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Brain organoids for the study of human neurobiology at the interface of in vitro and in vivo

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Brain development is an extraordinarily complex process achieved through the spatially and temporally regulated release of key patterning factors. In vitro neurodevelopmental models seek to mimic these processes to recapitulate the steps of tissue fate acquisition and morphogenesis. Classic two-dimensional neural cultures present higher homogeneity but lower complexity compared to the brain. Brain organoids instead have more advanced cell composition, maturation and tissue architecture. They can thus be considered at the interface of in vitro and in vivo neurobiology, and further improvements in organoid techniques are continuing to narrow the gap with in vivo brain development. Here we describe these efforts to recapitulate brain development in neural organoids and focus on their applicability for disease modeling, evolutionary studies and neural network research.

The human brain is one of the most complex organs in the body, making it particularly vulnerable, such that even subtle abnormalities lead to neurological dysfunction. The fetal period is a particularly sensitive time, when defects in neural stem cell proliferation[1,2], neuronal differentiation[1] and synapse formation[1–3] can lead to neurodevelopmental disorders[4]. Despite the growing number of studies on neurodevelopmental disorders, in 80% of cases the etiology is still unknown[1], suggesting there is still much to be learned about human brain development and disease.

Animal models have long been used as valid systems for disease modeling, behavioral paradigms and neuroanatomical studies. Nevertheless, the domestic mouse, the animal model par excellence, presents some striking differences with human in terms of brain development[5]. The mouse brain is much smaller, with just 13.7 million cortical neurons compared with 12 billion in humans, and is lissencephalic, while that of humans is gyrencephalic[6–8]. These macroscopic differences are supported by microscopic differences in cytoarchitecture and cellular behavior. For instance, while radial glial (RG) neural stem cells densely occupy the ventricular zone (VZ) in both species and serve as the first neural progenitor population[9–12], outer radial glial progenitors (oRGs) present in the outer subventricular zone (oSVZ) are significantly more abundant in primates compared to rodents, so much so that a defined and separate oSVZ layer is recognizable in humans[13–16]. Moreover, the proliferative potential of cortical progenitors before terminal differentiation into neurons is greatly expanded in humans compared to rodents, thus supporting the increase in cortical surface and the over-representation of upper cortical layers in primates[17]. As for the molecular signature, gene expression profiles are divergent between human and mouse for genes contributing to cortical development and function[18–20]. These important differences need to be taken into account when studying neurodevelopmental dynamics.

Because studies in humans are ethically and practically challenging, species-specific in vitro models could provide a complementary approach to animal studies for understanding the evolution and complexity of the human brain. In vitro models have seen significant progress in recent years, from the reprogramming of somatic cells to iPSCs[21,22] to organogenesis in a dish, including the generation of neural organoids[23,24] and cortical spheroids (CSs)[25] mimicking brain development in three dimensions. This review will discuss applications of neural organoids with a focus on how they fit in the context of in vitro and in vivo biology.

In vitro guided by in vivo

In vivo human brain development. Human brain development is an extraordinarily complex process, achieved through the meticulous spatial and temporal regulation of gene expression[26]. Brain organogenesis is orchestrated by a fine arrangement of autocrine and paracrine growth factors, mechanical forces and other cell–cell interactions[27,28] (Box 1 and Fig. 1a). Understanding these intricate events in vivo can provide a blueprint for in vitro organogenesis, enabling better models of human biology.

Neurogenesis begins when neuroepithelial (NE) cells, positioned adjacent to the ventricle in the VZ region, gradually shift into RG cells, elongated neural progenitors expressing certain astroglial markers[9,12,28]. The hallmark of neurogenesis is the switch from a symmetric to asymmetric division mode, when a RG cell generates an identical daughter stem cell and one cell committed to a more differentiated progenitor or neuronal fate. Neurogenic progenitors migrate basally from the VZ to the newly formed subventricular zone (SVZ). By the second trimester, the SVZ has become the major proliferative zone in the cortex as the VZ gradually shrinks and then disappears[29]. Two major populations of progenitors have been found in the SVZ: intermediate progenitors (IPs) and oRGs[14,30]. Both progenitors are capable of self-renewing and of generating terminally differentiated neurons.

Immature cortical neurons, generated from the VZ and SVZ, migrate along the radial glial fibers[9–12] to reach a primitive structure called the preplate. The preplate further splits into the upper marginal zone and the lower sub-plate (SP)[31]. Multiple sequential neurogenic waves allocate neurons between the SP and the marginal zone, in the cortical plate (CP), in an inside-out fashion. Neurons generated first migrate to constitute the deep layers of the cortex, whereas neurons generated later occupy the upper layers[32,33]. The final result is a complex cortical architecture where the SP has disappeared, the VZ is replaced by ependyma, and the SVZ is restricted to a thin layer, separated from the CP by the thick white matter. Importantly, neurons of the cortical plate appear organized within six layers, a topology unique to mammals and more elaborate in apes and humans.

Two-dimensional methods. A major breakthrough in in vitro studies was the establishment of embryonic stem (ES) cell culture conditions for both mouse[34,35] and human[36]. Some of the first attempts to generate neural cells from these involved floating aggregates of ES cells called embryoid bodies (EBs). EBs have the potential to generate all three germ-layer derivatives but fail to reproduce the

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Box 1 | Human brain development

The nervous system precursor, the neuroectoderm, is specified during gastrulation, when migration of cells through the primitive streak leads to the formation of a deep endoderm layer, an intermediate layer called mesoderm, and the remaining epiblast, which becomes the ectoderm (Fig. 1a). At this stage, the release of inductive or inhibiting factors is essential for the acquisition of axial polarity and for the maintenance of layer identity and boundaries. Studies in *Xenopus* demonstrated the importance of the TGFβ superfamily, specifically ACTIVIN-A and NODAL, for the induction of mesoderm in animal cap explants[130]. Notably, the role of TGFβ is evolutionarily conserved, as shown in mouse embryos, where *Nodal* expression is required for primitive streak formation[136] and mesendoderm development[127,128]. Observations that the blockade of TGFβ signaling was sufficient to specify neurectoderm fate led to the default theory of neural induction[55,56]. Indeed, neuroepithelia can be obtained intrinsically, in the absence of mesoderm, by inhibition of mesendoderm inducing factors. NOGGIN[137], CHORDIN[138] and FOLLISTATIN[139], produced by the streak node, act as neural inducers in the region of the epiblast where their gradient is the highest (Fig. 1a).

Interestingly, the aforementioned neural inducers were also shown to be potent inhibitors of bone morphogenetic proteins (BMPs)[140–142]. As BMPs promote epidermal identity and block neural fate[61], the combination of NOGGIN, CHORDIN and FOLLISTATIN acts to repress both epidermal and mesendodermal lineages through the parallel inhibition of BMPs and TGFβ. Other non-neural ectoderm inducers, in cooperation with BMPs, are WNT family proteins. The neuroectoderm is protected from WNT signaling through the secretion of WNT antagonists, among which DICKKOPF-1 (DKK1) has a well-established role in mammals[59,129] (Fig. 1a).

Release of growth factors has different and sometimes opposing effects, depending on the time window of embryogenesis. For instance, fibroblast growth factors (FGFs) have been shown to initiate neural induction before gastrulation[143], but also support mesoderm identity in the gastrula, together with WNTs and TGFβ. Indeed, dominant negative mutation of FGF receptor abolishes mesoderm formation in *Xenopus*[131].

