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# Using organoids to study human brain development and evolution

**Citation for published version:**
Chan, W-K, Fetit, R, Griffiths, R, Marshall, H, Mason, J & Price, D 2021, 'Using organoids to study human brain development and evolution', *Developmental neurobiology*, vol. 81, no. 5, pp. 608-622. https://doi.org/10.1002/dneu.22819

**Digital Object Identifier (DOI):**
10.1002/dneu.22819

**Link:**
Link to publication record in Edinburgh Research Explorer

**Document Version:**
Peer reviewed version

**Published In:**
Developmental neurobiology

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Download date: 24. Sep. 2022

Developmental
Neurobiology

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|---|---|
| Journal: | *Developmental Neurobiology* |
| Manuscript ID | DNEU-00071-2020.R1 |
| Wiley - Manuscript type: | Review Article |
| Date Submitted by the Author: | 18-Feb-2021 |
| Complete List of Authors: | Chan, Wai Kit; University of Edinburgh, Centre for Discovery Brain Sciences<br>Fetit, Rana; University of Edinburgh, Centre for Discovery Brain Sciences<br>Griffiths, Rosie; University of Edinburgh, Centre for Discovery Brain Sciences<br>Marshall, Helen; University of Edinburgh, Centre for Discovery Brain Sciences<br>Mason, John; University of Edinburgh, Centre for Discovery Brain Sciences<br>Price, David; University of Edinburgh, Centre for Discovery Brain Sciences |
| Key Words: | cerebral organoids, human cortical development, human cortical evolution, human embryonic neurodevelopment |
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1 # §Using organoids to study human brain development and evolution

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15 ## Abstract

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

Keywords: cerebral organoids; human cortical development; human cortical evolution; human embryonic neurodevelopment

## 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 to that of even our closest living relatives. During embryogenesis, important anatomical differences emerge between the developing brains of humans and other mammals (Lui et al., 2011; Florio and Huttner, 2014) . While 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 (Pollard et al., 2006a, 2006b; Prabhakar et al., 2006; O'Bleness et al., 2012; Hubisz and Pollard, 2014; Zhang and Long, 2014; Bae et al., 2015; Gittelman et al., 2015; Dennis and Eichler, 2016; Silver, 2016). These human-specific genomic features are almost certainly involved in human-specific gene regulatory mechanisms that account for the morphological, physiological, and behavioural divergence between our brains and those of our closest relatives (Britten and Davidson, 1969; King and 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 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; Pollen et al., 2019). Further examples will

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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.

## 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 (Paşca et al., 2015; Qian et al., 2016; Birey et al., 2017; Xiang et al., 2017, 2019; Giandomenico et al., 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 localised signalling centres has also been used to direct organoid differentiation (Takata et al., 2017; Cederquist et al., 2019). 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.

## Using organoids to probe early human neurodevelopmental processes



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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.

**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 signalling 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.

**Rostral-caudal regionalisation**

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 mechanisms by which R-C regionalization occurs in humans remains a mystery. In non-human species, one major signalling pathway implicated in R-C regionalization is Wnt signalling, with many Wnt signalling genes showing spatially restricted expression patterns during early brain development (Roelink and Nusse, 1991; Rowitch and McMahon, 1995; Hoang et al., 1998; Fischer et al., 2007). The addition of Wnt agonists caudalises neuroectodermal cells, including those derived from human stem cells, in a dose-dependent manner (Kiecker and Niehrs, 2001; Nordström et al., 2002; Rhinn



