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REVIEW ARTICLE

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Using organoids to study human brain development and evolution

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Abstract

Recent advances in methods for making cerebral organoids have opened a window of opportunity to directly study human brain development and disease, countering limitations inherent in non-human-based approaches. Whether freely patterned, guided into a region-specific fate or fused into assembloids, organoids have successfully recapitulated key features of in vivo neurodevelopment, allowing its examination from early to late stages. Although organoids have enormous potential, their effective use relies on understanding the extent of their limitations in accurately reproducing specific processes and components in the developing human brain. Here we review the potential of cerebral organoids to model and study human brain development and evolution and discuss the progress and current challenges in their use for reproducing specific human neurodevelopmental processes.

KEYWORDS

cerebral organoids, human cortical development, human cortical evolution, human embryonic neurodevelopment

1 | THE NEED FOR A HUMAN-CENTRIC APPROACH

Much of our knowledge about human brain development is extrapolated from studies of brain development in other species. It is undoubtedly true that many of these extrapolations are likely to be valid, given the high level of conservation of brain structure and function among mammalian species, but the approach has clear limitations. Most obvious is the high level of sophistication of the mature human brain compared with that of even our closest living relatives. During embryogenesis, important anatomical differences emerge between the developing brains of humans and other mammals such as the neocortex expansion and the arising of gyrification seen in higher evolved species (Florio & Huttner, 2014; Lui et al., 2011). Although many of the mechanisms that create the human brain are highly conserved, the generation of its unique attributes are likely to involve important genomic regions that show differences unique to humans, e.g., human accelerated regions (HARs), or human-specific genomic rearrangements (Bae et al., 2015; Dennis & Eichler, 2016; Gittelman et al., 2015; Hubisz & Pollard, 2014; O'Bleness et al., 2012; Pollard, Salama, King, et al., 2006; Pollard, Salama, Lambert, et al., 2006; Prabhakar et al., 2006; Silver, 2016; Zhang & Long, 2014). These human-specific genomic features are almost certainly involved in human-specific gene regulatory

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mechanisms that account for the morphological, physiological, and behavioral divergence between our brains and those of our closest relatives (Britten & Davidson, 1969; King & Wilson, 1975). These considerations lead to the inevitable conclusion that gaining a full understanding of the mechanisms regulating human brain development will require studies of human brain development.

The discovery that brain-like structures, cerebral organoids, can be made by differentiating stem cells that offers the potential to study aspects of human brain development directly. Cerebral organoids are 3D cell aggregates derived from pluripotent stem cells (PSCs) which contain many of the cell types found in embryonic brains, locally organized and behaving similarly to cells found in vivo (Eiraku et al., 2008; Kadoshima et al., 2013; Lancaster et al., 2013). Their use has already led to the identification of human-specific features of brain development, such as human-specific gene expression along cortical lineages (Kanton et al., 2019; Pollard, et al., 2006). Further examples will be discussed at the end of the review. Here we review the potential of cerebral organoids to model the development of the human brain, to explore its mechanisms of development and their evolution.

2 | STRATEGIES FOR MAKING CEREBRAL ORGANOIDS

Brain development is a complex process involving intricate spatiotemporal regional patterning, precisely choreographed cell migration and accurately directed axonal targeting. These processes result in the production of highly specialized brain regions, each containing a specific set of cells of diverse classes with distinct functions interacting via complex circuitry. Clearly, there is no expectation that organoids will deliver a model that faithfully reproduces all aspects of brain development in their entirety. The hope is that cerebral organoids will offer models reproducing specific processes occurring in, and specific components of, the developing human brain with sufficient accuracy to make them useful for studying human brain development and disease (Le Bail et al., 2021; Shi et al., 2021).

Protocols for generating human cerebral organoids fall into two main categories. In the first, PSCs are aggregated and allowed to free-pattern and differentiate in the absence of specific added differentiation cues. Such protocols, exemplified by Lancaster et al. (2013), produce organoids comprising multiple regions corresponding to various components of the brain, such as dorsal and ventral forebrain, hindbrain, hippocampus or choroid plexus (Lancaster et al., 2013). Alternatively, many protocols include the addition of specific cues that promote formation of a specific region of the brain (Birey et al., 2017; Giandomenico et al., 2019; Paşca et al., 2015; Qian et al., 2016; Xiang et al., 2017, 2019). In a hybrid approach, multiple organoids each representing a different specific brain region are combined to generate "assembloids", thereby achieving selective regional heterogeneity in a relatively controlled manner (Bagley et al., 2017; Birey et al., 2017; Xiang et al., 2017, 2019). Generation of localized signaling centers has also been used to direct organoid differentiation (Cederquist et al., 2019; Takata et al., 2017). We discuss some of the progress and current challenges in using cerebral organoids to model components of the developing human brain, to examine particular processes involved in human brain development, and to study human brain evolution.

3 | USING ORGANOIDS TO PROBE EARLY HUMAN NEURODEVELOPMENTAL PROCESSES

The generation of organoids whose heterogeneous cellular composition reflects that of human fetuses has allowed us to probe many stages of human brain development, some of which predate the emergence of the brain as a discrete structure.

3.1 | Gastrulation and neural induction

Early in development the embryo undergoes gastrulation, during which it reorganizes to form the endoderm, mesoderm, and ectoderm, after which neural induction occurs to generate the neuroectoderm. In humans, gastrulation occurs around the end of the second embryonic week. At this stage, human embryos are difficult to obtain and cannot be cultured, for technical, legal, and ethical reasons. In many stem cell in vitro systems, neural induction can be mimicked by blocking the TGFβ and BMP signaling pathway using dual SMAD inhibition (Chambers et al., 2009). Recently, human "gastruloids" with the ability to form the three germ layers and displaying patterned gene expression have been grown in culture for the first time (Moris et al., 2020). Gastruloids generate derivatives of the three germ layers and express markers of early neuroectoderm in the absence of SMAD inhibition. Gastruloids successfully elongate and establish and maintain axial patterning, with BMP pathway-related genes expressed preferentially anteriorly and WNT pathway genes expressed preferentially posteriorly (Moris et al., 2020). Due to technical limitations, gastruloids have only been cultured for up to 3 days, providing insufficient time for the neuroectoderm to generate cell types found in the developing brain. Nevertheless, they offer a powerful new three-dimensional

model of very early human developmental processes, predating the start of brain development, that have previously been difficult or impossible to study.

3.2 | Rostral-caudal regionalization

In vertebrates, gastrulation and generation of neuroectoderm is followed by folding and closure of the neural plate to form the neural tube. Regional specialization of the neural tube along its rostral-caudal (R-C) axis determines the prospective brain and other CNS regions. The mechanism by which R-C regionalization occurs in humans remains a mystery. In non-human species, one major signaling pathway implicated in R-C regionalization is Wnt signaling, with many Wnt signaling genes showing spatially restricted expression patterns during early brain development (Fischer et al., 2007; Hoang et al., 1998; Roelink & Nusse, 1991; Rowitch & McMahon, 1995). The addition of Wnt agonists caudalizes neuroectodermal cells, including those derived from human stem cells, in a dose-dependent manner (Fang et al., 2019; Imaizumi et al., 2015; Kiecker & Niehrs, 2001; Kirkeby et al., 2012; Nordström et al., 2002; Rhinn et al., 2005; Rifes et al., 2020). Notably, in the experiments of Rifes et al. (2020) on human cells, expression of regionalized markers appeared very early, after only a day of differentiation, a day before neural-specific genes became expressed, suggesting that regionalization occurred before neural specification. This interesting result needs further investigation to establish whether it is a consistent finding.

The importance of WNT signaling in the R-C regionalization of the human neuroectoderm remains unclear. In non-human species, the use of 3D culture systems in which aggregates of stem cells self-organize along an axis has made it possible to investigate these processes. Studies using aggregates of mouse embryonic stem cells (mESCs) revealed that after only 3 days of culture, aggregates showed localized Wnt activity (ten Berge et al., 2008) and self-organized along the R-C axis (Takata et al., 2017). This was preceded by expression of Fgf5, inhibition of which prevented polarization and produced rostralized aggregates. Activation of Fgf led to caudalization and increased expression of Wnt genes. This work revealed that an interplay between Fgf and Wnt signaling in neuroectoderm is key to R-C patterning, suggesting that the initial appearance of a localized population of $Fgf5^{+}$ cells induces and combines with localized expression of Wnt signaling components to repress rostral fates caudally. In the human gastruloid study (Moris et al., 2020), pre-treatment of hPSCs with the WNT activator CHIR99021 was essential for axis formation and could not be replaced with WNT3A and BMP4. Although it is possible to generate a R-C axis in in vitro models of human neural differentiation, these models all require extrinsic signals (Cederquist et al., 2019; Rifes et al., 2020). Further insights from these models may provide answers to the exact cellular signals required for axis formation during early human brain development. Furthermore, being able to recapitulate embryo-like axes within organoids may produce a better model of normal brain structure.

3.3 | Dorsal-ventral regionalization

It is suspected that endogenous forebrain organizing centers that direct dorsal-ventral (D-V) patterning arise spontaneously in some free-patterning forebrain human organoids (Lancaster et al., 2013; Renner et al., 2017). The evidence in favor of this possibility is the observation in such organoids of localized expression of signaling molecules important for D-V patterning such as WNT2B and BMP6 (Renner et al., 2017). Moreover, the presence of such signaling centers has been inferred from the fact that regions mimicking those specified along the D-V axis of the embryo are generated in these organoids. For example, regions with molecular identities typical of ventral telencephalic regions, including lateral, medial, and caudal ganglionic eminences (LGE, MGE and CGE), or regions corresponding to components of dorsal telencephalon, such as choroid plexus or hippocampus, have been identified (Hansen et al., 2013; Kim et al., 2019; Lancaster et al., 2013; Ma et al., 2013; Renner et al., 2017; Sloan et al., 2018). Even though free-patterning organoids can develop organizing centers and regions with the characteristics of those that develop at different locations along the D-V axis, current models have not yet succeeded in reproducing accurately the spatial layout of those centers and regions in the developing embryo. Achieving this will most likely require the application of external cues in a spatiotemporally controlled way.