After the acquisition of neural fate, the neuroectoderm is rapidly shaped into the neural tube through the sequential thickening of the neural plate, folding of the ridges and, finally, fusion of the two ends to form a hollow tube. Neural tube fusion is completed by day 32 after conception[144], when neural progenitors organize to form a single layer of cells surrounding the central lumen, which will become the ventricles of the brain and spinal cord. Just before the complete closure of the neural tube, the anterior region enlarges to form three brain vesicles, namely the prosencephalon, mesencephalon and rhombencephalon. By embryonic day 49, the prosencephalon (also called the forebrain) further divides into telencephalon and diencephalon, the mesencephalon (midbrain) remains un-split, and the rhombencephalon (hindbrain) forms the metencephalon and myelencephalon[31]. At this stage, gradients of growth factors play a pivotal role in establishing the correct brain regional identity along the rostrocaudal and dorsoventral neural tube axes (Fig. 1a).

The rostral forebrain is specified by the cooperative secretion of SHH from the neural tube floor plate, BMP4 from the roof plate and FGF8 from the anterior neural ridge, also called the rostral patterning center[145]. FGF8 acts as a potent telencephalon inducer[132], but it is also essential in setting up the midbrain–hindbrain boundary in the area called the isthmus[133], in cooperation with WNT1. Studies report that *Wnt1*-mutant mice are depleted of most of the midbrain and cerebellum, validating its role as a mesencephalon and caudal brain inducer[62].

Dorsoventral patterning along the neural tube length is established through the fine regulation of a SHH–BMP gradient, where SHH is the ventralizing factor and BMPs dorsalize. SHH is initially expressed by the notochord and anterior mesoderm, and it induces ventral identity in the neural plate[146]. Shortly after, the ventral neural plate differentiates into floor plate and becomes competent in secreting SHH[66]. Selective mutation of *Shh* in mouse embryos confirms its role in ventral patterning, as shown by depletion of motor neurons in the absence of a functional form of *Shh*[134]. The restriction of SHH signaling to the ventral pole, together with the opposing dorsalizing BMP gradient, has been shown to initiate the differentiation of dorsal cell types[64]. BMP4 and BMP7 are initially produced by the epidermal ectoderm, and subsequently the dorsal roof of the neural tube becomes itself a source of BMP4. Along the rostrocaudal axis, WNTs, FGFs and RA have been reported to contribute to the posteriorization of the neural tube[130]. Suppression of the RA pathway leads to the loss of posterior markers, whereas strengthening the signaling has the effect of caudalizing rostral tissues[135]. Growth factors thus have different effects depending on the time of secretion and on the target tissue, providing a variegated and intricate map of tissue fate induction (Fig. 1a).

organized spatial pattern of organogenesis[37]. In 1995, Bain et al. obtained a significant percentage of cells with neuronal identity from dissociated EBs when the EBs were plated as aggregates in the presence of retinoic acid (RA). Surprisingly, some 'neuron-like' cells were also observed in the non-treated cultures, but exclusively when cells were plated as aggregates and not when plated sparsely[38]. This result supports the notion that neural differentiation is a default mechanism and can be achieved in vitro through endogenous cell-secreted inducing or inhibiting factors whose signal is stronger in cell aggregates.

Interestingly, not only are ES cells intrinsically biased toward the neural lineage, but stem cells cultured in a chemically defined growth factor-free medium have the tendency to differentiate into anterior neuroectoderm and more specifically into forebrain identity[39]. Notably, neurogenesis in vitro recapitulates the multistep process happening in the developing embryo. Indeed, human EBs plated in a chemically defined medium in the presence of FGF2 organize into neural tube-like structures called neural rosettes[40], composed of NE cells with polarized apical and basal proteins[41]. Moreover, radial glia-like cells surrounding the lumen display characteristic inter-kinetic nuclear migration, thereby restricting mitotic divisions to the apical surface. In neural rosettes, RG cells next to the lumen are surrounded by a thin (though misaligned) SVZ, mainly populated by IPs at the basal feet of radial progenitors[41,42] with some oRG cells[41], highlighting remarkable similarities to human neural progenitor populations, but still lacking a more defined spatial organization.

As neural precursors differentiate, glutamatergic, GABAergic and a small percentage of midbrain neurons are generated, followed by astrogliogenesis[40]. Interestingly, neurogenesis timing in vitro matches the cell-intrinsic transcriptional clock observed in vivo. In neural rosettes, Cajal–Retzius cells are the first neuronal population to appear, followed by deep-layer neurons and later by upper-layer neurons[39,42]. Although synaptic contacts and neuronal processes