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3 114 et al., 2005; Kirkeby et al., 2012; Imaizumi et al., 2015; Fang et al., 2019; Rifes et al., 2020).
4
5 115 Notably, in the experiments of Rifes et al., (2020) on human cells, expression of regionalized
6
7 116 markers appeared very early, after only a day of differentiation, a day before neural-specific genes
8
9 117 became expressed, suggesting that regionalization occurred before neural specification. This
10
11 118 interesting result needs further investigation to establish whether it is a consistent finding.
12
13 119 The importance of WNT signalling in the R-C regionalization of the human neuroectoderm
14
15 120 remains unclear. In non-human species, the use of 3D culture systems in which aggregates of stem
16
17 121 cells self-organize along an axis has made it possible to investigate these processes. Studies using
18 122 aggregates of mouse embryonic stem cells (mESCs) revealed that after only three days of culture,
19
20 123 aggregates showed localised Wnt activity (ten Berge et al., 2008) and self-organized along the R-
21
22 124 C axis (Takata et al., 2017). This was preceded by expression of Fgf5, inhibition of which
23
24 125 prevented polarisation and produced rostralised aggregates. Activation of Fgf led to caudalisation
25
26 126 and increased expression of Wnt genes. This work revealed that an interplay between Fgf and Wnt
27
28 127 signalling in neuroectoderm is key to R-C patterning, suggesting that the initial appearance of a
29 128 localised population of $Fgf5^{+}$ cells induces and combines with localized expression of Wnt
30
31 129 signalling components to repress rostral fates caudally. In the human gastruloid study (Moris et
32
33 130 al., 2020), pre-treatment of hPSCs with the WNT activator CHIR99021 was essential for axis
34
35 131 formation and could not be replaced with WNT3A and BMP4. While it is possible to generate a
36
37 132 R-C axis in *in vitro* models of human neural differentiation, these models all require extrinsic
38
39 133 signals (Cederquist et al., 2019; Rifes et al., 2020). Further insights from these models may provide
40
41 134 answers to the exact cellular signals required for axis formation during early human brain
42
43 135 development. Furthermore, being able to recapitulate embryo-like axes within organoids may
44 136 produce a better model of normal brain structure.

45 137 **Dorsal-ventral regionalization**
46
47 138 It is suspected that endogenous forebrain organizing centres that direct dorsal-ventral (D-V)
48
49 139 patterning arise spontaneously in some free-patterning forebrain human organoids (Lancaster et
50
51 140 al., 2013; Renner et al., 2017). The evidence in favour of this possibility is the observation in such
52
53 141 organoids of localized expression of signalling molecules important for D-V patterning such as
54
55 142 WNT2B and BMP6 (Renner et al., 2017). Moreover, the presence of such signalling centres has
56 143 been inferred from the fact that regions mimicking those specified along the D-V axis of the
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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; Lancaster et al., 2013; Ma et al., 2013; Renner et al., 2017; Sloan et al., 2018; Kim et al., 2019). Even though free-patterning organoids can develop organizing centres 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 centres and regions in the developing embryo. Achieving this will most likely require the application of external cues in a spatiotemporally controlled way.

## Using organoids to probe later human neurodevelopmental events

### 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, suggesting that its production is a default state achieved in the absence of externally applied molecular cues (**Table 1**). 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 (**Figure 1**) (Götz and Huttner, 2005; Nowakowski et al., 2017; Mayer et al., 2018), 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 to 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**) (Quadrato et al., 2017; Xiang et al., 2017; Fiddes et al., 2018; Zhong et al., 2018; Giandomenico et al., 2019; Velasco et al., 2019; Bhaduri et al., 2020; Tanaka et al., 2020). 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.,





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173 2018, 2020). They present a valuable opportunity to investigate processes, such as the cellular
174 behaviour of the oRGC population, that are essential for human cortical expansion and might be
175 affected in neurodevelopmental disorders (Bershteyn et al., 2017).

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

184 **The development of other brain regions**

185 Unlike the free-patterning methods of generating cerebral organoids, adding growth factors or
186 morphogens allows for their directed differentiation into specific brain regions **(Figure 2)**. **Table**
187 **1** summarizes the patterning molecules used to generate region-specific human organoids, the
188 markers used to recognize them and their features.

189 ***Subpallium*** A subpallial identity is promoted in organoids subject to a combination of WNT
190 inhibition together with enhanced SHH signalling. This identity is evidenced by gene expression
191 profiles characteristic of the ganglionic eminence (GE) subregions that produce different
192 interneuron subtypes (Bagley et al., 2017). Cytoarchitectural examination of subpallial organoids
193 revealed SOX2+ radial glial-like progenitors arranged in a layer resembling the embryonic
194 ventricular zone (VZ) surrounded by an outer subventricular zone (OSVZ) and, at early stages, an
195 inner subventricular zone (ISVZ)-like layer. The thickness of the VZ-like layer decreased during
196 organoid development, mimicking a similar decrease during *in vivo* subpallial development
197 (Hansen et al., 2013; Birey et al., 2017; Xiang et al., 2017). The subpallial organoids generated
198 inhibitory interneuronal subtypes with functional activity in temporal patterns similar to those
199 observed *in vivo*, including somatostatin (SST), calretinin (CR), calbindin (CB) and parvalbumin
200 (PV) interneurons (Birey et al., 2017; Xiang et al., 2017; Sloan et al., 2018). The fusion of
201 subpallial organoids with cortical organoids to generate so-called assembloids has allowed the
202 study, with methods including time-lapse imaging, of the migration of inhibitory GABAergic





