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Learning about cell lineage, cellular diversity and evolution of the human brain through stem cell models

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ABSTRACT

Here, we summarize the current knowledge on cell diversity in the cortex and other brain regions from *in vivo* mouse models and *in vitro* models based on pluripotent stem cells. We discuss the mechanisms underlying cell proliferation and temporal progression that leads to the sequential generation of neurons dedicated to different layers of the cortex. We highlight models of corticogenesis from stem cells that recapitulate specific transcriptional and connectivity patterns from different cortical areas. We overview state-of-the art of human brain organoids modeling different brain regions, and we discuss insights into human cortical evolution from stem cells. Finally, we interrogate human brain organoid models for their competence to recapitulate the essence of human brain development.

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Introduction

The cerebral cortex is populated by a great diversity of glutamatergic and GABAergic neuron subtypes that display specific electrophysiological, molecular, morphological and hodological properties. Excitatory projection neurons are born from dorsal progenitors within the developing cortex, whereas GABAergic inhibitory interneurons are generated in the caudal and medial ganglionic eminences (CGE and MGE, respectively), as well as in the preoptic area (POA). Cortical neurons are organized into layers that arise from an inside-out migration pattern across time. Early born neurons populate deep layers whereas late-born neurons invade upper layers of the cortex. The laminar, molecular and a real identity of a given excitatory cortical neuron defines its specific pattern of connectivity within the rest of the brain [1]. Single-cell transcriptomic studies have highlighted the remarkable diversity of cortical excitatory neurons that is partly conserved in humans and mice [2]. The sequential generation of distinct neural subtypes is also conserved across mammals. However, developmental paths may differ according to the existence of species-specific types of neural progenitors and specific timing for progenitor pool expansion, neurogenesis and ultimately neuronal maturation. In addition, the laminar distribution of neuronal subtypes [2] and the relative size of each layer in different brain areas varies in primates and rodents [3].

In this review, we summarize the current knowledge of the molecular, cellular and functional basis of neuronal diversity within the cortex, as well as species-specific evolutionary features learnt from mouse and human stem cell models, including those obtained from cortical organoids. We also highlight the latest efforts made to model distinct brain regions using human organoids and discuss how closely these structures recapitulate human brain development and function, as well as their potential value to study brain region-associated diseases.

Generation of neuronal diversity from cortical progenitor subtypes: what can we learn from stem cell models?

Distinct types of cortical progenitors born sequentially in specialized germinal regions around the ventricles give rise to all excitatory neurons across layers (**Figure 1a**). Neuroepithelial cells (NE) are the earliest progenitors that divide symmetrically to expand their pool in the ventricular zone (VZ). At the onset of neurogenesis, they generate neurogenic radial glia cells (RGCs) that initially divide symmetrically. As corticogenesis proceeds, they switch to an asymmetric division mode in order to self-renew and to generate either a neuron, or a neurogenic progenitor (intermediate progenitor (IP), short neural precursor (SNP), or outer radial glia cell (oRG) [4]. IPs and oRGs settle at basal positions within the intermediate zone (IZ) and subventricular zone (SVZ) of the cortex and give rise to deep and upper layer neurons [5–8]. While being present in small number in the rodent cortex, oRGs are especially

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abundant in primates and other gyrencephalic non-primate species, including ferrets, and have been related to evolutionary differences in cortical size among species [7,8].

Initial *in vivo* retroviral lineage experiments suggested that neuronal diversity emerge from one single progenitor type that becomes fate restricted with time [9–11]. Accordingly, *in vitro* models from mouse and human embryonic stem cells (ESCs) recapitulated the sequential generation of deep and upper layer neurons from cortical progenitors [12–14], and clonal analysis showed the existence of multipotent progenitors that generate all layer subtype neurons in a time-dependent fashion [15,16]. Conversely, others have shown the existence of fate-restricted progenitors in the mouse cortex that generate solely callosal projection neurons [17–19].