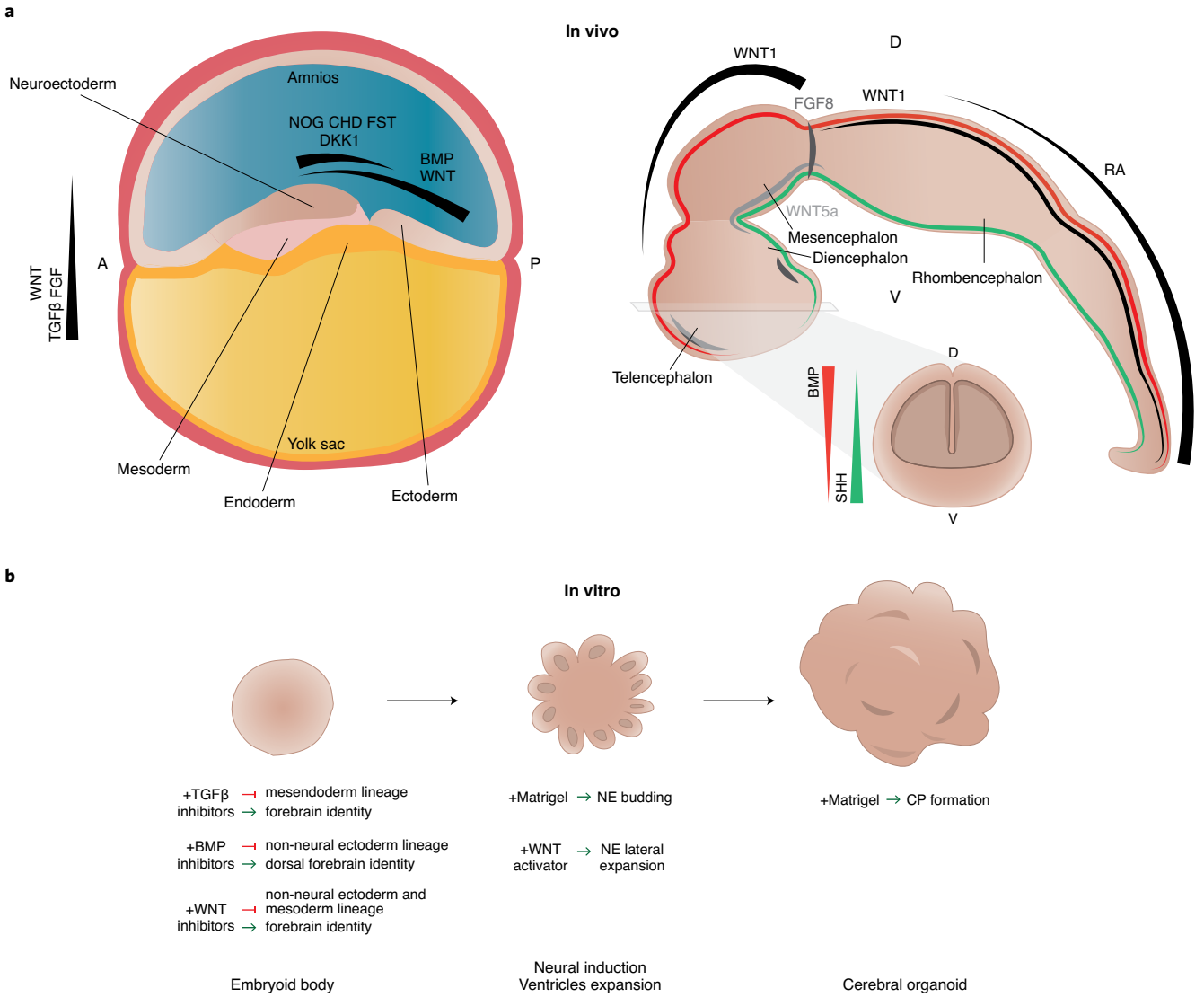

Fig. 1 | Signaling molecules acting during in vivo neurodevelopment and the cerebral organoid protocol. a, Schematic representation of signaling factor gradients in the human gastrula (left) and neural tube at embryonic day 49 (right). **b**, Overview of the main phases of cerebral organoid protocols and list of exogenous molecules added in different protocols and their respective effect. TGFβ inhibitors are used in refs. [1,23,25,50,51,53,54]. BMP inhibitors are used in refs. [1,25,51,53]. WNT inhibitors are used in refs. [1,23,50,51,54]. WNT activator is used in ref. [71]. Matrigel is used in refs. [23,24]. TGFβ, transforming growth factor β; FGF, fibroblast growth factor; NOG, Noggin; CHD, Chordin; FST, Follistatin; DKK1, Dickkopf-1; BMP, bone morphogenetic protein; SHH, Sonic hedgehog; A, anterior; P, posterior; D, dorsal; V, ventral.

are detectable in neural rosettes[39,41], the absence of a higher-order cortex-like architecture limits more complex studies on neuronal activity and circuitry establishment in vitro.

Organoids as a paradigm shift. Modeling brain organogenesis in vitro has continued to advance, first with the optimization of protocols for the neuralization of floating EBs in a serum-free medium, defined as SFEBq (serum-free floating culture of EB-like aggregates)[43,44]. Neural fate was shown to be uniformly acquired on the surface of aggregates, where multiple polarized neuroepithelial structures started appearing[44]. These neural tube-like units differ from neural rosettes by their larger lumen and expanded epithelium with self-determined dorsoventral–rostrocaudal axial polarity.

The next advance was the realization that neural tissues could be maintained as floating aggregates indefinitely without the need to plate them down. The breakthrough came with the introduction of extracellular matrix components in the form of Matrigel. Dissolved Matrigel led to the recreation of the three-dimensional (3D) optic cup, the first example of a fully 3D self-organizing human neural tissue in vitro[45]. Then, a combination of embedding in Matrigel as a polymerized gel, optimized media transitions and agitation of the tissues led to the formation of cerebral organoids with various brain region identities and discrete progenitor zones, including an SVZ with oRGs[24]. Similarly, long-term culture of SFEBq aggregates in suspension resulted in VZ and SVZ layering with oRGs[23] as well as a concrete cortical plate. These advances revealed the remarkable fidelity with which organogenesis can occur in vitro with very limited exogenous direction, leading to accurate modeling of events occurring during the first half of gestation[23,25,46]. Further molecular analyses have revealed the acquisition of cell identity in a timed manner, as well as gene regulatory networks and developmental processes that are preserved in organoids compared to fetal brain samples[46–50].

Table 1 | List of signaling factors involved in brain development and applied to cerebral organoids

Signaling factor	In vivo role	In vitro role
TFG-β	Mesendoderm induction[127,128]	TFGβ inhibition (SB-431542) in SFEBq[23], hCSs[25], cortical organoids[51], (A-8301+ SB-431542) in forebrain organoids[53]; promotes inhibition of mesendoderm differentiation
BMP	Non-neural ectoderm induction[61] Dorsal neural tube induction[64] Medial pallium induction[69]	BMP inhibition (dorsomorphin + LDN-193189) in hCSs[25], (dorsomorphin) in forebrain organoids[53], (LDN-193189) in cortical organoids[51]; promotes inhibition of non-neural ectoderm differentiation, induction of dorsolateral forebrain BMP activation (BMP4) later in SFEBq[44,67]; promotes medial pallium with hippocampus and choroid plexus (+WNT3a–CHIR99021)
WNT	Non-neural ectoderm induction[129] Mesoderm induction[130] Midbrain–hindbrain induction[62] Caudal neural tube induction[130] Cortical hem[69] Lateral expansion of NE[70] Specification into deep-layer neurons[74]	WNT inhibition (IWR1endo) in SFEBq[23], (XAV-939) in cortical organoids[51]; promotes inhibition of non-neural ectoderm and mesoderm, induction of forebrain WNT activation (CHIR99021) later in cerebral organoids[71]; promotes lateral expansion of NE buds (CHIR99021) in forebrain organoids (+WNT3a)[53]; promotes neural induction WNT activation later (WNT3a–CHIR99021) in SFEBq[44,67]; promotes induction of cortical hem, induction of hippocampal primordium (+ BMP4) WNT activation (CHIR99021) in sliced neocortical organoids[73]; promotes increase of deep-layer neurons WNT inhibition (IWR1endo) in sliced neocortical organoids[73]; increase of upper-layer neurons
FGF	Mesoderm induction[131] Telencephalon induction[132] Midbrain-hindbrain induction[133] Caudal neural tube induction[130]	FGF activation (FGF2) in cerebral organoids[24], hCSs[25]; promotes maintenance of stem cells' pluripotency, proliferation of neural progenitors FGF activation (FGF8) in SFEBq[23]; promotes induction of telencephalon
SHH	Ventral neural tube induction[134]	SHH activation (smoothened agonist, SAG) in SFEBq[23], cortical organoids[51], forebrain spheroids[76], cerebral organoids[75]; promotes induction of ventral forebrain SHH inhibition (cyclopamine A) in cerebral organoids[75]; promotes induction of dorsal forebrain
RA	Caudal neural tube induction[135] Neuronal and IP differentiation[79]	RA activation in cerebral organoids[24], cortical organoids[51]; promotes induction of neuronal differentiation
ECM	Proliferation of neural progenitors, cell shape, migration, differentiation[80]	ECM (Matrigel) in SFEBq[23], cerebral organoids[24,71]; promotes NE growth, NE budding, formation of CP

BMP, bone morphogenetic proteins; WNT, wingless-related integration site; FGF, fibroblast growth factor; SHH, sonic hedgehog; hCS, human CS.

Guiding further organoid methods based upon signaling pathways in vivo. Brain organoids are beginning to shift the way we study human brain development in vitro, particularly as the methods are continuously evolving to become ever more refined for answering specific questions. Many of the recent modifications have come about by taking a page from development (Table 1). While initial cerebral organoids spontaneously acquired forebrain, midbrain and hindbrain identities within the same organoid, newer protocols have focused on guiding or directing regional identity to more reliably generate particular regions of interest. For this reason, organoid protocols have been classified as directed or guided[23,25,51] and undirected or intrinsic[24,52].

Protocols for the generation of cortical identity have been developed and modified by several teams, often with overlapping approaches, thus highlighting which factors are most important[1,23–25,50–54] (Fig. 1b). Although neuralization happens by default[55,56], the addition of certain inhibitors has been shown to increase neural induction efficiency[43,57–60]. The blockade of both TFGβ and BMP leads to the prompt inhibition of mesendodermal and epidermal lineages in vivo[55,56,61] and in vitro[25,53]. Additionally, the early suppression of WNT–β-catenin signaling prevents the differentiation into non-neural ectoderm and mesoderm, similarly to the effect of endogenous WNT inhibitors during gastrulation[43,59].