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3 203 interneurons from the subpallium into the cerebral cortex (Bagley et al., 2017; Birey et al., 2017;
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5 204 Xiang et al., 2017; Sloan et al., 2018).
6
7 205 ***Striatum*** Using a combination of Activin A, agonist of retinoid X receptors and inhibiting the
8
9 206 WNT pathway using IWP-2 generates 3D spheroids that expressed high levels of the forebrain
10
11 207 marker FOXG1 and LGE (which gives rise to the striatum) markers of DLX5, GSX2, CTIP2 and
12
13 208 MEIS2. After culturing these spheroids for more than 80 days, scRNA-sequencing, morphological
14
15 209 and electrophysiological analyses showed that these spheroids contain medium spiny neurons that
16
17 210 were electrophysiologically active. These striatal organoids were then co-cultured with cortical
18
19 211 organoids to form cortico-striatal assembloids. In these assembloids, they found axonal projections
20
21 212 from the cortical organoid which then formed synaptic connections (Miura et al., 2020).
22
23 213 ***Hippocampus*** The manipulation of WNT and bone morphogenetic protein (BMP) signalling can
24
25 214 induce cerebral organoids to adopt a fate mimicking that of dorsomedial telencephalon, where the
26
27 215 hippocampus forms (Chen et al., 1997; Xie et al., 2010; Sakaguchi et al., 2015). Upon dissociation,
28
29 216 these organoids can generate hippocampal-like mature CA3 and granule neurons resembling those
30
31 217 of the CA and DG, as well as astrocyte-like cells (Arnold and Trojanowski, 1996; Sakaguchi et
32
33 218 al., 2015). The neurons in hippocampal organoids are also able to form neuronal networks with
34
35 219 functional synaptic connections (Sakaguchi et al., 2015).
36
37 220 ***Thalamus*** The addition of BMP7 and MEK inhibitors generates organoids that contains neurons
38
39 221 with thalamic identity that are functionally active (Shiraishi et al., 2017; Xiang et al., 2019). Single
40
41 222 cell RNA sequencing (scRNA-seq) revealed that thalamic organoids contain cells that express high
42
43 223 levels of OTX2, TCF7L2 and GBX2, markers of thalamic identity. However, markers of other
44
45 224 brain regions such as cortex or medial ganglionic eminence (MGE) are also expressed. The
46
47 225 thalamus and MGE form in relative proximity and these findings suggest overlap in the molecular
48
49 226 cues that induce cell fates in these regions.
50
51 227 ***Midbrain*** Applying a combination of SHH agonists, FGF8, SMAD inhibitors and GSK3β inhibitor
52
53 228 induces the differentiation of human iPSCs into organoids with characteristics of midbrain (Arenas
54
55 229 et al., 2015; Jo et al., 2016; Marton and Paşca, 2016). At later stages, these organoids produce
56
57 230 mature, functional tyrosine hydroxylase-expressing (TH+) dopaminergic neurons. A population of
58
59 231 TH+ cells also express additional markers that are enriched in A9 and A10 midbrain dopaminergic
60
232 neurons (Jo et al., 2016; Marton and Paşca, 2016).



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***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 (Raman and Bean, 1999; Williams et al., 2002; Morales and Hatten, 2006; Muguruma et al., 2010, 2015).

**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 showed 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, since it 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 showed that neurons in organoids show spontaneous network formation producing periodic and regular oscillatory events dependent on glutamatergic and GABAergic signalling reaffirming that neurons in organoids develop axons, synapses and mature functional properties (Birey et al., 2017; Xiang et al., 2017, 2019; Trujillo et al., 2019; Izsak et al., 2020). A recent study pushed the boundaries of using organoids to model neural circuit formation even further by making cortico-motor assembloids that comprised of 3 organoids fused together (cortical-spinal-muscle). They showed that corticofugal neurons project and connect with spinal spheroids, while 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



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shows 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) while brain organoids are established by induction of the neuroectoderm.