Further research is thus needed to better understand the contribution of fate-restricted progenitors versus multipotent ones to the cellular diversity of the cortex. Coupling *in vitro* models with advanced lineage tracing may bring new insights into human progenitor biology.

Expansion and temporal progression of progenitors: novel insights from *in vivo* and *in vitro* models

Progenitor cell identity is dictated by a dynamic interplay between intrinsic and extrinsic factors accruing along the neural tube and across developmental stages that results in specific transcriptomic networks and molecular identities [20,21,22••].

Figure 1. Mechanisms controlling proliferation and cell fate progression of cortical progenitors. **(a)** Scheme showing intrinsic and extrinsic cues controlling corticogenesis resulting in normal development and generation of different types of progenitors and neurons. **(b)** Loss-of-function of PTEN/hyperphosphorylation of AKT results in shortened cell-cycle, increased pool of progenitors, delayed neurogenesis and larger human brain organoids [28]. **(c)** GSK3β inhibition/Wnt activation results in expansion of the progenitor pool, disruption of cell polarity, decrease of IPs and oRGs, and overall increase of organoid size [33]. **(d)** On the contrary, reduced Wnt signaling leads to a premature switch from symmetric to asymmetric mode of division, altered RGC morphology, increase of IPs and overall smaller organoids [34^]. **(e)** Areal identity of transplanted human PSC-derived cortical neurons following current models. (Left) Visual-patterned progenitors transplanted into the mouse adult visual cortex project axons following a visual pattern of connectivity, whereas the same progenitors placed into the rostral motor cortex retain their visual identity but fail to efficiently reestablish axonal tracts within the host brain [46^^,47]. (Right) Non-patterned (early) progenitors are plastic and can be respecified following grafting, resulting in endogenous patterns of axonal connectivity depending on the transplant location [48,49] (Areal color code: Yellow: prefrontal cortex; Red: Motor cortex; green: somatosensory cortex; blue: visual cortex). VZ: ventricular zone, SVZ: subventricular zone; oSVZ: Outer subventricular zone; SP: subplate; CP: cortical plate; MZ: marginal zone, SC: superior colliculus, LG: lateral geniculate, CP: cerebral peduncle.