Later, WNTs act as posteriorizing signals in the neural tube[62]. Therefore, WNT inhibitors have been used in organoid protocols to obtain a more rostral brain identity[1,23,50,51,54] and, together with BMP inhibitors[1,51] or TFGβ inhibitors[1,23,50,51,54], to strengthen the neural commitment. As neural development proceeds, WNTs, as well as BMPs, act as dorsalizing factors throughout the neural tube, including in the forebrain[63–66]. Thus, once rostral identity has been established, exposure to WNT or to a WNT agonist and BMP4 was shown to pattern dorsomedial identities, including the pallial hem with hippocampus[67] and the choroid plexus[68], consistent with the secretion of WNTs and BMPs from the cortical hem observed in the embryo[69]. The choroid plexus is responsible for generating cerebrospinal fluid, an important and nutrient-rich supportive fluid, and likewise, choroid plexus organoids produce a fluid highly similar to cerebrospinal fluid. This system thus offers ways to study the contribution of cerebrospinal fluid for tissue nourishment, survival and maturation.

After patterning is established, WNT–β-catenin signaling then promotes horizontal expansion of neural progenitor cells by delaying neurogenesis[70]. Along these lines, a pulse of WNT activator after the neuroepithelial budding of brain organoids can enlarge NE and ventricle lumen size[71]. In addition, recent studies suggest the contribution of WNT signaling in layer-specific fate acquisition in human pluripotent stem cell-derived SP neurons in vitro[72] and cortical neurons of neocortical organoids[73]. Similarly to what is seen in vivo[74], WNT7B is enriched in deep CP neurons in organoids and promotes the differentiation to subcortical projection neurons[73]. Thus, the treatment of organoids with WNT agonist results in the increase of deep-layer neurons, whereas the inhibition of WNT enlarges the pool of upper-layer neurons.

Other key signaling factors include SHH, FGFs and RA. SHH is antagonistic to WNTs and BMPs, acting as a potent ventralizing factor. It has thus been adopted in vitro for the induction of ventral forebrain[23,51,75,76], while SHH antagonists promote dorsal identity[75]. The role of FGFs changes according to the isoform secreted and the developmental stage. FGF8, responsible for rostral specification in vivo, boosts telencephalic identity in vitro[23]. FGF2 (also called bFGF) is important for the maintenance of pluripotent human ES cells[77]; therefore, it has been used to prevent premature lineage fate

acquisition before neural induction in brain organoids[24]. Later, FGF2 and EGF act as mitogens for neural stem cells[78], capable of expanding the NE. Both growth factors in combination have been used for the generation of human CSs[25]. In the developing mouse embryo, the meninges play a pivotal role in controlling the timing of neurogenesis. Specifically, the release of RA, or vitamin A, from dorsal forebrain meninges is important for cell-cycle exit in neural progenitors and for the differentiation into IPs and neurons[79]. Therefore, RA was added to the differentiation medium to promote neurogenesis and neuronal migration in cerebral organoids[24] and cortical organoids[51]. Furthermore, BDNF and NT3, two growth factors present in later neurogenesis, were shown to support neuronal differentiation and the neurogenesis–astrogliogenesis switch observed in vivo[25].

Further crucial signals for brain development and growth are the interactions of neural progenitor cells and neurons with the extracellular matrix (ECM). Specifically, ECM major components, proteoglycans, laminins and integrins, have been known to regulate the proliferation of progenitors, their distribution along the NE layer, cell shape, migration and differentiation[80]. Thus, the introduction of ECM-like hydrogels (e.g., Matrigel) to the organoid protocols not only provided a scaffold but also contributed to the establishment of tissue polarity. Indeed, the addition of dissolved Matrigel to SFEBq promotes the growth of cortical NE tissue[23], and Matrigel embedding leads to formation of neural tube-like buds of polarized NE[24]. At later stages, addition of dissolved Matrigel promotes the proper positioning of neurons into a discrete CP[23,71]. Thus, growth factors and signaling pathways share a conserved role in nervous system development in vivo and in vitro (Table 1), and future modifications should be informed by findings in vivo.

Organoids at the interface between in vitro and in vivo

Tissue architecture. Brain organoids recapitulate many features of the fetal human brain, including cytoarchitecture, cell diversity and maturation. Early on, they exhibit the proliferative layers typical of the embryonic cortex, with a VZ-like band that is distinguishable from the overlying SVZ-like region. RG progenitors maintain apical–basal polarity and display typical inter-kinetic nuclear migration[23,24,81]. The IPs occupy an inner SVZ, while oRG cells are positioned along the outer region of the SVZ[23,24,73] (Fig. 2). However, despite the expression of oRG markers, organoids still lack an authentic oSVZ-like layer as present in primary samples by gestational week 15 (ref. [82]).

In the maturing human neocortex, neurons are generated in a specific timed manner, and they also position themselves at discrete locations within the cortical plate. In vivo, Cajal–Retzius cells located in the marginal zone guide neuronal migration and layer establishment through the release of the glycoprotein Reelin[83]. In neural rosettes, a CP and neuronal layers are not distinguishable, but in organoids neurons properly migrate externally, forming a defined and separate CP[23,71] (Fig. 2). Reelin$^+$ neurons were abundantly identified along the basal surface of brain organoids[23–25,51,53,71,84,85], suggesting that Cajal–Retzius cells might contribute to cortical plate architecture similar to in vivo. Later, organoids exhibit some degree of spatial separation of upper and deep layers, which becomes more evident when organoids are cultured for more than 3 months in optimized conditions of oxygenation and nutrient diffusion[73,86]. This is likely a result of the fact that neurons of the uppermost layer (II/III) in the human cortex are generated even beyond gestational week 18 (ref. [87]), and layering is not defined before the perinatal period[88]. Thus, a longer culture of organoids would be beneficial for the observation of cortical layers.

Cell diversity. Recent studies have comprehensively analyzed the single-cell transcriptomes of organoids generated from self-patterning[52,86] and growth-factor-guided protocols[23,50,51,54,76,89–93], highlighting how cell repertoires are conserved in different cortical organoids[46], as well as certain deficiencies in cell subtype resolution in proliferative cells and neurons in organoids[82]. Notably, self-patterned organoids display more heterogeneity in cell composition due to the non-guided differentiation[46]. Among the various organoid protocols, glutamatergic neurons are the most abundant mature neurons. Nevertheless, interneurons have been found in gui ded[51,54,76,91,93] and non-guided protocols[52,86], providing a resource for studying the debated origin of GABAergic neurons[46]. To this aim, ventral forebrain organoids resembling the medial ganglionic eminence, the site of production of interneurons[94], have been generated and fused to cortical organoids to study the tangential migration guiding inhibitory neurons to the neocortex[51,75,76]. Interestingly, brain organizing centers have been observed in cerebral organoids, such as cortical hem and pallial–subpallial boundary, suggesting a scenario in which regional identity might be acquired intrinsically within unguided organoids[84].

Considering non-neuronal cells in the brain, glial cells are at least equally abundant (if not more) compared to neurons, and they contribute to neuronal activity, synaptogenesis and circuit remodeling. Astrocytes are the first glial type to be produced after the neurogenic–gliogenic transition of neural progenitors, happening during the second trimester. Long-term culture of brain organoids and spheroids promotes gliogenic fate, to the point where astrocytes are abundantly present[86,89] and display mature features[89]. Interestingly, different morphologies of astrocytes have been reported in neocortical organoids, and they mimic the distribution within the cortex[73]. Moreover, astrocytes derived from human CSs contribute to neuronal depolarization, synapse formation and pruning[89]. Oligodendrocyte progenitors and cells with oligodendrocyte identity have also been found in cortical organoids[46]. These cells could be further supported by the application of signaling factors in long-term culture of organoids, resulting in their maturation and partial myelination of axons[91,92]. However, a complex organization of myelin sheets, including nodes of Ranvier, has not been reported, and functional myelination influencing nerve impulses has yet to be observed.