## 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.

### Cellular stress

Bhaduri et al., showed 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 analysed transcriptomic data from most of the published cortical organoid protocols (Tanaka et al., 2020). In 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 vascularisation (Pollen et al., 2019; Tanaka et al., 2020). A recent study showed a possible solution involved 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



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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 and Knoblich, 2014; Qian et al., 2016). Hybrid neurovascular spheroids have been generated and they might provide insight into such interactions (Song et al., 2019).

## 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 (Birey et al., 2017; Quadrato et al., 2017; Velasco et al., 2019; Xiang et al., 2019; Bhaduri et al., 2020; Qian et al., 2020; Tanaka et al., 2020), but an important question for understanding developmental mechanisms is whether equivalent compositions are achieved through different routes. Analysing 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 post-mitotic 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 of cells compared to 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.

## Scalability

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

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

## 334 The evolution of neurodevelopmental mechanisms

335 Cerebral organoids are emerging as a powerful tool to study the differences between developing
336 human and non-human primate (NHP) brains (Mostajo-Radji et al., 2020). Neurogenesis takes
337 significantly longer in humans than in NHPs (Zhu et al., 2018), demonstrated by comparisons
338 between human with NHP in both organoid and 2D culture systems, which have consistently
339 shown that human-derived neural cells undergo more rounds of cell division, slower rates of
340 migration and maturation, and delayed onset of neural activity (Otani et al., 2016; Marchetto et al.,
341 2019; Muchnik et al., 2019). Cerebral organoids offer the potential to explore the mechanisms
342 responsible for intrinsic differences such as these in the cells of humans versus those of other
343 species. Here we illustrate this potential with some recent examples.

344 The ability of organoids to preserve gene regulatory networks and developmental processes allows
345 human-specific gene expression patterns to be investigated. Pollen et al., (2019) compared gene
346 expression in human fetal tissue, human cerebral organoids, chimp cerebral organoids and
347 macaque fetal tissue. They identified 261 genes whose expression levels were different in both
348 human fetal and organoid samples when compared to both chimpanzee organoids and macaque
349 fetal tissue; many of these genes were overlapping recent genomic segmental duplications (Pollen
350 et al., 2019). Interestingly, the expression of 85% of these genes was specific to cortical
351 development. They included regulators of PI3K-AKT-mTOR signalling - a complex intracellular

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signalling pathway important in regulating cell growth, differentiation, migration and survival - that are up-regulated in both human organoids and fetal cells (Sánchez-Alegría et al., 2018; Jafari et al., 2019; Pollen et al., 2019). Pollen et al., (2019) provided evidence that the PI3K-AKT-mTOR pathway is particularly strongly activated in the oRGCs of human organoids compared to 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). Since 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 signalling, and the higher proportion of oRGCs it regulates, has contributed to expansion of the human cortex.

Radial glia are also reliant on NOTCH signalling, 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 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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**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)

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**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**.

**Figure 3: Comparison between organoid and human foetal 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 to a fully developed embryonic brain and the lack of cross-talk with non-neuronal tissue, alternative differentiation routes are selectively

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695 induced during organoid development to achieve this broad cell diversity. It is, therefore, evident
696 that the cellular specification and diversification processes are highly constrained, even *ex vivo*,
697 despite the adoption of developmental bypasses which could be a result or a cause of the elevation
698 of cellular stress pathways due to culture conditions (Tanaka et al., 2020). However, this renders
699 the exact recapitulation of appropriate progenitor maturation and specific subtype specification
700 difficult to achieve using organoids. Figure adapted from (Kelava and Lancaster, 2016).

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**Table1.** 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.<br><br>Generate diverse electrophysiologically active neuronal subtypes of all six cortical layers.<br><br>Differentiate into upper and deep cortical layers. | (Paşca et al., 2015; Qian et al., 2016; Lancaster et al., 2017; Renner et al., 2017; Xiang et al., 2017; Krefft et al., 2018; Yoon et al., 2019) |
| Sub-pallium | WNT inhibition, enhanced SHH signalling | 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.<br><br>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; Xiang et al., 2017; Sloan et al., 2018) |
| Hippocampus | manipulation of Wnt and bone | Nrp2, Lef1 (NE layers); Zbtb20 (hippocampal | Prox1 (granule neurons); KA1 (CA3 pyramidal neurons); | Neurons capable of forming neuronal networks with functional synaptic connections: voltage-dependent Na–K | (Sakaguchi et al., 2015) |