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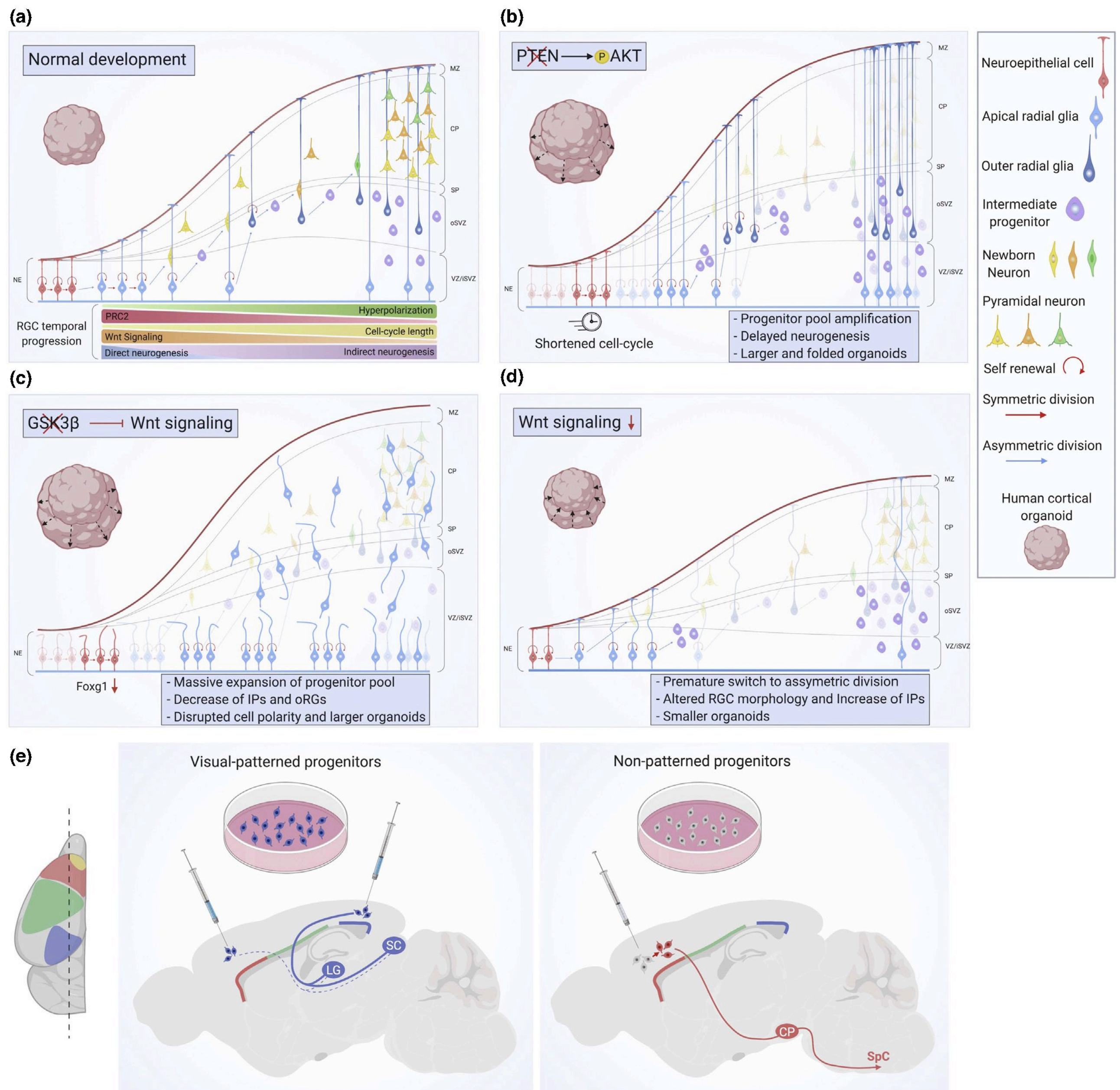

Among these signals, Notch plays an important role for the maintenance of progenitor cells in the developing cortex [23,24]. Mouse and hESC-derived cortical progenitors respond to Notch signaling inhibition by reducing the formation of rosette-like neuroepithelia structures and by increasing neurogenesis rate [12,25]. In addition, growth factor signaling through the Serine and Threonine kinase AKT (PKB) integrates multifunctional downstream signaling networks to control proliferation, survival, metabolism and growth [26]. Consistently, *in vivo* loss-of-function of the tumor suppressor gene *Pten* leads to AKT hyperphosphorylation, thereby increasing neuronal progenitor proliferation, partially by shortening their cell cycle length [27]. Interestingly, *Pten* mutations also result in larger and folded hESC-derived cortical organoids. These organoids are characterized by accumulation of cycling progenitors

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and delayed neuronal differentiation, leading to the expansion of the VZ and SVZ [28] (**Figure 1a–b**).