Microglia are immune cells derived from yolk sac and mesoderm. Because brain organoids are neuroectoderm-derived, microglia are not usually observed. Nonetheless, microglia have been shown to spontaneously develop in unguided cerebral organoids that unintentionally develop some mesoderm tissue[95]. A more controlled experimental approach is the combination of independently derived brain organoids and microglia, to more accurately mimic the invasion of microglia during development[96]. Overall, organoids exhibit a variegate cell composition, comparable to some extent to the brain, but still lacking important features, such as endothelial cells and the co-presence of all the glial cell types (Fig. 2).

Maturation. Cerebral organoids recapitulate the brain developmental transcriptional profile up to 24 weeks after conception, as shown by the comparison of organoid age and brain development stage[46]. Long-term culture of cerebral organoids does not further expand this time window, but rather leads to necrosis in the core of the organoid, due to hypoxia[73,86], and to heterogeneity in the tissue composition of self-patterned organoids[52]. Oxygenation and nutrient diffusion are important limiting factors in the process of maturation. To overcome these barriers, organoids were initially grown in spinning bioreactors[24], with later modifications making use of BDNF to promote neuronal survival and maturation[52]. Recently, transplantation of brain organoids into adult mouse brain resulted in vascularization from the host, with improved survival of neurons within the graft, as well as the presence of astroglia, oligodendrocytes and mouse-derived microglia[97]. Alternatively, sliced organoids maintained floating in orbital shakers[73] or at the air–liquid interface[86] exhibited improved survival, presumably due to increased nutrient supply to internal regions.

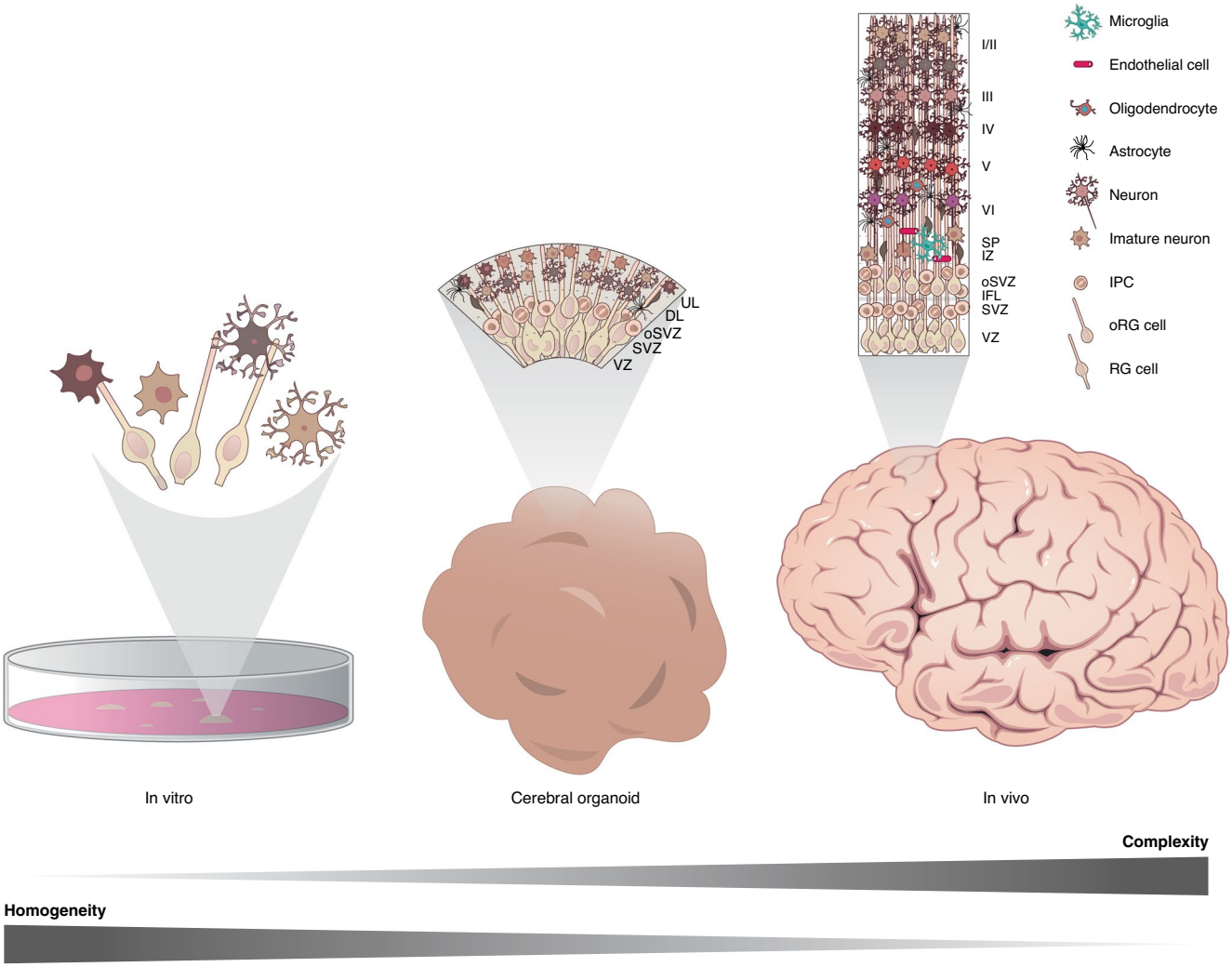

Fig. 2 | Cerebral organoid at the interface of in vitro and in vivo. Cerebral organoids display intermediate features of homogeneity (higher in in vitro 2D systems) and complexity (higher in the brain). Schematic focus on cell composition and architecture for each of the three models. UL, upper-layer neurons; DL, deep-layer neurons; I–VI, cortical layers; IZ, intermediate zone; IFL, inner fiber layer; IPC, intermediate progenitor cell.

The hallmark of neuronal maturation is the acquisition of spontaneous firing activity and the emergence of dendritic spines and synaptic contacts, enabling the transmission of nerve impulses along the network. Both inhibitory and excitatory synapses have been observed in brain organoids[52,86], together with functionally relevant presynaptic vesicles[52]. Spontaneous neuronal activity has been detected in brain organoids[24] and SFEBq[44] through calcium dye imaging. Remarkably, concomitant calcium waves characterize largely distant neurons[44], suggesting the existence of functional communication among neuronal cells. Excitatory synaptic transmission, selectively mediated by non-NMDA glutamatergic receptors, has been measured in extracellular recordings of mature whole-brain organoids[52].

Current neural organoid capabilities and limitations

Disease modeling. One of the most practical applications of brain organoids is disease modeling. Thus far, most human-related diseases have been studied in genetically modified animals that would not naturally develop the pathology but do share certain disease-responding mechanisms with humans due to their phylogenetic relatedness. The introduction of in vitro human-specific models has the potential to overcome interspecies differences, help reduce the demand for animal facilities and accelerate the process of drug screening. Organoids offer an unprecedented advantage in studying disease in vitro, in a 3D environment at least partly resembling the affected tissue. A growing number of reports of disease modeling in organoids have recently been published (reviewed in ref. [98]), largely made possible by patient-derived cells as a starting material for organoids.