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| | morphogenetic protein (BMP) signalling | neurons and their precursors) | CaMKII (mature hippocampal neurons) | currents, action potential following injection of depolarizing currents, spontaneous excitatory postsynaptic currents present. | |
| Thalamus | BMP7 and MEK inhibitors, dual SMAD inhibition | OTX2, TCF7L2 and GBX2 (thalamic identity) | | Reciprocal thalamocortical (TC) connections observed when fused with cortical organoids.<br><br>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: NE neuroepithelium, CP cortical plate, IZ intermediate zone, MZ marginal zone, SP subplate, VZ ventricular zone, SVZ subventricular, RGs radial glia, IPs intermediate progenitors, dLGE dorsal lateral ganglionic eminence, CGE caudal ganglionic eminence, MGE medial ganglionic eminence, IN interneuron, DCN deep cerebellar nuclei.

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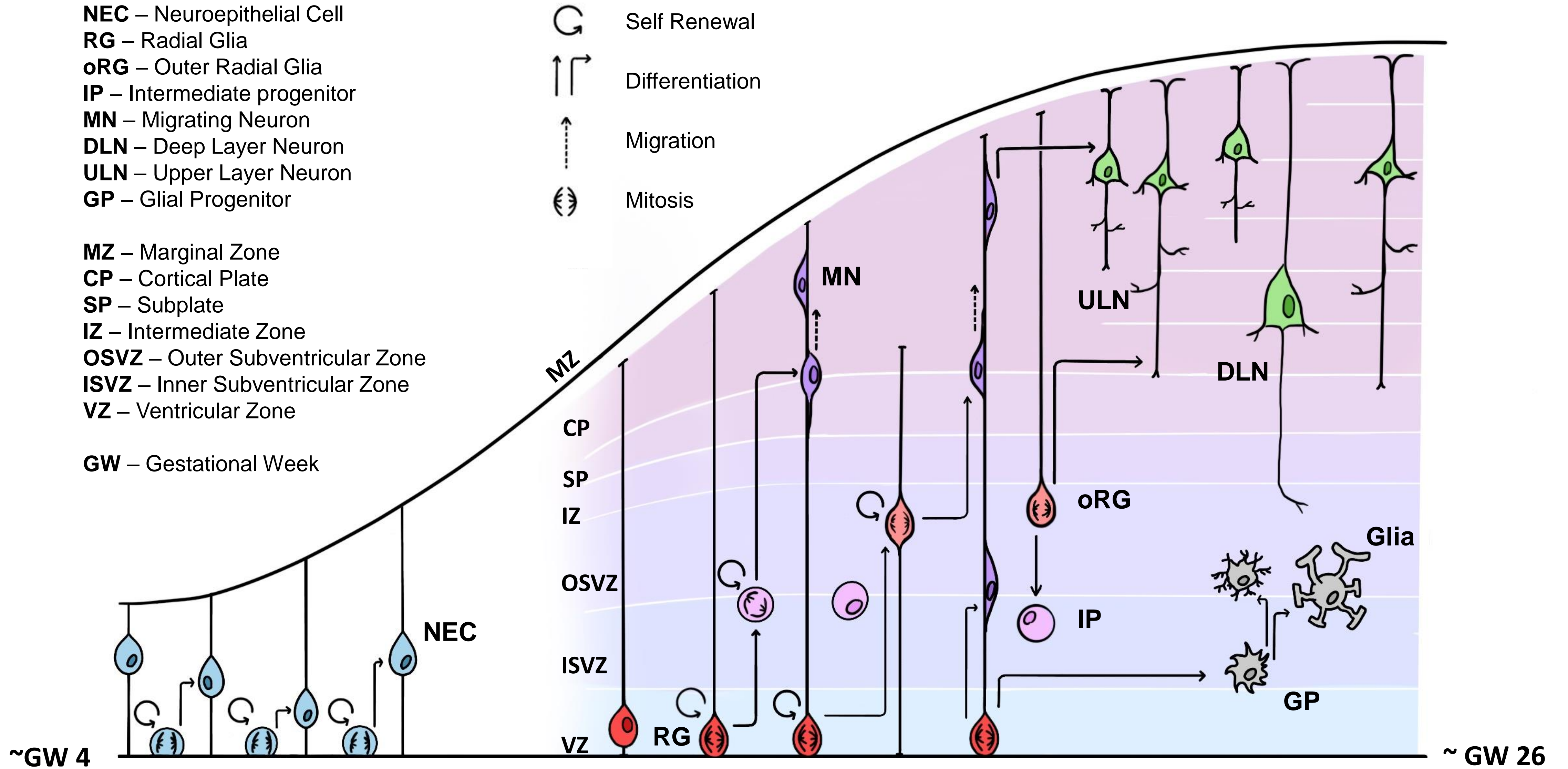