Glycogen synthase kinase 3 (GSK3β integrates multiple proliferation and differentiation signals, and its conditional deletion in mice leads to VZ progenitor hyperproliferation and reduction of IPs and neurons, associated to deregulation of β-catenin, Shh, Notch and fibroblast growth factor (FGF) pathways [29]. Interestingly, high level of Wnt lock RGCs into an early like state, driving direct neurogenesis and precluding temporal maturation towards the generation of late born neurons in mouse [30,31••,32]. Moreover, a progressive decrease of RGCs membrane potential repressed the Wnt pathway and induced a shift towards the generation of upper layer neurons [31••]. Consistently with *in vivo* data, inhibition of GSK3β in hESC-derived cortical organoids reduced the formation of ventricle-like structures, disrupted cell polarity, increased proliferation, and resulted in larger organoids. As in mice models, GSK3β inhibition led to fewer number of IPs, but also oRG cells, suggesting an important role of the GSK3β signaling for the generation of basal progenitors [33] (**Figure 1a,c**). Conversely, attenuation of Wnt signaling led to smaller organoid size, switch from symmetric to asymmetric cell division, and increased number of IPs in Miller-Dieker syndrome (MDS)–derived cortical organoids [34•] (**Figure 1a,d**). Restoration of the N-cadherin/β-catenin signaling axis rescued the mode of division, thereby increasing the size of MDS–derived cortical organoids [34•].

Intrinsic factors regulating the cell cycle also control the choice of proliferative versus neurogenic progenitor divisions [35,36]. Deletion of the microcephaly related gene *WDR62* was associated with increased cell cycle length and cell cycle exit, earlier neurogenesis and reduced number of oRG cells and organoid size [37]. Epigenetic changes also drive progenitor progression [38], and the polycomb repressive complex 2 (PRC2) is progressively downregulated in developing RGCs [22••]. Interestingly, depletion of PRC2 in mouse RGCs forces a premature switch to upper layer neuron generation, resulting in reduced cortical thickness [22••].

Thus, a balance between extrinsic and intrinsic factors controls proliferation and temporal progression of cortical progenitors. Future studies using human pluripotent stem cells may help to discover additional factors and species-specific differences in corticogenesis.

Lessons on genetic programs and areal-specific connectivity of the cortex from *in vitro* models

In addition to temporal drivers of neural diversity, the topographical distribution of cortical progenitors correlates with specialized neuronal subtypes and connectivity patterns (**Figure 1e**). Specialized brain areas, such as motor, somatosensory or visual areas also exhibit differential lamination patterns, with varying proportions of neuronal subtypes and connectivity profiles [39,40].

Areal-specific diversity is correlated with rostro-caudal gradients of expression of transcription factors such as COUPTF1, Sp8 and Pax6 in cortical progenitors which regulate the size and molecular specificity of cortical areas [40,41]. Furthermore, broadly expressed

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transcription factors may regulate the activity of small enhancer elements active in discrete cortical regions as early as from E12.5 in the mouse contributing to the emergence of sharp cortical area boundaries [42]. Recent developments in single-cell transcriptomics on micro-dissected frontal and posterior murine cortex have confirmed molecular differences across cortical areas over the course of development [39,43]. Similar experiments performed in fetal human brain demonstrated that RGCs isolated from prefrontal and primary visual cortex show few transcriptional differences that cascade into distinct transcriptomic profiles in mature neurons [21,44,45]. Thus, spatial organization of cortical progenitors, and consequently neurons, give rise to specific molecular signatures that contribute to the arealization process and the acquisition of cortical functions.

In vitro differentiation of mouse and human embryonic stem cells revealed that neural cortical progenitors generated by default conditions acquire visual identity based on their molecular and hodological properties [13,16,46••,47] (**Figure 1e** left). Indeed, progenitors exhibited a typical visual/limbic projection pattern after grafting into the mouse brain [13,16,46••,47]. Conversely, other studies using hESC-derived cortical cells transplanted into different brain areas have shown a pattern of axonal connectivity with the host corresponding to the transplant location [48,49] (**Figure 1e** right). These discrepancies may result from different temporal degrees in fate acquisition of transplanted human cortical progenitors, in agreement with previous *in vivo* data from cortical embryonic progenitors [50].

Interestingly, two studies showed polarized structures with opposite gradients of expression of caudal and rostral cortical markers in hESC-derived organoids [51,52]. In addition, a recent single-cell RNAseq study revealed a broad representation of cortical areas in human cortical organoids, with stark resemblance to primary tissue. However areal identity lacked clear spatial organization in this model [53•].

