to increase at later stages [8,9], and Shh has been shown to promote this, both for basal intermediate progenitors (bIPs) and bRG [35].

III) BPs are first generated in the VZ from APs. Pax6– & $Tbr2^+$ BPs, that is, bIPs, have been observed in early forebrain organoid models [7]. Other BPs that, like APs, remain $Pax6^+$ & $Sox2^+$, but lose apical attachment, that is, bRG, were found in more advanced models with improved cortical wall development [[28••],9]. Also, organoid BP characterization has been complemented by scRNA-seq in human [11••,18] and chimpanzee [28••] cerebral organoids.

Pending task I) Endogenous blood vessel development has not been achieved in brain organoids. Blood vessels provide ECM via their basal lamina, and become necessary in all DCW zones when simple nutrient and oxygen diffusion can no longer support corticogenesis, leading to extensive cell death [8]. Brain organoids typically cannot generate the non-ectodermal blood vessels, although spurious endothelial differentiation has been detected, likely from the original ESCs/iPSCs [18]. Incorporating vasculature precursors into developing organoids may become a viable alternative, but even if vessels can form, a functional blood flow remains a formidable task, even for microfluidics technology.

Alternatively, **exogenous vascularisation** has recently been achieved by grafting brain organoids into a specific site of the live adult mouse brain. Importantly, this allowed the development of germinal zones and active neurons with less cell death than in previous non-vascularized organoids [36••]. Beyond what can be learned from organoid-host brain integration, grafting experiments may lead to insights on how to achieve a functional endogenous vascularisation.

Pending task II) Microglia are another non-neural cell type not yet achieved in brain organoids. This will be important to further approach physiological brain development, especially for late corticogenesis events, such as synapse pruning. Microglial precursors generated from iPSCs [37,38] are also candidates to be included in future brain organoid protocols.

The subventricular zone

In the DCW, the zones basal to the VZ form by accumulation of cells, notably BPs and neurons. This eventually gives rise to the 6-layered mammalian neocortex [23,24,25].

I) BP accumulation in the SVZ, of both **bIPs** [7] and **bRG**, has been shown [8,9] and improved [10••,11••,12••,16••] in human brain organoids. However, the BP numbers and proportions still do not match the *in vivo* situation.

Pending task I) The SVZ subdivision, into an inner and outer SVZ separated by an inner fibre layer, typical of many mammals with large brains including primates [24,39], has not been replicated in brain organoids. Nevertheless, the SVZ in human organoids has been shown to be larger and with more cells than in rodent organoids [8,9]. Improved protocols are having increasing success in emulating the physiological SVZ [10••,11••,12••,13,16••].

The cortical plate

During cortical neurogenesis *in vivo*, Cajal-Retzius cells are the first to accumulate in the marginal zone. They express and secrete reelin, thereby helping to guide the formation of the CP and its characteristic layering

('inside-out', where upper-layer neurons migrate past the firstly generated deep-layer neurons [25]).

I) A spatiotemporal sequence of neurogenesis reminiscent of that *in vivo* has been shown in human brain organoids by IF, including an early basal-most presence of reelin-positive cells and some tendency of upper-layer neurons to appear later and more basally than deep-layer neurons [[11••],9]. Aspects of this temporal sequence have also been described by scRNA-seq [11••].

II) Most major types of glial cells and neurons from the different layers have been documented in organoids, thanks to ongoing protocol improvements [7,8,9,11••,12••,13,40].

III) An increasing scope of neuronal morphology, activity and connectivity has been achieved [7,[10••],9,10••,11••,16••,36••], reflecting improvements in organoid architecture and longevity. Nevertheless, much work remains to approach the full scope of neuronal characteristics [25]. Migration of inhibitory interneurons may be improved by combining the growth of region-specific organoids (see above), on scaffolds [10••] and in static culture [41], to simplify the delivery of combinations of spatiotemporal morphogenic cues.

Pending task I) The canonical six-layer CP architecture remains largely missing from human brain organoids, despite ongoing progress in SVZ and CP development [[11••],9,11••,12••,13,16••,36••]. Interestingly, this is also true for mouse organoids. Mice have a much thinner DCW *in vivo*, suggesting that a layered architecture is not more easily generated in smaller brains. Brain size *per se* may not be a main issue. Also, extending human cerebral organoids culture without high cell death improved neuron and glia diversity and function, but not layering, suggesting that time *per se* is also not a main issue [11••].