4 | USING ORGANOIDS TO PROBE LATER HUMAN NEURODEVELOPMENTAL EVENTS

4.1 | Cerebral cortical development

The cerebral cortex forms in the dorsal part of the forebrain. Cerebral cortex-like tissue is a consistently generated component of free-patterning organoids and stem cell-derived neuronal models, suggesting that its production is a default state achieved in the absence of externally applied molecular cues (Table 1) (Gaspard et al., 2008; Smukler et al., 2006). A large amount of research using human cerebral organoids has focused on the analysis of cerebral cortical development.

In the developing human cortex, the generation of neurons from progenitors known as radial glia follows a highly conserved intrinsically regulated differentiation program

TABLE 1 Summary of patterning molecules, markers, and features in region-specific organoids

Region	Externally applied patterning molecules	General early markers	Neuronal markers	Notable features	References
Pallium	–	PAX6, OTX2, FOXG1 (Forebrain); Emx1 (Dorsal cortex); PAX6, SOX2 (RGs/VZ); FAM107A (oRGs); TBR2 (IPs)	Tuj1 (new-born); NeuN (differentiating); CTIP2, TBR1 (early born, deep-layers); SATB2 (late born, upper-layers); CUX1, BRN2 (layer II/III); REELIN (Cajal-Retzius cells)	Display well-defined laminar neuronal zones: VZ, SVZ and CP Generate diverse electrophysiologically active neuronal subtypes of all six cortical layers Differentiate into upper and deep cortical layers	Krefft et al. (2018); Lancaster et al. (2017); Paşca et al. (2015); Qian et al. (2016); Renner et al. (2017); Xiang et al. (2017); Yoon et al. (2019)
Sub-pallium	WNT inhibition, enhanced SHH signaling	GSX2 (dLGE, CGE); NKX2-1 (MGE); LHX6 (subregion of MGE)	GABA, GAD67 (GABAergic markers); SST, CR, CB, PV (IN subtypes); SYN1, VGAT (synaptogenesis)	Develop ventral forebrain GE subregions that produce different interneuron subtypes with an ongoing process of synaptogenesis Generate functional neurons: ~75% of neurons generated action potentials in response to depolarization and ~60% exhibited spontaneous inhibitory postsynaptic currents (IPSCs) that reverse in direction around the chloride reversal potential and are abolished by gabazine, a GABAA receptor antagonist	Bagley et al. (2017); Birey et al. (2017); Sloan et al. (2018); Xiang et al. (2017)
Hippocampus	manipulation of Wnt and bone morphogenetic protein (BMP) signaling	Nrp2, Lef1 (NE layers); Zbtb20 (hippocampal neurons and their precursors)	Prox1 (granule neurons); KA1 (CA3 pyramidal neurons); CaMKII (mature hippocampal neurons)	Neurons capable of forming neuronal networks with functional synaptic connections: voltage-dependent Na–K currents, action potential following injection of depolarizing currents, spontaneous excitatory postsynaptic currents present	Sakaguchi et al. (2015)

(Continues)

TABLE 1 (Continued)

Region	Externally applied patterning molecules	General early markers	Neuronal markers	Notable features	References
Thalamus	BMP7 and MEK inhibitors, dual SMAD inhibition	OTX2, TCF7L2, and GBX2 (thalamic identity)		Reciprocal thalamocortical (TC) connections observed when fused with cortical organoids Functionally active neurons with synchronized calcium surges without stimuli	Shiraishi et al. (2017); Xiang et al. (2019)
Midbrain	SHH agonists, FGF-8, dual-SMAD inhibition, GSK3b inhibitor	FOXA2, NESTIN (floor plate progenitor), MASH1, OTX2 (midbrain progenitors), NURR1 (IZ postmitotic cells)	MAP2 (mantle zone neurons); TH, FOXA2, DAT, NURR1, PITX3 (dopaminergic neurons); TH, GIRK2, CB (A9 and A10 midbrain dopaminergic neurons)	Generate functional neurons: fire rhythmically, respond to treatment with dopamine D2/D3 receptor agonists, form intricate neural networks that can be probed by electrical stimulation	Jo et al. (2016); Qian et al. (2018)
Cerebellum	FGF2, insulin, FGF19, SDF1	EN2, GBX2 (midbrain-hindbrain markers); FGF8, WNT1 (isthmic organizer); PTF1A, KIRREL2 (cerebellar plate neuroepithelium)	L7, SKOR2, OLIG2, LHX5 (Purkinje cells); NRGN, PAX2, GAD65 (Golgi cells); ATOH1, BARHL1, PAX6 (Granule cells); LHX2, TBR1, SMI32 (DCN); PV, CB, GAD65 (INs)	Generate mature Purkinje cells after long-term culture that exhibit functional electrophysiological properties: spontaneous repetitive firing, I_h currents and AMPA-receptor-dominant response to glutamate input	Muguruma et al. (2010, 2015)

Abbreviations: CGE, caudal ganglionic eminence; CP, cortical plate; DCN, deep cerebellar nuclei; dLGE, dorsal lateral ganglionic eminence; IN, interneuron; IPs, intermediate progenitors; IZ, intermediate zone; MGE, medial ganglionic eminence; MZ, marginal zone; NE, neuroepithelium; RGs, radial glia, SP, subplate, SVZ, subventricular, VZ, ventricular zone.

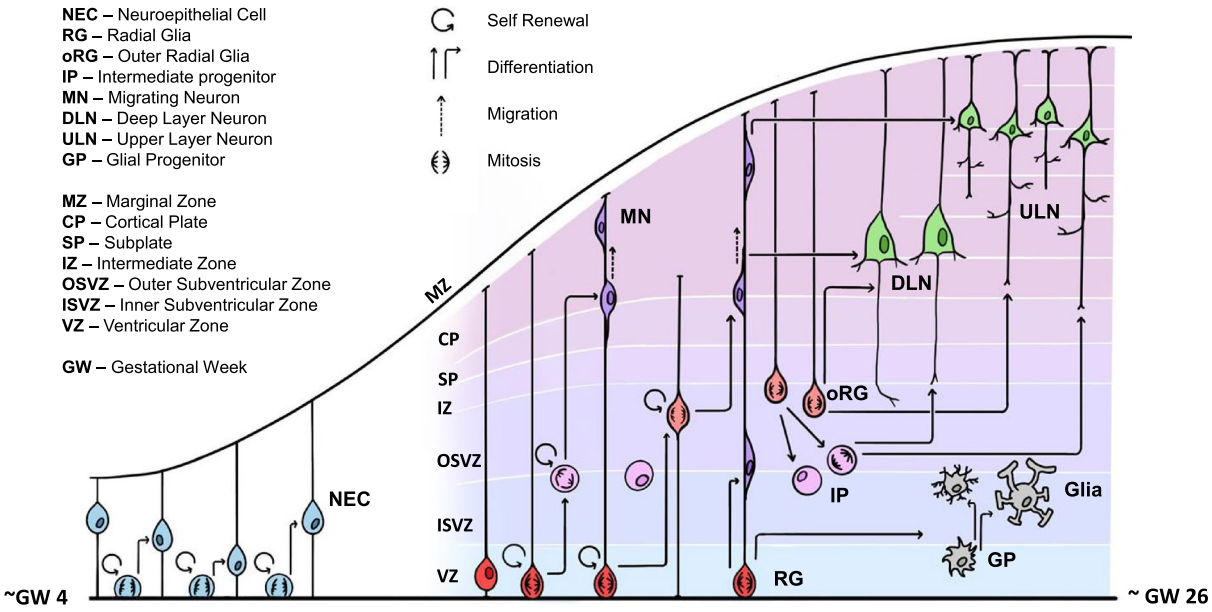

FIGURE 1 Cell types involved in corticogenesis. During corticogenesis, neurons are born in the ventricular zone (VZ), where radial glial cells (RGCs) undergo asymmetric divisions to self-renew and generate intermediate progenitors, these in turn go on to divide into migratory neurons. These migratory neurons are guided by RGC projections to their final positions within the six layered cortex in an inside-out fashion, where earlier born neurons populate the deeper layers, and later born neurons populate the upper layers. The outer subventricular zone (OSVZ) is a germinal area unique to primates and contains outer radial glial cells (oRGs) which constitute around half of the progenitors in primates, but only a fraction in rodents (Dehay et al., 2015). This progenitor is pool widely held to be responsible for brain expansion in primate species. Figure adapted from Molnár et al. (2019)

(Figure 1) (Götz & Huttner, 2005; Mayer et al., 2018; Nowakowski et al., 2017), with interneurons (appearing in the subpallium before migrating into the cerebral cortex) and maturing later than excitatory neurons (around gestational weeks 26 and 16 respectively) (Zhong et al., 2018). Organoid protocols can generate the major cell types identified in the developing human cortex between gestational weeks 6 and 26, including neuroepithelial cells, radial glia, outer radial glial cells (oRGCs), intermediate progenitors, immature neurons, cortical excitatory and inhibitory neurons, astrocytes, and oligodendrocytes (Figure 1) (Bhaduri et al., 2020; Fiddes et al., 2018; Giandomenico et al., 2019; Quadrato et al., 2017; Tanaka et al., 2020; Velasco et al., 2019; Xiang et al., 2017; Zhong et al., 2018). Organoids also recapitulate known cellular features of in vivo neurogenesis such as interkinetic nuclear migration and radial migration (Figure 1) (Bershteyn et al., 2017; Qian et al., 2018, 2020). They present a valuable opportunity to investigate processes, such as the cellular behavior of the oRGC population, that are essential for human cortical expansion and might be affected in neurodevelopmental disorders (Bershteyn et al., 2017).