Brain organoids have been used to model neurological disease and, importantly, neurodevelopmental disorders, given the resemblance with developing brain up to mid-gestation[23,25,46]. The first neurological disorder modeled in this fashion was a genetic form of microcephaly using patient-derived iPSCs with loss-of-function mutations on *CDK5RAP2* (ref. [24]). Patient-derived organoids were smaller in size, with premature neuronal differentiation. Moreover, neurodevelopmental disorders, such as autism spectrum disorders (ASDs), represent unique human disruptions in the cognitive and behavioral sphere, frequently accompanied by brain growth abnormalities. Telencephalic organoids derived from individuals with idiopathic ASD with macrocephaly displayed an initial transient increase in size, followed by expanded neuronal maturation and synaptic overgrowth[1]. Overall, the multifactorial nature of ASD and the pathophysiological heterogeneity make clinical studies on affected individuals particularly challenging. iPSC-derived brain organoids from ASD cohorts offer the chance to zoom in

on cell-autonomous and non-cell-autonomous effects in a human background[1,24,76,99]. Moreover, organoids generated from isogenic lines upon gene editing for a specific ASD risk-associated gene can help understand the contribution of a specific gene in the complex scheme of ASD genetics through a monogenic approach[100].

Brain organoids were also shown to be a valid model for the study of infectious diseases. Indeed, Zika virus (ZIKV) effects on brain development have been extensively tested in vitro[53,101–103]. Interestingly, Qian et al. demonstrated the tropism of ZIKV for neural progenitors in the VZ and oSVZ of forebrain organoids and a dramatic reduction in VZ and neuronal layer thickness typical of microcephaly[53]. High-throughput drug screening for potential anti-ZIKV compounds in human neural stem cells and the subsequent validation in forebrain organoids led to the individuation of an antiviral therapeutic candidate[104]. This example highlights the remarkably powerful applications of in vitro compound screening.

Neurodegenerative disorders are particularly challenging to model with brain organoids, because of the late onset of the disease and the limited maturity the organoid can reach. Nevertheless, attempts to study Alzheimer's disease in organoids derived from cells of people with familial Alzheimer's disease resulted in interesting findings. Amyloid plaques and phosphorylated Tau aggregates, two distinct hallmarks of the disease, have been detected in organoids together with some degree of apoptosis[105]. Unlike in vitro two-dimensional (2D) methods, organoids can provide a complex extracellular environment to support the characteristic protein aggregates seen in the adult brain of patients[106].

Disease modeling in brain organoids can be particularly powerful when combined with gene editing techniques (reviewed in ref. [107]). The expression of a single, targeted gene can be abolished or restored by means of viral delivery, transposon systems or the popular CRISPR–Cas9 system. Alternatively, transient gene expression perturbation can be achieved through plasmid-mediated overexpression or RNA interference knock-down[107]. CRISPR–Cas9 has been adopted in organoids with the aim of simulating loss-of-function found in affected individuals, such as *PTEN* heterozygous deletion associated with human macrocephaly[100] or *LIS1* heterozygous mutation associated with lissencephaly[108]. The reverse approach is the rescue of a pathological phenotype in patient-derived brain organoids by CRISPR–Cas9-mediated introduction of the wild-type gene, as is the case for *LIS1* and *YWHAE* for Miller–Dieker syndrome[109] or the overexpression of the wild-type *CDKRAP2* in microcephaly patient-derived brain organoids[24]. Altogether, the powerful combination of gene editing and brain organogenesis in vitro enables the selective and bidirectional manipulation of specific genes responsible for pathological phenotypes in a time- and space-controlled manner, which facilitates the readout of the gene function.

Neural networks. The developing cortex is characterized by waves of spontaneous electrical activity in neurons and neural precursors, even before synapses are formed[110]. As neurons begin to mature and project neurites at long distances to establish synaptic contacts, the spontaneous activity becomes synchronized across distant cells. Quadrato et al. detected such synchronized population bursts in mature whole-brain organoids. This temporal structure of electrical activity indicates that the nerve impulse travels across interconnected neurons, and the presence of a lag time in signal transmission points to a communication through chemical synapses[52]. Oriented axon bundles were shown in sliced organoids, where callosal projection-like neurons point their axons toward the core of the organoid, whereas functionally active subcortical projection-like neurons extend their axons away[86].

Recently, Trujillo et al. shed light on the development of network-like activity as cortical organoids mature. In particular, highly synchronous oscillations and cross-frequency coupling, and thus the interaction between activities at different frequencies, speak to the presence of network communication[93]. A higher order of network complexity might perhaps be achieved if the organoid contained multiple identities of cortical regions functionally connected as in the brain. The lack of a clear directionality of axons as in the cerebral white matter could be attributable to the absence of axial planes in cerebral organoids. Overall, the complex neural network activity recorded in brain organoids suggests they could potentially be adopted to study neuronal activity abnormalities, as in the glutamatergic–GABAergic imbalance reported in people with ASD[111].

Evolution. Brain scaling and differences in cerebral structure and functional connectivity are thought to underlie human-specific cognitive capacity. The human brain can be considered a scaled-up primate brain[8], three times larger than the brain of a chimpanzee, our closest relative[112]. Therefore, it is of major importance to consider the primate evolutionary landscape when studying human-specific brain features. Given the scarce availability of human and primate fetal brain samples, in vitro models can once again offer a valid alternative. The establishment of primate iPSCs[113] encouraged the production of brain organoids from different species of apes to identify the subtle cellular and molecular differences accounting for brain growth and increased number of neurons in humans. Additionally, human iPSC-derived organoids from different individuals can be used to study the rate of introgression of archaic hominin DNA into the human genome and the contribution of Neanderthal-derived variants on brain development[114].

Neurogenesis is protracted in human compared to macaque and other non-primate mammals, thus enabling the expansion of proliferative progenitors and the accumulation of larger numbers of neurons, even in the late-born upper cortical layers[115]. Importantly, in vitro systems accurately replicate species-specific intrinsic neurogenic timing. Indeed, lineage analysis has revealed that human progenitor cells expand for over 30 days, compared to 15 days in macaque, extending to the deep-layer neurogenic phase and giving rise to a remarkably increased neurogenic potential and clonal size amplification[116]. The balance between proliferation and neuronal fate acquisition is a cell-autonomous mechanism, as demonstrated by transplantations of human progenitor cells into macaque neural rosettes, resulting in human-specific clonal size and vice versa[116]. The increased neural proliferative amplitude in human was further confirmed by the comparison of cell-cycle length in human and chimpanzee brain organoids. Mora-Bermudez et al. observed a longer prometaphase–metaphase in the VZ progenitors in human, likely an index of proliferative rather than neurogenic divisions[112].

Transcriptome analysis can also provide insights into developmental modules conserved across primate evolution or differentially regulated in human. Single-cell RNA-sequencing and gene-network analysis revealed that expression modules, enriched in human brain compared to macaque, were also upregulated in human brain organoids compared to chimpanzee organoids, confirming the validity of organoids as models for evolutionary studies[50].