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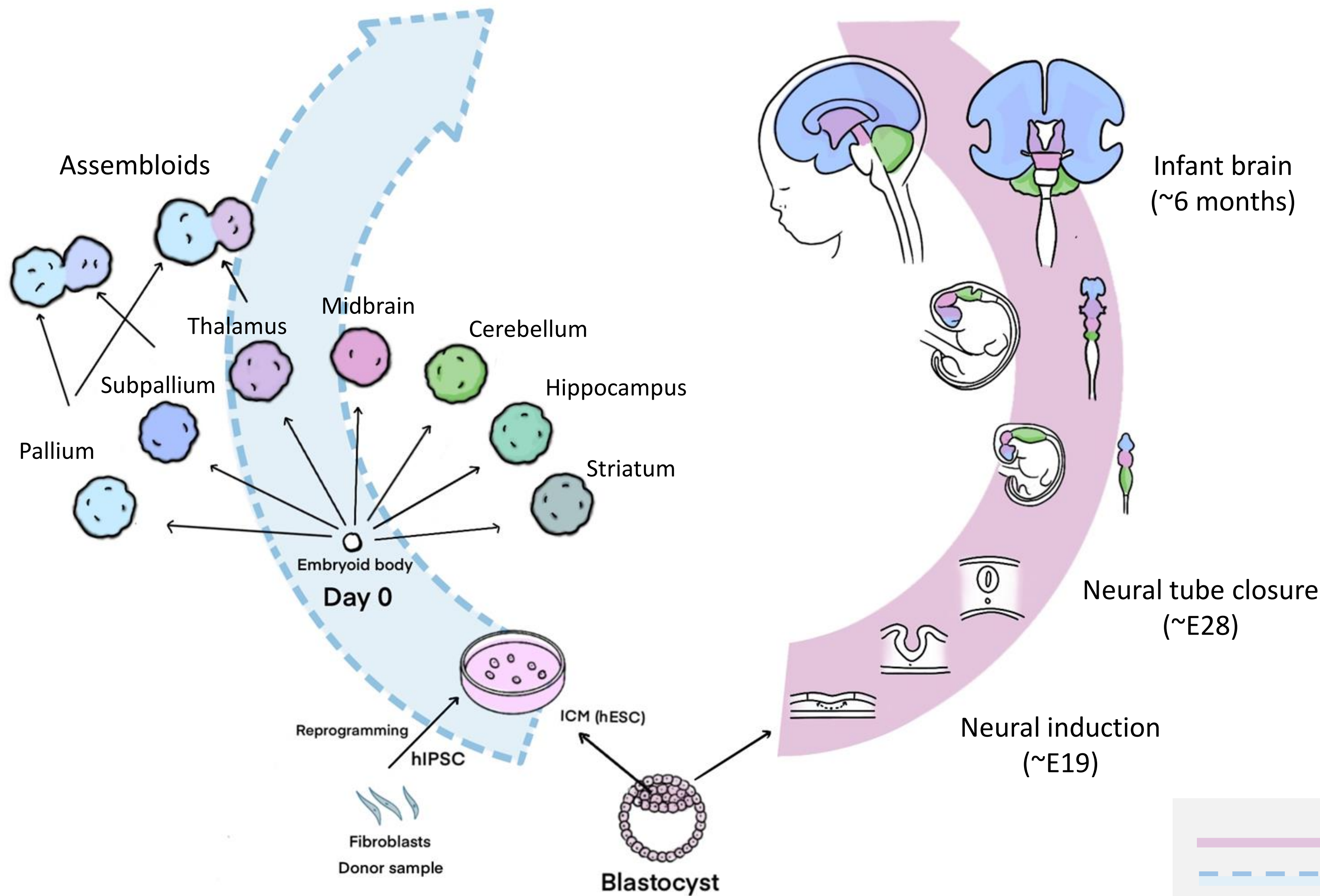


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