Although these studies found broad correspondence between events unfolding in cortical organoids and embryonic cortex, future work will need to address the challenge raised by recent work suggesting that, at a more detailed level, the molecular identities of layer-specific and other differentiating cell populations in cortical organoids do not recapitulate the molecular signatures of the equivalent cell populations in vivo (Bhaduri et al., 2020). Furthermore, these authors found that cortical layers in organoids only display a rudimentary spatial separation, suggesting that neuronal migration is not optimized. Existing protocols appear to generate cortical organoids with important limitations for the study of later events in corticogenesis.

4.2 | The development of other brain regions

Unlike the free-patterning methods of generating cerebral organoids, adding growth factors or morphogens allows for their directed differentiation into specific brain regions (Figure 2). Table 1 summarizes the patterning molecules used to generate region-specific human organoids, the markers used to recognize them and their features.

4.2.1 | Subpallium

A subpallial identity is promoted in organoids subject to a combination of WNT inhibition together with enhanced SHH signaling. This identity is evidenced by gene expression profiles characteristic of the ganglionic eminence (GE) subregions that produce different interneuron subtypes (Bagley et al., 2017). Cytoarchitectural examination of subpallial

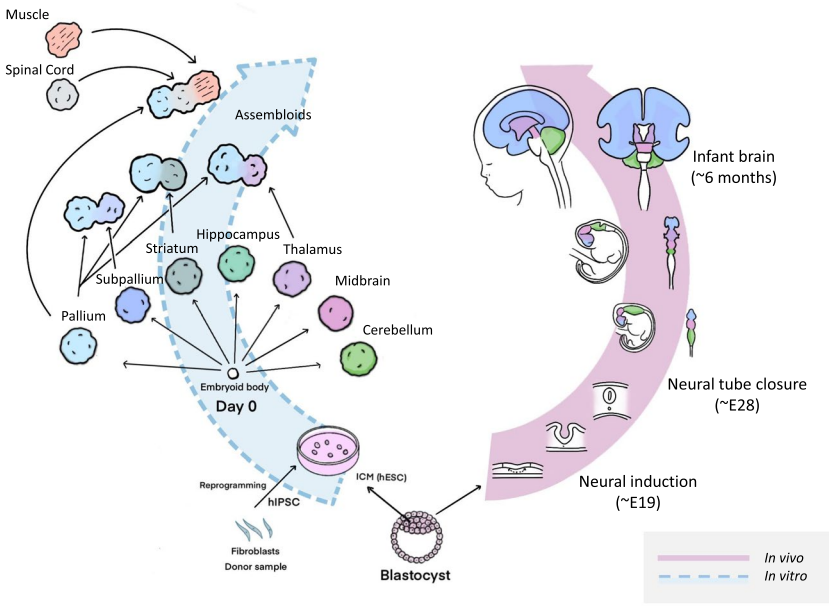

FIGURE 2 Generation of region-specific brain organoids to model regional interactions of the human brain. Despite the advancements in organoid protocols, cerebral organoids reaching a few millimeters in size are still far from resembling a fully functional human brain with interconnected regions. Using growth factors or morphogens to direct the differentiation of PSCs into region-specific organoids, however, has enabled us to recapitulate individual brain regions separately, which can then be fused into assembloids to study cellular interactions and connectivity between the different regions, bringing us one step closer to mimicking the spatial complexity of the human brain, albeit the limitations. It might be then worth generating assembloids from more than two different brain regions to gain further insights into how well regional organoids resemble the developing brain and expand on the uses of this in vitro model. For region-specific patterning molecules, markers, and features please see Table 1

organoids revealed SOX2+ radial glial-like progenitors arranged in a layer resembling the embryonic ventricular zone (VZ) surrounded by an outer subventricular zone (OSVZ) and, at early stages, an inner subventricular zone (ISVZ)-like layer. The thickness of the VZ-like layer decreased during organoid development, mimicking a similar decrease during in vivo subpallial development (Birey et al., 2017; Hansen et al., 2013; Xiang et al., 2017). The subpallial organoids generated inhibitory interneuronal subtypes with functional activity in temporal patterns similar to those observed in vivo, including somatostatin (SST), calretinin (CR), calbindin (CB), and parvalbumin (PV) interneurons (Birey et al., 2017; Sloan et al., 2018; Xiang et al., 2017). The fusion of subpallial organoids with cortical organoids to generate so-called assembloids has allowed the study, with methods including time-lapse imaging, of the migration of inhibitory GABAergic interneurons from the subpallium into the cerebral cortex (Bagley et al., 2017; Birey et al., 2017; Sloan et al., 2018; Xiang et al., 2017).

4.2.2 | Striatum

Using a combination of Activin A, agonist of retinoid X receptors and inhibition of the WNT pathway using IWP-2 generate 3D spheroids that expressed high levels of the forebrain marker FOXG1 and LGE (which gives rise to the striatum) markers of DLX5, GSX2, CTIP2, and MEIS2. After culturing these spheroids for more than 80 days, scRNA-sequencing, morphological, and electrophysiological analyses showed that these spheroids contain medium spiny neurons that were electrophysiologically active. These striatal organoids were then co-cultured with cortical organoids to form cortico-striatal assembloids. In these assembloids, they found axonal projections from the cortical organoid which then formed synaptic connections (Miura et al., 2020).

4.2.3 | Hippocampus

The manipulation of WNT and bone morphogenetic protein (BMP) signaling can induce cerebral organoids to adopt a fate mimicking that of dorsomedial telencephalon, where the hippocampus forms (Chen et al., 1997; Sakaguchi et al., 2015; Xie et al., 2010). Upon dissociation, these organoids can generate hippocampal-like mature CA3 and granule neurons resembling those of the CA and DG, as well as astrocyte-like cells (Arnold & Trojanowski, 1996; Sakaguchi et al., 2015). The neurons in hippocampal organoids are also able to form

neuronal networks with functional synaptic connections (Sakaguchi et al., 2015).

4.2.4 | Thalamus

The addition of BMP7 and MEK inhibitors generates organoids that contain neurons with thalamic identity, which are functionally active (Shiraishi et al., 2017; Xiang et al., 2019). Single-cell RNA sequencing (scRNA-seq) revealed that thalamic organoids contain cells that express high levels of OTX2, TCF7L2, and GBX2, markers of thalamic identity. However, markers of other brain regions such as cortex or medial ganglionic eminence (MGE) are also expressed. The thalamus and MGE form in relative proximity, and these findings suggest overlap in the molecular cues that induce cell fates in these regions.

4.2.5 | Midbrain

Application of a combination of SHH agonists, FGF8, SMAD inhibitors, and GSK3β inhibitor induces the differentiation of human iPSCs into organoids with characteristics of midbrain (Arenas et al., 2015; Jo et al., 2016; Marton & Paşca, 2016). At later stages, these organoids produce mature, functional tyrosine hydroxylase-expressing (TH+) dopaminergic neurons. A population of TH+cells also express additional markers that are enriched in A9 and A10 midbrain dopaminergic neurons (Jo et al., 2016; Marton & Paşca, 2016).

4.2.6 | Cerebellum

In the presence of high fibroblast growth factor 2 (FGF2), insulin, FGF19, and SDF1, cellular aggregates develop into cerebellum-like organoids displaying an elongated, polarized cerebellar neuroepithelium with a rhombic-lip region containing granule progenitors at one end and a three-layered cytoarchitecture that is reminiscent of the embryonic cerebellum in the first trimester (Muguruma et al., 2015). They develop cerebellar cell types including Golgi cells, granule cells, deep cerebellar nuclei (DCN) projection neurons, and Purkinje cells (Muguruma et al., 2015; Pasca, 2018). After long-term culture, mature GABA-releasing Purkinje cells bearing single long axons, elaborate dendritic branches and spines with functional electrophysiological properties appear, mimicking mature Purkinje cells in embryonic cerebellar plate (Morales & Hatten, 2006; Muguruma et al., 2010, 2015; Raman & Bean, 1999; Williams et al., 2002).

4.3 | Neural circuit formation

Functional neural circuits are formed through the stepwise process of cell migration, axonal projection, synapse formation, and synapse elimination (Matsui et al., 2020). Recent studies have found that several of those steps can be studied using organoids. The integration of inhibitory GABAergic interneurons into functional neural circuits with excitatory glutamatergic neurons in fused pallial-subpallial assembloids has been demonstrated (Birey et al., 2017; Xiang et al., 2017), showing that neurons in organoids are capable of cell migration, axonal projection, dendritic growth, and synapse formation. Similarly, other studies have reported reciprocal connections between thalamic and cortical organoids in thalamocortical assembloids. This work produced the hypothesis that neuronal circuits play a role in the maturation of the intrinsic properties of thalamic neurons, because it was found that fusion of thalamic organoids with cortical organoids increased the frequency of electrophysiologically mature neurons in the thalamic tissue (Xiang et al., 2019). Furthermore, other studies found that neurons in organoids show spontaneous network formation producing periodic and regular oscillatory events dependent on glutamatergic and GABAergic signaling, reaffirming that neurons in organoids develop axons, synapses, and mature functional properties (Birey et al., 2017; Izsak et al., 2020; Trujillo et al., 2019; Xiang et al., 2017, 2019). A recent study has pushed the boundaries of using organoids to model neural circuit formation even further by making cortico-motor assembloids that comprised of three organoids fused together (cortical-spinal-muscle). They showed that corticofugal neurons project and connect with spinal spheroids, whereas spinal-derived motor neurons connect with muscle cells. When they stimulated the cortical spheroids, they found that it triggers robust contraction of the muscle cells. Their work found the possibility of using organoids to model a multi-synaptic circuit (Andersen et al., 2020). However, organoids have not been used to investigate the process of synapse elimination or synaptic pruning. This is because during the process of neural circuit formation, inactive synapses are removed mainly by microglial cells (Paolicelli et al., 2011; Schafer et al., 2012), and brain organoids are not expected to have microglial cells as they are yolk sac-derived mesodermal cells (Ginhoux et al., 2013) whereas brain organoids are established by induction of the neuroectoderm.