Although evolution manifests through an intricate accumulation of genetic variations, including single-nucleotide polymorphisms, copy number variations, duplications and deletions, the single-gene approach can help reduce the complexity and provide clues as to species-specific differences. Brain organoids offer remarkable flexibility for genetic engineering, to the point where mouse or primate organoids can be 'humanized' with the introduction of human unique genes. Based on this, Fiddes et al. introduced human *NOTCH2NL* into mouse cortical organoids, resulting in the upregulation of genes involved in the negative regulation of neuron differentiation and downregulation of neuronal differentiation genes. Indeed, *NOTCH2NL* has been shown to be absent in the non-human primate lineage and to promote RG expansion in

Table 2 | List of current capabilities and future perspectives of cerebral organoids

	Current capabilities and limitations	Future perspectives
Reproducibility	Inter-batch differences (more pronounced in unguided protocols versus guided and semi-guided protocols)	Increased batch homogeneity by working out and controlling key initial patterning events
Survival	Long-term culture of whole-organoids up to 9 months (ref. [52]) and 10 months (ref. [93]). Long-term culture of sliced organoids up to 365 days[73,86]. Transplantation of brain organoids into adult mouse brain promotes cell maturation and survival[97]	Further long-term maintenance of over 1 year to several years of sliced and plated organoids to look at later maturation
Nutrients diffusion and oxygenation	Hyperoxic culture conditions[23], use of gas permeable dishes[23,50], agitation in orbital shaker[51,54,82,99], spinning bioreactors[24,53] and organoid slicing[73,86] help provide nutrients and oxygenation to the entire organoid. No functional vascularization (to date) demonstrated in vitro	Vascularized 3D organoids with a constant supply of nutrients and oxygen to prevent the formation of a necrotic core
Tissue architecture	Cerebral organoids with a thick cortical plate and established segregation of deep- and upper-layer neurons[73]. Fully organized six-layer cytoarchitecture still lacking	Cerebral organoids recapitulating six-layered laminar neocortical architecture and columnar organization
Gliogenesis	Cerebral organoids with microglia[95,96], spheroids and organoids with astrocytes[73,86,89], and spheroids with maturing oligodendrocytes[91,92] enable the investigation of neuron–glia interaction	Morphologically and functionally mature glia subpopulations (astrocytes, oligodendrocytes and microglia) developing all in the same cerebral organoid. Complex structures underlying functional neuron–glia interaction (e.g., nodes of Ranvier in myelinated axons)
Neuronal activity	Synchronized oscillatory network events in mature cortical organoids dependent on glutamate and GABA signaling recorded on multielectrode arrays[93]	Further maturation of neuronal activity dependent on glutamate and GABA and fine-tuned by neuromodulators. Organoids with a well-established connectome and network-based activity

human. Furthermore, *NOTCH2NL* deletion in human brain organoids leads to the development of smaller organoids, with accelerated neuronal differentiation[90]. Thus, brain organoids are a valuable tool for the analysis of brain evolution in different species and for testing the function of genes whose sequence or expression profile have human unique features.

Current weaknesses. Although brain organoids represent an elegant technique for disease modeling, developmental and evolutionary studies, there are still several limitations. Standing at the interface between in vivo and in vitro, organoids have, to some extent, advantages and disadvantages from both systems. We have already mentioned some drawbacks that the organoid protocols collectively share, such as limited oxygen and nutrient diffusion with consequent necrosis, limited maturation and the absence of some cell types found in the brain (Table 2). Additionally, organoids notoriously suffer from the 'batch effect', with variation in efficiency of differentiation, morphology and variability in cell composition across different batches of organoids[99], and this heterogeneity is higher in self-patterned organoids[46,52]. Attempts to increase the homogeneity include the addition of exogenous patterning factors[23,25,50,51,53,54,89], the use of micro-scaffolds to produce specific cellular configurations[71] and the use of mini spinning bioreactors with smaller volumes of media and better-controlled conditions[53]. Moreover, reproducibility is hampered by the variable organoid size and morphology attained with different pluripotent stem cell lines[117]. Thus, the establishment of stringent controls when studying brain growth defects with patient-derived iPSCs is pivotal to distinguish true pathological phenotypes.

Interspecies comparisons using brain organoids require an attentive evaluation of developmental timing. In vivo mouse development is overall faster compared to human and characterized by a neurogenic phase of only about 7 days (ref. [87]). Similarly, neuronal differentiation in vitro takes in general 5 days for murine embryonic stem cells and roughly 20 days for human stem cells[118]. Thus, the protocol for generating brain organoids needs to be adapted to the species-specific intrinsic timing. Overall, current weaknesses in organoid models do not undermine the broad applicability of this technique (Fig. 3), and future studies will help improve and enrich this relatively young research field (Box 2).

Future improvements

Less than a decade since the emergence of cerebral organoids, we have already seen an exponential growth in innovations aimed at optimizing in vitro culture conditions to narrow the gap with in vivo models. Of particular interest are the efforts taken to increase the applicability of organoids for transplantation, drug screening and toxicology. For this purpose, homogeneity and reproducibility, scalability and automation are essential requirements. The introduction of 3D printers, microfluidic devices, bioreactors and robotic devices will help drive advances in this direction. Though cells can survive in such bioengineered cultures, they need the controlled release of growth factors for proliferation and differentiation.

Signaling molecules also contribute to the establishment of different tissue identities and axial polarity, still missing in brain organoids. Indeed, we believe the lack of uniform axial cues in organoids to be responsible for the absence of organization of different brain-like regions along the rostrocaudal–ventrodorsal axes. In a recent study aimed at creating spatial patterning within the organoid, introducing a cell-based signaling center for the release of the ventralizing factor SHH resulted in a spatial topography similar to that of the human developing forebrain[119]. Future approaches could include organoid culture on microfluidic devices dispensing morphogens in a temporally and spatially regulated manner. Microfluidic chips have initially been designed for spheroid cultures[120] and further optimized for brain organoids. Although organoid-on-chip technologies[121] can open the way for more homogenous and reproducible cultures, with better results in the efficiency of nutrient diffusion within the tissue, they might compromise organoid morphology. In fact, the reduced space available for tissue growth in the device can potentially limit its 3D expansion.

The establishment of complex tissue architecture represents perhaps the most important accomplishment of brain organoids compared to 2D systems. We expect future studies to further improve the spatial segregation of neuronal layers within the organoid CP, perhaps with the aid of engineered extracellular scaffolds that could