Pending task II) Gyrification, the folding of the outer layers and surface of the cerebral cortex *in vivo*, also remains elusive for brain organoids. Recent protocols achieved some folding by genetic [42] and physical [41] manipulations. However, the folding reported involved also [42], or mostly [41], the inner, germinal zones, which is absent *in vivo*. Similar to other complex aspects of corticogenesis, true gyrification, restricted to the outer layers of the cortex, will likely depend on a more physiological expansion of the zones basal to the VZ.

Pending task III) Myelination, a key feature of mature neurons *in vivo* [43], is missing from organoid neurons. Progress may be achieved by favouring oligodendrocyte development, or by adding them, at defined maturation time points.

Brain organoids to study pathophysiological development and evolution of the human cerebral cortex

Pathophysiology. Over the past five years, brain organoids have also helped uncover mechanisms underlying pathophysiological development of human cerebral cortex. A striking application of human brain organoids in pathophysiology studies caused by extrinsic factors is the Zika virus epidemic. Here, organoids served as models to study the tropism and the pathomechanism of Zika infection and to identify potential drug treatments [12••,16••,44,45•,46–49]. Despite their small size and lack of gyrencephaly, human brain organoids also serve as models to study the effects of intrinsic, genetic factors on neuropsychiatric [12••,66] and neurodevelopmental disorders, for example, primary microcephaly [8,50] or Miller-Dieker syndrome (MDS). MDS brain organoid studies suggested that the pathophysiology of MDS lissencephaly is more complex than previously thought [51,52].

Evolution. One avenue to understand human neocortex evolution is to compare human vs. other great apes, and organoids offer this without the need of primary tissue. Symmetric vs. asymmetric cell division could underlie evolutionary differences in brain size across primates, as it is key for the spatiotemporal control of proliferation vs. differentiation in the DCW [23,24,27,53–55]. In this context, detailed spindle orientation variability in live tissue can be used to estimate the asymmetry of AP divisions [56]. Interestingly, despite the ~3-fold larger brain in humans vs. chimpanzees, their cerebral organoid APs did not show differences in spindle orientation. AP mitosis did differ in the duration of metaphase, which was ~50% longer in human than chimpanzee or orangutan [28••]. Also, some differentiating progenitors ($Pax6^+$, $Tbr2^+$) were more abundant in chimpanzee than human cerebral organoids, suggesting an earlier switch from proliferation to differentiation in chimpanzees [28••], consistent with findings in neural rosettes [31•]. In addition, scRNA-seq has revealed interesting differences in gene expression between human and chimpanzee neural cells [28••]. Together, these findings could help explain the larger human brain. Cell cycle and proliferation control via tumor suppressors have also been implicated in human-rodent differences involving organoid BP abundance [42,57].

Furthermore, organoids will likely be useful tools for studying human-specific genomic changes that may have contributed to human neocortex evolution [22,58–61]. Several studies have identified such changes [62–65] and future studies could either remove them from human brain organoids and/or introduce them into non-human primate ones.

Concluding remarks and future directions

Ten years of brain organoid development have yielded impressive progress in modelling human *in vivo*-like 3D cerebral cortex tissue and, despite the distinction between pre-patterning and self-patterning protocols, key improvements have also come from clever combinations of both. Organoids can now recapitulate most hallmarks of the early DCW, especially for its apical-most zone. Most remaining challenges are linked to the structured, radial expansion of the more basal zones. For example, gyrification is not properly recapitulated by current organoid technology. The limits to how far-reaching brain organoid self-organization can be, lie therefore mostly on the basal side. Here, decisive progress is likely to come from strategies that improve the basal-most architecture of the tissue (e.g. meninges). Also important is a more physiological vascularization of the tissue, both for improved oxygen and nutrient delivery, and for improved interaction between progenitor and endothelial cells, potentially providing important signals for an improved brain organoid development. Finally, organoid technology offers novel avenues to study the development and function not only of the human brain, but also potentially of any mammalian species where brain tissue, notably of fetal origin, is hard to obtain.

Conflict of interest statement

Nothing declared.

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