5 | CURRENT CHALLENGES

Organoids have enormous potential, but their effective use depends critically on an understanding of existing limitations. Here we highlight some major current challenges.

5.1 | Cellular stress

Bhaduri et al., found that the molecular signatures of developing cortical layer-specific cell populations are not recapitulated in cortical organoids, and proposed that this was due partly to environmentally induced cellular stress. Their evidence was that cells in the organoids activated glycolysis and endoplasmic reticulum (ER) stress genes, whereas this was reversed when the organoid cells were transplanted into living brains (Bhaduri et al., 2020). Their conclusion was supported by another recent study that has analyzed transcriptomic data from most of the published cortical organoid protocols (Tanaka et al., 2020). By contrast, in vivo fetal tissue showed little expression of stress-related genes at corresponding developmental stages (Bhaduri et al., 2020; Tanaka et al., 2020).

Dysregulation of glycolysis and ER stress genes in organoids has been attributed to the depletion of nutrients and inadequate oxygen diffusion to the organoid core due to the lack of vascularization (Pollard, et al., 2006; Tanaka et al., 2020). A recent study has found a possible solution to this and it involves slicing forebrain organoids during the culture period, thereby exposing the core (Qian et al., 2020). This addressed the problem of interior hypoxia and reduced cell death. The cytoarchitecture of the cortical layers was much improved, with clear separation of layers and improved cellular subtype marker gene expression. Furthermore, they found morphologically distinct astrocyte subtypes in laminar distributions resembling those in the developing human cerebral cortex (Qian et al., 2020). Another possible solution might involve the use of microfluidic perfusion systems and the addition of vascular endothelial cells, not only to overcome the limits on organoid growth potential but also to provide a means to investigate the cross-talk between neuronal and non-neuronal tissue, a feature that current models lack (Lancaster & Knoblich, 2014; Qian et al., 2016). Hybrid neurovascular spheroids have been generated and they might provide insight into such interactions (Song et al., 2019).

5.2 | Developmental bypasses

As discussed above, the broad categories of early developing cell types produced in human cortical organoids represent those found in human embryonic cortex (Bhaduri et al., 2020; Birey et al., 2017; Qian et al., 2020; Quadrato et al., 2017; Tanaka et al., 2020; Velasco et al., 2019; Xiang et al., 2019), but an important question for understanding developmental mechanisms is whether equivalent compositions are achieved through different routes. Analysis of scRNA datasets from different cortical organoid protocols alongside those from human fetal tissue suggested that multiple protocol-dependent developmental bypasses occur in the organoids (Figure 3), most likely outlining alternative differentiation routes that could exist during brain development (Tanaka et al., 2020). Three different bypasses with distinct transcriptional dynamics have been highlighted by Tanaka et al., during the differentiation trajectory into postmitotic neurons and glia from proliferative neuroepithelial cells. Although some differentiation routes, such as those enriched for cell cycle regulators or insulin response genes, seemed to be protocol-specific, another bypass enriched for proliferation genes and early neurogenesis transcription factors was universally adopted in all protocols and considered a "major" neuronal differentiation route. Given the shorter developmental periods, the absence of cross-talk with non-neuronal tissue and the evidently smaller size and number

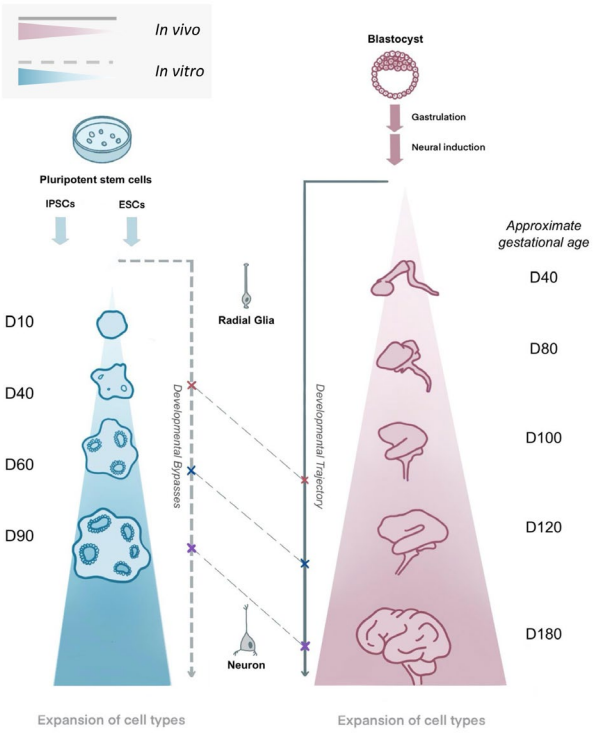

FIGURE 3 Comparison between organoid and human fetal brain development. Organoids mimic the terminal cellular composition of primary tissue, with a relatively consistent transcriptional profile for the broader cell types that recapitulates major developmental timepoints and areal signature of the brain. However, owing to the shorter developmental periods, the evidently smaller size and number of cells compared with a fully developed embryonic brain and the lack of cross-talk with non-neuronal tissue, alternative differentiation routes are selectively induced during organoid development to achieve this broad cell diversity. It is, therefore, evident that the cellular specification and diversification processes are highly constrained, even ex vivo, despite the adoption of developmental bypasses which could be a result or a cause of the elevation of cellular stress pathways due to culture conditions (Tanaka et al., 2020). However, this renders the exact recapitulation of appropriate progenitor maturation and specific subtype specification difficult to achieve using organoids. Figure adapted from Kelava and Lancaster (2016)

of cells compared with a fully developed embryonic brain, it is possible that such alternative differentiation routes are selectively induced during organoid development to achieve this broad cell diversity, highlighting a highly constrained cell-fate transition. We do need to caution that despite the stark resemblance between region-specific brain organoids and the human brain, it remains unclear whether the in vitro developmental trajectory exactly recapitulates in vivo development and maturation processes especially during later development.

5.3 | Scalability

The need for laborious manual manipulation of organoid cultures, their heterogeneity, and batch-to-batch variation make it difficult to adapt current organoid culturing protocols for high-throughput applications (Ao et al., 2020; Lancaster et al., 2017; Marton & Paşca, 2016). To try to overcome this, some laboratories have directed their efforts toward the design of synthetic matrices that correctly mimic the human brain's external niche. Information on the mechanical properties of brain tissues as well as the molecular composition of the extracellular matrix (ECM) has led to the synthesis of new synthetic hydrogels that can help produce organoids with less variability and higher reproducibility (Oksdath et al., 2018; Tekin et al., 2018). Microglia are critical components of the brain niche, and their absence in organoids remains a limitation that needs to be overcome (Bejoy et al., 2019; Song et al., 2019). This may be overcome by allowing them to develop within the organoid by omitting SMAD inhibition (Ormel et al., 2018) or by co-culturing organoids with iPSCs-derived microglia (Song et al., 2019).

6 | THE EVOLUTION OF NEURODEVELOPMENTAL MECHANISMS

Cerebral organoids are emerging as a powerful tool to study the differences between developing human and non-human primate (NHP) brains (Mostajo-Radji et al., 2020). Neurogenesis takes significantly longer in humans than in NHPs (Zhu et al., 2018), demonstrated by comparisons between human with NHP in both organoid and 2D culture systems, which have consistently shown that human-derived neural cells undergo more rounds of cell division, slower rates of migration and maturation, and delayed onset of neural activity (Marchetto et al., 2019; Muchnik et al., 2019; Otani et al., 2016). Cerebral organoids offer the potential to explore the mechanisms responsible for intrinsic differences such as these in the cells of humans versus those of other species. Here we illustrate this potential with some recent examples.

The ability of organoids to preserve gene regulatory networks and developmental processes allows human-specific gene expression patterns to be investigated. Pollard and et al. (2006) compared gene expression in human fetal tissue, human cerebral organoids, chimp cerebral organoids and macaque fetal tissue. They identified 261 genes whose expression levels were different in both human fetal and organoid samples when compared with both chimpanzee organoids and macaque fetal tissue; many of these genes were overlapping recent genomic segmental duplications (Pollard, et al., 2006). Interestingly, the expression of 85% of these genes was specific to cortical development. They included regulators of PI3K-AKT-mTOR signaling—a complex intracellular signaling pathway important in regulating cell growth, differentiation, migration and survival—that are up-regulated in both human organoids and fetal cells (Jafari et al., 2019; Pollard, et al., 2006; Sánchez-Alegría et al., 2018). Pollard and et al. (2006) provided evidence that the PI3K-AKT-mTOR pathway is particularly strongly activated in the oRGCs of human organoids compared with those in chimpanzee organoids. This has been investigated further with primary human brain samples and cortical organoids, where mTOR signaling was found to play a key role in regulating the morphology and migration of human oRGCs, contributing to normal radial architecture in the human cortex (Andrews et al., 2020). As the oRGCs are thought to be responsible for the increased brain size of humans (Noctor et al., 2001; Rakic, 2003), these findings suggest that changes in the regulation of mTOR signaling, and the higher proportion of oRGCs it regulates, have contributed to expansion of the human cortex.