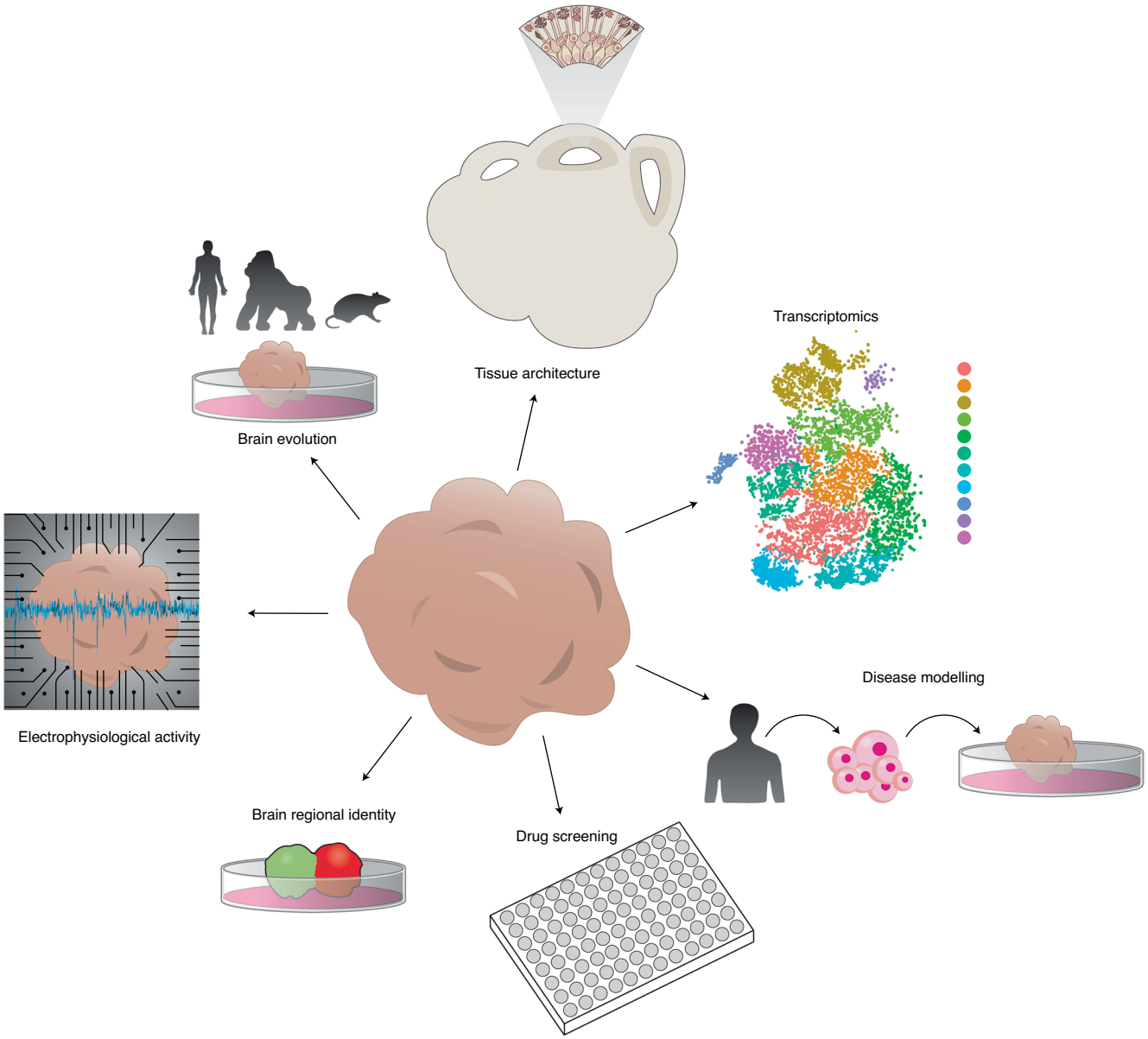

Fig. 3 | Main applications of cerebral organoids. Schematic illustration of the broad applicability of cerebral organoids based on studies cited in the text. Cerebral organoids are suitable for studying cell composition and tissue architecture with a higher degree of complexity than is available in 2D cultures. Transcriptomic analysis can provide insights into cell subpopulations, developmental stage, interspecies differences and reproducibility within batches and cell lines. Evolutionary comparison of organoids from humans, non-human primates and other mammals can shed light on human-specific developmental features. Human-derived cerebral organoids are widely used for modeling neurodevelopmental disorders as well as infectious, psychiatric and neurodegenerative diseases. Current advances in fast mass-production and reproducibility within organoids are opening the way for high-throughput drug screening, toxicology tests and personalized medicine. Protocols for generating brain region-specific organoids can help understanding important questions, such as those around the inter-neurogenesis and migration of GABAergic neurons. Functional communication within cerebral organoids is observable from neural network dynamics of highly synchronous firing cells, thus offering a substrate for the study of electrophysiological abnormalities during brain development.

reproduce the role of meninges, known to be important for neuronal migration in the brain[79]. The organoid field would benefit from such an integration of biophysics and biomechanics with cell biology.

Brain organoids are far from resembling the size of the actual human brain. One reason for this is the lack of vasculature that nourishes and oxygenates the tissue in vivo. While organoids have been transplanted to rodent hosts to achieve vascularization in vivo[97], a fully in vitro system would be more powerful in the long run for disease modeling and drug testing. So far, experiments trying to reproduce blood vessels in vitro have included co-culture of endothelial and mesenchymal stem cells with tissue-specific progenitors on an extracellular substrate[122], microfluidic perfusion[123] and engineered cortical organoids with ES cells ectopically expressing the endothelial-inducing transcription factor *ETV2*[124]. Just recently, blood vessel organoids with capillary networks were generated in vitro from pluripotent stem cells[125]. One could foresee that brain organoids of the future might count on the fusion with in vitro-derived vasculature.

Box 2 | Integrating organoids with state-of-the-art technologies in neuroscience

Cerebral organoids represent a powerful tool per se, mainly for neurodevelopmental studies. Nevertheless, the combination with advanced technologies, normally applied to animal models, can open up unprecedented scenarios for in vitro research. Since its introduction in 2005[147], optogenetics has found large applicability in behavioral and electrophysiological studies. In brief, the selective introduction of a light-sensitive ion channel (i.e., opsin) in specific cell populations allows for a rapid switch on–switch off of the cellular electrical activity when a light stimulus is delivered locally. As extensively discussed by Shiri et al., the synthesis of optogenetics and brain organoids could be useful for studying neural network dynamics, the functional communication between different regions within the organoid, synaptic plasticity, and pathological abnormalities in neuronal firing, such as in epilepsy or Huntington's disease[148]. Steps toward the combination of brain organoids with optogenetics have already been taken. They include the selective expression of channelrodopsin2 (ChR2) in neurons[85] and the induction of action potentials in hCS neurons expressing ChR2 (ref. [149]).

A major interest of the neuroscience community is now on providing a map of all neuronal connections within the brain, the connectome, starting from animals with very small brains. Recently, researchers working in the FlyEM project at Janelia Research Campus, succeeded in reconstructing the whole circuitry of a large part of the *Drosophila* brain, by means of electron microscopy[150]. The process is extremely time-intensive and currently beyond the realm of possibility for the human brain. Given the small size of brain organoids, future connectome studies might contemplate organoids as eligible models. Although still far from resembling the complexity of the human brain map, they could provide insight into human neuronal connectivity.

Finally, we have reviewed above the potential applications of brain organoids for disease modeling and drug screening. However, further efforts need to be made to ensure better automation and scaling-up in the protocol. A fully automated protocol for the production of kidney organoids for high-throughput screening was recently published[126]. Similarly, the use of robotic devices for making brain organoids would guarantee higher homogeneity between batches and would allow fast mass production.

Conclusion

One of the most ambitious goals in science is translating in vivo models to a dish, both for their ease of accessibility and their scalability. The result is clearly a balance between the complexity of animal models and the homogeneity of in vitro cultures. Because reductionism is often the key to unravelling intricate biological questions, in vitro models can be vital to scientific discovery. Stem cell methods have contributed to the establishment of paradigms of neurodevelopment in a dish, from 2D neural rosettes to more recent brain organoids. Compared to rosettes, organoids display remarkably organized tissue architecture, with distinct neural progenitor layers and inside-out migration of neurons. Indeed, upper-layer and deep-layer neurons are distinguishable in mature brain organoids, an index of a temporally and spatially regulated neurogenesis. Furthermore, organoids recapitulate species-specific brain features, such as the presence of numerous oRGs in human. RNA-sequencing analysis has revealed conserved gene expression profiles and developmental trajectories in brain organoids and fetal brain samples. Recent optimizations of the original protocols have enabled the production of organoids with mature electrophysiological properties and neural networks.

Future improvements will likely focus on further refinement of tissue identity and patterning, as well as in vitro vascularization. Learning from past advances, where understanding development has proven key to production of novel methods, future modifications should also be built on the attentive evaluation of in vivo examples. In addition, the combination of organoid techniques with cutting-edge technologies in neuroscience can offer great promise. Overall, organoids have great potential but they still lack certain features, such as the six-layered human brain cortical architecture, the massive expansion of neural progenitors and the extraordinary human brain connectome. This places them somewhere between more traditional 2D in vitro methods and in vivo in terms of their abilities and limitations. Their position at this interface makes them a unique model that is highly complementary to more reductionist in vitro methods and in vivo animal models.

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Competing interests

M.A.L. is an inventor on several brain organoid patents, as well as co-founder and scientific advisor of a:head bio AG.

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