Radial glia are also reliant on NOTCH signaling, which regulates cortical progenitor proliferation and determines the final number of neurons in the mammalian cortex. When the human-specific Notch homolog NOTCH2NL was deleted in human cortical organoids, their sizes were reduced and there was premature differentiation of neuronal progenitors (Fiddes et al., 2018). This supported the role of NOTCH2NL in delaying differentiation, thereby prolonging the proliferation of cortical progenitors and increasing their neuronal production (Suzuki et al., 2018).

These types of studies will provide insight into our fundamental understanding of the mechanisms unique to humans that explain the complexity of our brains. They should also highlight, and allow the study of, human-specific molecular pathways whose disruption is likely to increase the risk of neurodevelopmental disorders. This will be particularly important for disorders such as autism spectrum disorders that affect high level cognitive functions and are difficult to model adequately in animal models, a topic we have recently reviewed elsewhere (Chan et al., 2020).

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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REFERENCES

Andersen, J., Revah, O., Miura, Y., Thom, N., Amin, N. D., Kelley, K. W., Singh, M., Chen, X., Thete, M. V., Walczak, E. M., Vogel, H., Fan, H. C., & Paşca, S. P. (2020). Generation of functional human 3D cortico-motor assembloids. *Cell*, *183*, 1913–1929.e26. https://doi.org/10.1016/j.cell.2020.11.017

Andrews, M. G., Subramanian, L., & Kriegstein, A. R. (2020). mTOR signaling regulates the morphology and migration of outer radial glia in developing human cortex. *eLife*, *9*, e58737https://doi.org/10.7554/eLife.58737

Ao, Z., Cai, H., Havert, D. J., Wu, Z., Gong, Z., Beggs, J. M., Mackie, K., & Guo, F. (2020). One-stop microfluidic assembly of human brain organoids to model prenatal cannabis exposure. *Analytical Chemistry*, *92*, 4630–4638. https://doi.org/10.1021/acs.analchem.0c00205

Arenas, E., Denham, M., & Villaescusa, J. C. (2015). How to make a midbrain dopaminergic neuron. *Development*, *142*, 1918–1936. https://doi.org/10.1242/dev.097394

Arnold, S. E., & Trojanowski, J. Q. (1996). Human fetal hippocampal development: I. Cytoarchitecture, myeloarchitecture, and neuronal morphologic features. *The Journal of Comparative Neurology*, *367*, 274–292.

Bae, B.-I., Jayaraman, D., & Walsh, C. A. (2015). Genetic changes shaping the human brain. *Developmental Cell*, *32*, 423–434. https://doi.org/10.1016/j.devcel.2015.01.035

Bagley, J. A., Reumann, D., Bian, S., Levi-Strauss, J., & Knoblich, J. A. (2017). Fused cerebral organoids model interactions between brain regions. *Nature Methods*, *14*(7), 743–751. https://doi.org/10.1038/nmeth.4304

Bejoy, J., Yuan, X. G., Song, L. Q., Hua, E., Jeske, R., Sart, S., Sang, Q. X. A., & Li, Y. (2019). Genomics analysis of metabolic pathways of human stem cell-derived microglia-like cells and the integrated cortical spheroids. *Stem Cells International*, *2019*, 1–21. https://doi.org/10.1155/2019/2382534

Bershteyn, M., Nowakowski, T. J., Pollen, A. A., Di, L. E., Nene, A., Wynshaw-Boris, A., & Kriegstein, A. R. (2017). Human iPSC-derived cerebral organoids model cellular features of lissencephaly and reveal prolonged mitosis of outer radial glia. *Cell Stem Cell*, *20*, 435–449.e4. https://doi.org/10.1016/j.stem.2016.12.007

Bhaduri, A., Andrews, M. G., Mancia Leon, W., Jung, D., Shin, D., Allen, D., Jung, D., Schmunk, G., Haeussler, M., Salma, J., Pollen, A. A., Nowakowski, T. J., & Kriegstein, A. R. (2020). Cell stress in cortical organoids impairs molecular subtype specification. *Nature*, *578*, 142–148. https://doi.org/10.1038/s41586-020-1962-0

Birey, F., Andersen, J., Makinson, C. D., Islam, S., Wei, W. U., Huber, N., Fan, H. C., Metzler, K. R. C., Panagiotakos, G., Thom, N., O'Rourke, N. A., Steinmetz, L. M., Bernstein, J. A., Hallmayer, J., Huguenard, J. R., & Paşca, S. P. (2017). Assembly of functionally integrated human forebrain spheroids. *Nature*, *545*, 54–59. https://doi.org/10.1038/nature22330

Britten, R. J., & Davidson, E. H. (1969). Gene regulation for higher cells: A theory. *Science*, *165*(3891), 349–357. https://doi.org/10.1126/science.165.3891.349

Cederquist, G. Y., Asciolla, J. J., Tchieu, J., Walsh, R. M., Cornacchia, D., Resh, M. D., & Studer, L. (2019). Specification of positional identity in forebrain organoids. *Nature Biotechnology*, *37*, 436–444. https://doi.org/10.1038/s41587-019-0085-3

Chambers, S. M., Fasano, C. A., Papapetrou, E. P., Tomishima, M., Sadelain, M., & Studer, L. (2009). Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nature Biotechnology*, *27*, 275–280. https://doi.org/10.1038/nbt.1529

Chan, W. K., Griffiths, R., Price, D. J., & Mason, J. O. (2020). Cerebral organoids as tools to identify the developmental roots of autism. *Molecular Autism*, *11*(1). 58https://doi.org/10.1186/s13229-020-00360-3

Chen, H., Chédotal, A., He, Z., Goodman, C. S., & Tessier-Lavigne, M. (1997). Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. *Neuron*, *19*, 547–559. https://doi.org/10.1016/S0896-6273(00)80371-2

Dehay, C., Kennedy, H., & Kosik, K. S. (2015). The outer subventricular zone and primate-specific cortical complexification. *Neuron*, *85*, 683–694. https://doi.org/10.1016/j.neuron.2014.12.060

Dennis, M. Y., & Eichler, E. E. (2016). Human adaptation and evolution by segmental duplication. *Current Opinion in Genetics & Development*, *41*, 44–52. https://doi.org/10.1016/j.gde.2016.08.001

Eiraku, M., Watanabe, K., Matsuo-Takasaki, M., Kawada, M., Yonemura, S., Matsumura, M., Wataya, T., Nishiyama, A., Muguruma, K., & Sasai, Y. (2008). Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals. *Cell Stem Cell*, *3*, 519–532. https://doi.org/10.1016/j.stem.2008.09.002

Fang, Z., Liu, X., Wen, J., Tang, F., Zhou, Y., Jing, N., & Jin, Y. (2019). SOX21 ensures rostral forebrain identity by suppression of WNT8B during neural regionalization of human embryonic stem cells. *Stem Cell Reports*, *13*, 1038–1052. https://doi.org/10.1016/j.stemcr.2019.10.013

Fiddes, I. T., Lodewijk, G. A., Mooring, M., Bosworth, C. M., Ewing, A. D., Mantalas, G. L., Novak, A. M., van den Bout, A., Bishara, A., Rosenkrantz, J. L., Lorig-Roach, R., Field, A. R., Haeussler, M., Russo, L., Bhaduri, A., Nowakowski, T. J., Pollen, A. A., Dougherty, M. L., Nuttle, X., … Haussler, D. (2018). Human-specific NOTCH2NL genes affect notch signaling and cortical neurogenesis. *Cell*, *173*, 1356–1369.e22. https://doi.org/10.1016/j.cell.2018.03.051

Fischer, T., Guimera, J., Wurst, W., & Prakash, N. (2007). Distinct but redundant expression of the Frizzled Wnt receptor genes at signaling centers of the developing mouse brain. *Neuroscience*, *147*, 693–711. https://doi.org/10.1016/j.neuroscience.2007.04.060

Florio, M., & Huttner, W. B. (2014). Neural progenitors, neurogenesis and the evolution of the neocortex. *Development*, *141*, 2182–2194. https://doi.org/10.1242/dev.090571

Gaspard, N., Bouschet, T., Hourez, R., Dimidschstein, J., Naeije, G., Van Den, A. J., Espuny-Camacho, I., Herpoel, A., Passante, L., Schiffmann, S. N., Gaillard, A., & Vanderhaeghen, P. (2008). An intrinsic mechanism of corticogenesis from embryonic stem cells. *Nature*, *455*, 351–357. https://doi.org/10.1038/nature07287

Giandomenico, S. L., Mierau, S. B., Gibbons, G. M., Wenger, L. M. D., Masullo, L., Sit, T., Sutcliffe, M., Boulanger, J., Tripodi, M., Derivery, E., Paulsen, O., Lakatos, A., & Lancaster, M. A. (2019). Cerebral organoids at the air-liquid interface generate diverse nerve tracts with functional output. *Nature Neuroscience*, *22*, 669–679. https://doi.org/10.1038/s41593-019-0350-2

Ginhoux, F., Lim, S., Hoeffel, G., Low, D., & Huber, T. (2013). Origin and differentiation of microglia. *Frontiers in Cellular Neuroscience*, *7*, 45.

Gittelman, R. M., Hun, E., Ay, F., Madeoy, J., Pennacchio, L., Noble, W. S., Hawkins, R. D., & Akey, J. M. (2015). Comprehensive identification and analysis of human accelerated regulatory DNA. *Genome Research*, *25*, 1245–1255. https://doi.org/10.1101/gr.192591.115

Götz, M., & Huttner, W. B. (2005). The cell biology of neurogenesis. *Nature Reviews Molecular Cell Biology*, *6*, 777–788. https://doi.org/10.1038/nrm1739

Hansen, D. V., Lui, J. H., Flandin, P., Yoshikawa, K., Rubenstein, J. L., Alvarez-Buylla, A., & Kriegstein, A. R. (2013). Non-epithelial stem cells and cortical interneuron production in the human ganglionic eminences. *Nature Neuroscience*, *16*, 1576–1587. https://doi.org/10.1038/nn.3541

Hoang, B. H., Thomas, J. T., Abdul-Karim, F. W., Correia, K. M., Conlon, R. A., Luyten, F. P., & Ballock, R. T. (1998). Expression pattern of two Frizzled-related genes, Frzb-1 and Sfrp-1, during mouse embryogenesis suggests a role for modulating action of Wnt family members. *Developmental Dynamics: an Official Publication of the American Association of Anatomists*, *212*, 364–372.

Hubisz, M. J., & Pollard, K. S. (2014). Exploring the genesis and functions of Human Accelerated Regions sheds light on their role in human evolution. *Current Opinion in Genetics & Development*, *29*, 15–21. https://doi.org/10.1016/j.gde.2014.07.005

Imaizumi, K., Sone, T., Ibata, K., Fujimori, K., Yuzaki, M., Akamatsu, W., & Okano, H. (2015). Controlling the regional identity of hPSC-derived neurons to uncover neuronal subtype specificity of neurological disease phenotypes. *Stem Cell Reports*, *5*, 1010–1022. https://doi.org/10.1016/j.stemcr.2015.10.005

Izsak, J., Seth, H., Theiss, S., Hanse, E., & Illes, S. (2020). Human cerebrospinal fluid promotes neuronal circuit maturation of human induced pluripotent stem cell-derived 3D neural aggregates. *Stem Cell Reports*, *14*, 1044–1059. https://doi.org/10.1016/j.stemcr.2020.05.006

Jafari, M., Ghadami, E., Dadkhah, T., & Akhavan-Niaki, H. (2019). PI3k/AKT signaling pathway: Erythropoiesis and beyond. *Journal of Cellular Physiology*, *234*, 2373–2385. https://doi.org/10.1002/jcp.27262

Jo, J., Xiao, Y., Sun, A. X., Cukuroglu, E., Tran, H.-D., Göke, J., Tan, Z. Y., Saw, T. Y., Tan, C.-P., Lokman, H., Lee, Y., Kim, D., Ko, H. S., Kim, S.-O., Park, J. H., Cho, N.-J., Hyde, T. M., Kleinman, J. E., Shin, J. H., … Ng, H.-H. (2016). Midbrain-like organoids from human pluripotent stem cells contain functional dopaminergic and neuromelanin-producing neurons. *Cell Stem Cell*, *19*, 248–257. https://doi.org/10.1016/j.stem.2016.07.005

Kadoshima, T., Sakaguchi, H., Nakano, T., Soen, M., Ando, S., Eiraku, M., & Sasai, Y. (2013). Self-organization of axial polarity, inside-out layer pattern, and species-specific progenitor dynamics in human ES cell-derived neocortex. *Proceedings of the National Academy of Sciences*, *110*, 20284–20289. https://doi.org/10.1073/pnas.1315710110

Kanton, S., Boyle, M. J., He, Z., Santel, M., Weigert, A., Sanchís-Calleja, F., Guijarro, P., Sidow, L., Fleck, J. S., Han, D., Qian, Z., Heide, M., Huttner, W. B., Khaitovich, P., Pääbo, S., Treutlein, B., & Camp, J. G. (2019). Organoid single-cell genomic atlas uncovers human-specific features of brain development. *Nature*, *574*, 418–422. https://doi.org/10.1038/s41586-019-1654-9

Kelava, I., & Lancaster, M. A. (2016). Dishing out mini-brains: Current progress and future prospects in brain organoid research. *Developmental Biology*, *420*, 199–209. https://doi.org/10.1016/j.ydbio.2016.06.037

Kiecker, C., & Niehrs, C. (2001). A morphogen gradient of Wnt/beta-catenin signalling regulates anteroposterior neural patterning in Xenopus. *Development*, *128*, 4189–4201.

Kim, H., Xu, R. J., Padmashri, R., Dunaevsky, A., Liu, Y., Dreyfus, C. F., & Jiang, P. (2019). Pluripotent stem cell-derived cerebral organoids reveal human oligodendrogenesis with dorsal and ventral origins. *Stem Cell Reports*, *12*, 890–905. https://doi.org/10.1016/j.stemcr.2019.04.011

King, M. C., & Wilson, A. C. (1975). Evolution at two levels in humans and chimpanzees. *Science*, *188*, 107–116. https://doi.org/10.1126/science.1090005

Kirkeby, A., Grealish, S., Wolf, D. A., Nelander, J., Wood, J., Lundblad, M., Lindvall, O., & Parmar, M. (2012). Generation of regionally specified neural progenitors and functional neurons from human embryonic stem cells under defined conditions. *Cell Reports*, *1*, 703–714. https://doi.org/10.1016/j.celrep.2012.04.009

Krefft, O., Jabali, A., Iefremova, V., Koch, P., & Ladewig, J. (2018). Generation of standardized and reproducible forebrain-type cerebral organoids from human induced pluripotent stem cells. *Journal of Visualized Experiments*. *131*, e56768https://doi.org/10.3791/56768

Lancaster, M. A., Corsini, N. S., Wolfinger, S., Gustafson, E. H., Phillips, A. W., Burkard, T. R., Otani, T., Livesey, F. J., & Knoblich, J. A. (2017). Guided self-organization and cortical plate formation in human brain organoids. *Nature Biotechnology*, *35*, 659–666. https://doi.org/10.1038/nbt.3906

Lancaster, M. A., & Knoblich, J. A. (2014). Generation of cerebral organoids from human pluripotent stem cells. *Nature Protocols*, *9*, 2329–2340. https://doi.org/10.1038/nprot.2014.158

Lancaster, M. A., Renner, M., Martin, C. A., Wenzel, D., Bicknell, L. S., Hurles, M. E., Homfray, T., Penninger, J. M., Jackson, A. P., & Knoblich, J. A. (2013). Cerebral organoids model human brain development and microcephaly. *Nature*, *501*(7467), 373–379. https://doi.org/10.1038/nature12517

Le Bail, R., Bonafina, A., Espuny-Camacho, I., & Nguyen, L. (2021). Learning about cell lineage, cellular diversity and evolution of the human brain through stem cell models. *Current Opinion in Neurobiology*, *66*, 166–177.

Lui, J. H., Hansen, D. V., & Kriegstein, A. R. (2011). Development and evolution of the human neocortex. *Cell*, *146*, 18–36. https://doi.org/10.1016/j.cell.2011.06.030

Ma, T., Wang, C., Wang, L., Zhou, X., Tian, M., Zhang, Q., Zhang, Y., Li, J., Liu, Z., Cai, Y., Liu, F., You, Y., Chen, C., Campbell, K., Song, H., Ma, L., Rubenstein, J. L., & Yang, Z. (2013). Subcortical origins of human and monkey neocortical interneurons. *Nature Neuroscience*, *16*, 1588–1597. https://doi.org/10.1038/nn.3536

Marchetto, M. C., Hrvoj-Mihic, B., Kerman, B. E., Yu, D. X., Vadodaria, K. C., Linker, S. B., Narvaiza, I., Santos, R., Denli, A. M., Mendes, A. P. D., Oefner, R., Cook, J., McHenry, L., Grasmick, J. M., Heard, K., Fredlender, C., Randolph-Moore, L., Kshirsagar, R., Xenitopoulos, R., … Gage, H. (2019). Species-specific maturation profiles of human, chimpanzee and bonobo neural cells. *eLife*, *8*, e37527.

Marton, R. M., & Paşca, S. P. (2016). Neural differentiation in the third dimension: Generating a human midbrain. *Cell Stem Cell*, *19*, 145–146. https://doi.org/10.1016/j.stem.2016.07.017

Matsui T. K., Tsuru Y., Kuwako K.-I. (2020). Challenges in Modeling Human Neural Circuit Formation via Brain Organoid Technology.

Frontiers in Cellular Neuroscience, *14*, 607399https://doi.org/10.3389/fncel.2020.607399.

Mayer, C., Hafemeister, C., Bandler, R. C., Machold, R., Batista Brito, R., Jaglin, X., Allaway, K., Butler, A., Fishell, G., & Satija, R. (2018). Developmental diversification of cortical inhibitory interneurons. *Nature*, *555*, 457–462. https://doi.org/10.1038/nature25999

Miura, Y., Li, M. Y., Birey, F., Ikeda, K., Revah, O., Thete, M. V., Park, J. Y., Puno, A., Lee, S. H., Porteus, M. H., & Paşca, S. P. (2020). Generation of human striatal organoids and cortico-striatal assembloids from human pluripotent stem cells. *Nature Biotechnology*, *38*, 1421–1430. https://doi.org/10.1038/s41587-020-00763-w

Molnár, Z., Clowry, G. J., Šestan, N., Alzu'bi, A., Bakken, T., Hevner, R. F., Hüppi, P. S., Kostović, I., Rakic, P., Anton, E. S., Edwards, D., Garcez, P., Hoerder-Suabedissen, A., & Kriegstein, A. (2019). New insights into the development of the human cerebral cortex. *Journal of Anatomy*, *235*, 432–451. https://doi.org/10.1111/joa.13055

Morales, D., & Hatten, M. E. (2006). Molecular markers of neuronal progenitors in the embryonic cerebellar anlage. *Journal of Neuroscience*, *26*, 12226–12236. https://doi.org/10.1523/JNEUROSCI.3493-06.2006

Moris, N., Anlas, K., van den Brink, S. C., Alemany, A., Schröder, J., Ghimire, S., Balayo, T., van Oudenaarden, A., & Martinez, A. A. (2020). An in vitro model of early anteroposterior organization during human development. *Nature*, *582*, 410–415. https://doi.org/10.1038/s41586-020-2383-9

Mostajo-Radji, M. A., Schmitz, M. T., Montoya, S. T., & Pollen, A. A. (2020). Reverse engineering human brain evolution using organoid models. *Brain Research*, *1729*, 146582. https://doi.org/10.1016/j.brainres.2019.146582

Muchnik, S. K., Lorente-Galdos, B., Santpere, G., & Sestan, N. (2019). Modeling the evolution of human brain development using organoids. *Cell*, *179*, 1250–1253. https://doi.org/10.1016/j.cell.2019.10.041

Muguruma, K., Nishiyama, A., Kawakami, H., Hashimoto, K., & Sasai, Y. (2015). Self-organization of polarized cerebellar tissue in 3D culture of human pluripotent stem cells. *Cell Reports*, *10*, 537–550. https://doi.org/10.1016/j.celrep.2014.12.051

Muguruma, K., Nishiyama, A., Ono, Y., Miyawaki, H., Mizuhara, E., Hori, S., Kakizuka, A., Obata, K., Yanagawa, Y., Hirano, T., & Sasai, Y. (2010). Ontogeny-recapitulating generation and tissue integration of ES cell–derived Purkinje cells. *Nature Neuroscience*, *13*, 1171–1180. https://doi.org/10.1038/nn.2638

Noctor, S. C., Flint, A. C., Weissman, T. A., Dammerman, R. S., & Kriegstein, A. R. (2001). Neurons derived from radial glial cells establish radial units in neocortex. *Nature*, *409*, 714–720. https://doi.org/10.1038/35055553

Nordström, U., Jessell, T. M., & Edlund, T. (2002). Progressive induction of caudal neural character by graded Wnt signaling. *Nature Neuroscience*, *5*, 525–532. https://doi.org/10.1038/nn0602-854

Nowakowski, T. J., Bhaduri, A., Pollen, A. A., Alvarado, B., Mostajo-Radji, M. A., Di Lullo, E., Haeussler, M., Sandoval-Espinosa, C., Liu, S. J., Velmeshev, D., Ounadjela, J. R., Shuga, J., Wang, X., Lim, D. A., West, J. A., Leyrat, A. A., Kent, W. J., & Kriegstein, A. R. (2017). Spatiotemporal gene expression trajectories reveal developmental hierarchies of the human cortex. *Science*, *358*, 1318–1323. https://doi.org/10.1126/science.aap8809

O'Bleness, M., Searles, V. B., Varki, A., Gagneux, P., & Sikela, J. M. (2012). Evolution of genetic and genomic features unique to the human lineage. *Nature Reviews Genetics*, *13*, 853–866. https://doi.org/10.1038/nrg3336

Oksdath, M., Perrin, S. L., Bardy, C., Hilder, E. F., DeForest, C. A., Arrua, R. D., & Gomez, G. A. (2018). Review: Synthetic scaffolds to control the biochemical, mechanical, and geometrical environment of stem cell-derived brain organoids. *APL Bioengineering*, *2*(4), 041501. https://doi.org/10.1063/1.5045124

Ormel, P. R., Vieira de Sá, R., van Bodegraven, E. J., Karst, H., Harschnitz, O., Sneeboer, M. A. M., Johansen, L. E., van Dijk, R. E., Scheefhals, N., Berdenis van Berlekom, A., Ribes Martínez, E., Kling, S., MacGillavry, H. D., van den Berg, L. H., Kahn, R. S., Hol, E. M., de Witte, L. D., & Pasterkamp, R. J. (2018). Microglia innately develop within cerebral organoids. *Nature Communications*, *9*, 1–14. https://doi.org/10.1038/s41467-018-06684-2

Otani, T., Marchetto, M. C., Gage, F. H., Simons, B. D., & Livesey, F. J. (2016). 2D and 3D stem cell models of primate cortical development identify species-specific differences in progenitor behavior contributing to brain size. *Cell Stem Cell*, *18*, 467–480. https://doi.org/10.1016/j.stem.2016.03.003

Paolicelli, R. C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., Giustetto, M., Ferreira, T. A., Guiducci, E., Dumas, L., Ragozzino, D., & Gross, C. T. (2011). Synaptic pruning by microglia is necessary for normal brain development. *Science*, *333*(6048), 1456–1458. https://doi.org/10.1126/science.1202529

Paşca, A. M., Sloan, S. A., Clarke, L. E., Tian, Y., Makinson, C. D., Huber, N., Kim, C. H., Park, J.-Y., O'Rourke, N. A., Nguyen, K. D., Smith, S. J., Huguenard, J. R., Geschwind, D. H., Barres, B. A., & Paşca, S. P. (2015). Functional cortical neurons and astrocytes from human pluripotent stem cells in 3D culture. *Nature Methods*, *12*, 671–678. https://doi.org/10.1038/nmeth.3415

Pasca, S. P. (2018). The rise of three-dimensional human brain cultures. *Nature*, *553*, 437–445. https://doi.org/10.1038/nature25032

Pollard, K. S., Salama, S. R., King, B., Kern, A. D., Dreszer, T., Katzman, S., Siepel, A., Pedersen, J. S., Bejerano, G., Baertsch, R., Rosenbloom, K. R., Kent, J., & Haussler, D. (2006). Forces shaping the fastest evolving regions in the human genome. *PLoS Genetics*, *2*, e168. https://doi.org/10.1371/journal.pgen.0020168

Pollard, K. S., Salama, S. R., Lambert, N., Lambot, M.-A., Coppens, S., Pedersen, J. S., Katzman, S., King, B., Onodera, C., Siepel, A., Kern, A. D., Dehay, C., Igel, H., Ares, M., Vanderhaeghen, P., & Haussler, D. (2006). An RNA gene expressed during cortical development evolved rapidly in humans. *Nature*, *443*, 167–172. https://doi.org/10.1038/nature05113

Pollen, A. A., Bhaduri, A., Andrews, M. G., Nowakowski, T. J., Meyerson, O. S., Mostajo-Radji, M. A., Di Lullo, E., Alvarado, B., Bedolli, M., Dougherty, M. L., Fiddes, I. T., Kronenberg, Z. N., Shuga, J., Leyrat, A. A., West, J. A., Bershteyn, M., Lowe, C. B., Pavlovic, B. J., Salama, S. R., … Kriegstein, A. R. (2019). Establishing cerebral organoids as models of human-specific brain evolution. *Cell*, *176*, 743–756.e17. https://doi.org/10.1016/j.cell.2019.01.017

Prabhakar, S., Noonan, J. P., Pääbo, S., & Rubin, E. M. (2006). Accelerated evolution of conserved noncoding sequences in humans. *Science*, *314*, 786. https://doi.org/10.1126/science.1130738

Qian, X., Jacob, F., Song, M. M., Nguyen, H. N., Song, H., & Ming, G.-L. (2018). Generation of human brain region-specific organoids using a miniaturized spinning bioreactor. *Nature Protocols*, *13*, 565–580. https://doi.org/10.1038/nprot.2017.152

Qian, X., Nguyen, H. N., Song, M. M., Hadiono, C., Ogden, S. C., Hammack, C., Yao, B., Hamersky, G. R., Jacob, F., Zhong, C., Yoon, K.-J., Jeang, W., Lin, L. I., Li, Y., Thakor, J., Berg, D. A.,

Zhang, C. E., Kang, E., Chickering, M., … Ming, G.-L. (2016). Brain-region-specific organoids using mini-bioreactors for modeling ZIKV exposure. *Cell*, *165*, 1238–1254. https://doi.org/10.1016/j.cell.2016.04.032

Qian, X., Su, Y., Adam, C. D., Deutschmann, A. U., Pather, S. R., Goldberg, E. M., Su, K., Li, S., Lu, L. U., Jacob, F., Nguyen, P. T. T., Huh, S., Hoke, A., Swinford-Jackson, S. E., Wen, Z., Gu, X., Pierce, R. C., Wu, H., Briand, L. A., … Ming, G.-L. (2020). Sliced human cortical organoids for modeling distinct cortical layer formation. *Cell Stem Cell*, *26*(5), 766–781.e9. https://doi.org/10.1016/j.stem.2020.02.002

Quadrato, G., Nguyen, T., Macosko, E. Z., Sherwood, J. L., Min Yang, S., Berger, D. R., Maria, N., Scholvin, J., Goldman, M., Kinney, J. P., Boyden, E. S., Lichtman, J. W., Williams, Z. M., McCarroll, S. A., & Arlotta, P. (2017). Cell diversity and network dynamics in photosensitive human brain organoids. *Nature*, *545*, 48–53. https://doi.org/10.1038/nature22047

Rakic, P. (2003). Developmental and evolutionary adaptations of cortical radial glia. *Cerebral Cortex*, *13*, 541–549. https://doi.org/10.1093/cercor/13.6.541

Raman, I. M., & Bean, B. P. (1999). Ionic currents underlying spontaneous action potentials in isolated cerebellar Purkinje neurons. *Journal of Neuroscience*, *19*, 1663–1674. https://doi.org/10.1523/JNEUROSCI.19-05-01663.1999

Renner, M., Lancaster, M. A., Bian, S., Choi, H., Ku, T., Peer, A., Chung, K., & Knoblich, J. A. (2017). Self-organized developmental patterning and differentiation in cerebral organoids. *EMBO Journal*, *36*, 1316–1329. https://doi.org/10.15252/embj.201694700

Rhinn, M., Lun, K., Luz, M., Werner, M., & Brand, M. (2005). Positioning of the midbrain-hindbrain boundary organizer through global posteriorization of the neuroectoderm mediated by Wnt8 signaling. *Development*, *132*(6), 1261–1272. https://doi.org/10.1242/dev.01685

Rifes, P., Isaksson, M., Rathore, G. S., Aldrin-Kirk, P., Møller, O. K., Barzaghi, G., Lee, J., Egerod, K. L., Rausch, D. M., Parmar, M., Pers, T. H., Laurell, T., & Kirkeby, A. (2020). Modeling neural tube development by differentiation of human embryonic stem cells in a microfluidic WNT gradient. *Nature Biotechnology*, *38*(11), 1265–1273.

Roelink, H., & Nusse, R. (1991). Expression of two members of the Wnt family during mouse development–restricted temporal and spatial patterns in the developing neural tube. *Genes & Development*, *5*, 381–388. https://doi.org/10.1101/gad.5.3.381

Rowitch, D. H., & McMahon, A. P. (1995). Pax-2 expression in the murine neural plate precedes and encompasses the expression domains of Wnt-1 and En-1. *Mechanisms of Development*, *52*, 3–8. https://doi.org/10.1016/0925-4773(95)00380-J

Sakaguchi, H., Kadoshima, T., Soen, M., Narii, N., Ishida, Y., Ohgushi, M., Takahashi, J., Eiraku, M., & Sasai, Y. (2015). Generation of functional hippocampal neurons from self-organizing human embryonic stem cell-derived dorsomedial telencephalic tissue. *Nature Communications*, *6*, 1–11. https://doi.org/10.1038/ncomms9896

Sánchez-Alegría, K., Flores-León, M., Avila-Muñoz, E., Rodríguez-Corona, N., & Arias, C. (2018). PI3K signaling in neurons: A central node for the control of multiple functions. *International Journal of Molecular Sciences*, *19*, 3725. https://doi.org/10.3390/ijms19123725

Schafer, D. P., Lehrman, E. K., Kautzman, A. G., Koyama, R., Mardinly, A. R., Yamasaki, R., Ransohoff, R. M., Greenberg, M. E., Barres, B. A., & Stevens, B. (2012). Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. *Neuron*, *74*, 691–705. https://doi.org/10.1016/j.neuron.2012.03.026

Shi, Y., Wu, Q., & Wang, X. (2021). Modeling brain development and diseases with human cerebral organoids. *Current Opinion in Neurobiology*, *66*, 103–115.

Shiraishi, A., Muguruma, K., & Sasai, Y. (2017). Generation of thalamic neurons from mouse embryonic stem cells. *Development*, *144*(7), 1211–1220. https://doi.org/10.1242/dev.144071

Silver, D. L. (2016). Genomic divergence and brain evolution: How regulatory DNA influences development of the cerebral cortex. *BioEssays*, *38*, 162–171. https://doi.org/10.1002/bies.201500108

Sloan, S. A., Andersen, J., Pasca, A. M., Birey, F., & Pasca, S. P. (2018). Generation and assembly of human brain region-specific three-dimensional cultures. *Nature Protocols*, *13*, 2062–2085. https://doi.org/10.1038/s41596-018-0032-7

Smukler, S. R., Runciman, S. B., Xu, S., & Van Der, K. D. (2006). Embryonic stem cells assume a primitive neural stem cell fate in the absence of extrinsic influences. *Journal of Cell Biology*, *172*, 79–90. https://doi.org/10.1083/jcb.200508085

Song, L. Q., Yuan, X. G., Jones, Z., Vied, C., Miao, Y., Marzano, M., Hua, T., Sang, Q. X. A., Guan, J. J., Ma, T., Zhou, Y., & Li, Y. (2019). Functionalization of brain region-specific spheroids with isogenic microglia-like cells. *Scientific Reports*, *9*, 1–18. https://doi.org/10.1038/s41598-019-47444-6

Suzuki, I. K., Gacquer, D., Van, H. R., Kumar, D., Wojno, M., Bilheu, A., Herpoel, A., Lambert, N., Cheron, J., Polleux, F., Detours, V., & Vanderhaeghen, P. (2018). Human-specific NOTCH2NL genes expand cortical neurogenesis through delta/notch regulation. *Cell*, *173*, 1370–1384.e16. https://doi.org/10.1016/j.cell.2018.03.067

Takata, N., Sakakura, E., Eiraku, M., Kasukawa, T., & Sasai, Y. (2017). Self-patterning of rostral-caudal neuroectoderm requires dual role of Fgf signaling for localized Wnt antagonism. *Nature Communications*, *8*, 1339. https://doi.org/10.1038/s41467-017-01105-2

Tanaka, Y., Cakir, B., Xiang, Y., Sullivan, G. J., & Park, I. H. (2020). Synthetic analyses of single-cell transcriptomes from multiple brain organoids and fetal brain. *Cell Reports*, *30*(6), 1682–1689.e3. https://doi.org/10.1016/j.celrep.2020.01.038

Tekin, H., Simmons, S., Cummings, B., Gao, L., Adiconis, X., Hession, C. C., Ghoshal, A., Dionne, D., Choudhury, S. R., Yesilyurt, V., Sanjana, N. E., Shi, X. I., Lu, C., Heidenreich, M., Pan, J. Q., Levin, J. Z., & Zhang, F. (2018). Effects of 3D culturing conditions on the transcriptomic profile of stem-cell-derived neurons. *Nature Biomedical Engineering*, *2*(7), 540–554. https://doi.org/10.1038/s41551-018-0219-9

ten Berge, D., Koole, W., Fuerer, C., Fish, M., Eroglu, E., & Nusse, R. (2008). Wnt signaling mediates self-organization and axis formation in embryoid bodies. *Cell Stem Cell*, *3*, 508–518. https://doi.org/10.1016/j.stem.2008.09.013

Trujillo, C. A., Gao, R., Negraes, P. D., Gu, J., Buchanan, J., Preissl, S., Wang, A., Wu, W., Haddad, G. G., Chaim, I. A., Domissy, A., Vandenberghe, M., Devor, A., Yeo, G. W., Voytek, B., & Muotri, A. R. (2019). Complex oscillatory waves emerging from cortical organoids model early human brain network development. *Cell Stem Cell*, *25*, 558–569.e7. https://doi.org/10.1016/j.stem.2019.08.002

Velasco, S., Kedaigle, A. J., Simmons, S. K., Nash, A., Rocha, M., Quadrato, G., Paulsen, B., Nguyen, L., Adiconis, X., Regev, A., Levin, J. Z., & Arlotta, P. (2019). Individual brain organoids reproducibly form cell diversity of the human cerebral cortex. *Nature*, *570*, 523–527. https://doi.org/10.1038/s41586-019-1289-x

Williams, S. R., Christensen, S. R., Stuart, G. J., & Häusser, M. (2002). Membrane potential bistability is controlled by the hyperpolarization-activated current IH in rat cerebellar Purkinje neurons in vitro. *Journal of Physiology*, *539*, 469–483.

Xiang, Y., Tanaka, Y., Cakir, B., Patterson, B., Kim, K.-Y., Sun, P., Kang, Y.-J., Zhong, M., Liu, X., Patra, P., Lee, S.-H., Weissman, S. M., & Park, I.-H. (2019). hESC-derived thalamic organoids form reciprocal projections when fused with cortical organoids. *Cell Stem Cell*, *24*(3), 487–497.e7. https://doi.org/10.1016/j.stem.2018.12.015

Xiang, Y., Tanaka, Y., Patterson, B., Kang, Y.-J., Govindaiah, G., Roselaar, N., Cakir, B., Kim, K.-Y., Lombroso, A. P., Hwang, S.-M., Zhong, M., Stanley, E. G., Elefanty, A. G., Naegele, J. R., Lee, S.-H., Weissman, S. M., & Park, I.-H. (2017). Fusion of regionally specified hPSC-derived organoids models human brain development and interneuron migration. *Cell Stem Cell*, *21*(3), 383–398.e7. https://doi.org/10.1016/j.stem.2017.07.007

Xie, Z., Ma, X., Ji, W., Zhou, G., Lu, Y., Xiang, Z., Wang, Y. X., Zhang, L., Hu, Y., Ding, Y.-Q., & Zhang, W. J. (2010). Zbtb20 is essential for the specification of CA1 field identity in the developing hippocampus. *Proceedings of the National Academy of Sciences*, *107*, 6510–6515. https://doi.org/10.1073/pnas.0912315107

Yoon, S.-J., Elahi, L. S., Paşca, A. M., Marton, R. M., Gordon, A., Revah, O., Miura, Y., Walczak, E. M., Holdgate, G. M., Fan, H. C., Huguenard, J. R., Geschwind, D. H., & Paşca, S. P. (2019). Reliability of human cortical organoid generation. *Nature Methods*, *16*, 75–78. https://doi.org/10.1038/s41592-018-0255-0

Zhang, Y. E., & Long, M. (2014). New genes contribute to genetic and phenotypic novelties in human evolution. *Current Opinion in Genetics & Development*, *29*, 90–96. https://doi.org/10.1016/j.gde.2014.08.013

Zhong, S., Zhang, S., Fan, X., Wu, Q., Yan, L., Dong, J. I., Zhang, H., Li, L., Sun, L. E., Pan, N. A., Xu, X., Tang, F., Zhang, J., Qiao, J., & Wang, X. (2018). A single-cell RNA-seq survey of the developmental landscape of the human prefrontal cortex. *Nature*, *555*, 524–528. https://doi.org/10.1038/nature25980

Zhu, Y., Sousa, A. M. M., Gao, T., Skarica, M., Li, M., Santpere, G., Esteller-Cucala, P., Juan, D., Ferrández-Peral, L., Gulden, F. O., Yang, M. O., Miller, D. J., Marques-Bonet, T., Imamura Kawasawa, Y., Zhao, H., & Sestan, N. (2018). Spatiotemporal transcriptomic divergence across human and macaque brain development. *Science*, *362*(6420), eaat8077. https://doi.org/10.1126/science.aat8077

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