Altogether these data suggest that neural stem cells differentiated *in vitro* may show intrinsic 'default' preference towards specific cortical area identities, defined early in the dish. Extrinsic signals, such as those released in tridimensional cultures or *in vivo*, following transplantation, may shape areal identity. Further research is thus needed to identify the molecular cues that promote cortical a realization and topographical distribution in organoid models.

Evolution of the cerebral cortex: lessons learnt from stem cell models

The cortex has experienced a remarkable increase in size and complexity through evolution from lower mammals to primates and humans (**Figure 2a**) [23]. It has become evident that the origin of such expansion in cortical area and thickness is an increase in the total number of neurons, which reflects a longer and considerably more important and complex proliferative phases in higher species [23,54]. Several mechanisms have been proposed to explain this tremendous size expansion, such as the number of cell division rounds, the associated time of cell divisions and the length of cell cycle that are different between macaques and rhesus monkeys when compared to the mouse [35,55–59] (**Figure 2a-b**).

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Likewise, human PSCs-derived progenitors have extended proliferation time, prolonged neurogenesis and higher neuronal production when compared to those derived from mouse or non-human primates [13,15,16,60–64]. Species-specific differences in proliferative capacity are cell autonomous, as demonstrated with co-cultures of non-human primate and human ESC-derived cortical cells [15,62–64].

Interestingly, Notch signaling, has been postulated to play a critical role in cortical evolution. Two recent reports described the function of a human-specific duplication of the *NOTCH* gene that generates *NOTCH2NLB*, a paralog associated with cortical development [65,66••]. Clonal expression of *NOTCH2NLB* in hESC-derived cortical progenitors *in vitro* led to their corresponding clonal expansion with slower exhaustion, ultimately leading to production of greater number of neurons [66••]. This mechanism involves enhancement of the NOTCH pathway activity with decrease in cell-cycle exit and an increase in cell-cycle re-entry [66••]. Expression of NOTCH2NLB induced a delay in differentiation in mouse ESC-derived organoids and conversely its deletion in hESC-derived organoids led to precocious differentiation [65].

On the other hand oRG cells, which are abundant in gyrencephalic species, have been correlated to a higher production of cortical neurons, and particularly of upper layer neurons among higher mammals [7,8]. Brain organoids derived from human cells include oRGs that conserve the specific mode of division and gene expression pattern observed in the human fetal brain [51,52,67,68]. Interestingly, organoids derived from Miller-Dieker syndrome patients, who suffer from impaired cortical folding and microcephaly, showed oRGs with mitotic defects that were rescued upon correction of the chromosomal deletion [68].

Figure 2. Cortical development in mouse and human brain and human organoid models. (a) Timeline of mouse, human and human organoid for cortical development. Equivalent stages between mouse and human development, and between human fetal brain and human organoids are indicated by dashed lines. (b) Center: Comparison of cortical area spatial distribution in mouse and human cortex and in current human organoid models. Areal color code: yellow: prefrontal cortex; red: motor cortex; green: somatosensory cortex; blue: visual cortex. Lateral: Comparison of cellular subtype composition and organization of the cortical wall in mouse, human and human organoid models. (c) Schematic representation of layer 2/3 pyramidal neurons and dendritic spine morphology in mouse, human cortex and human organoids (hCO). Mouse and human cortex representations are based on the morphology at mature stages, respectively P21 and 16 years old. Human organoid representation is based on imaging of pyramidal neurons in late stages organoids (10 months) [111]. GW: gestational week; PD: protocol days; FL: fiber layer; IFL: inner fiber layer; OFL: outer fiber layer; MZ: marginal zone; CP: cortical plate; SP: subplate; IZ: intermediate zone; SVZ: subventricular zone; oSVZ: outer subventricular zone; iSVZ: inner subventricular zone; VZ: ventricular zone; hCO: human cortical organoid.

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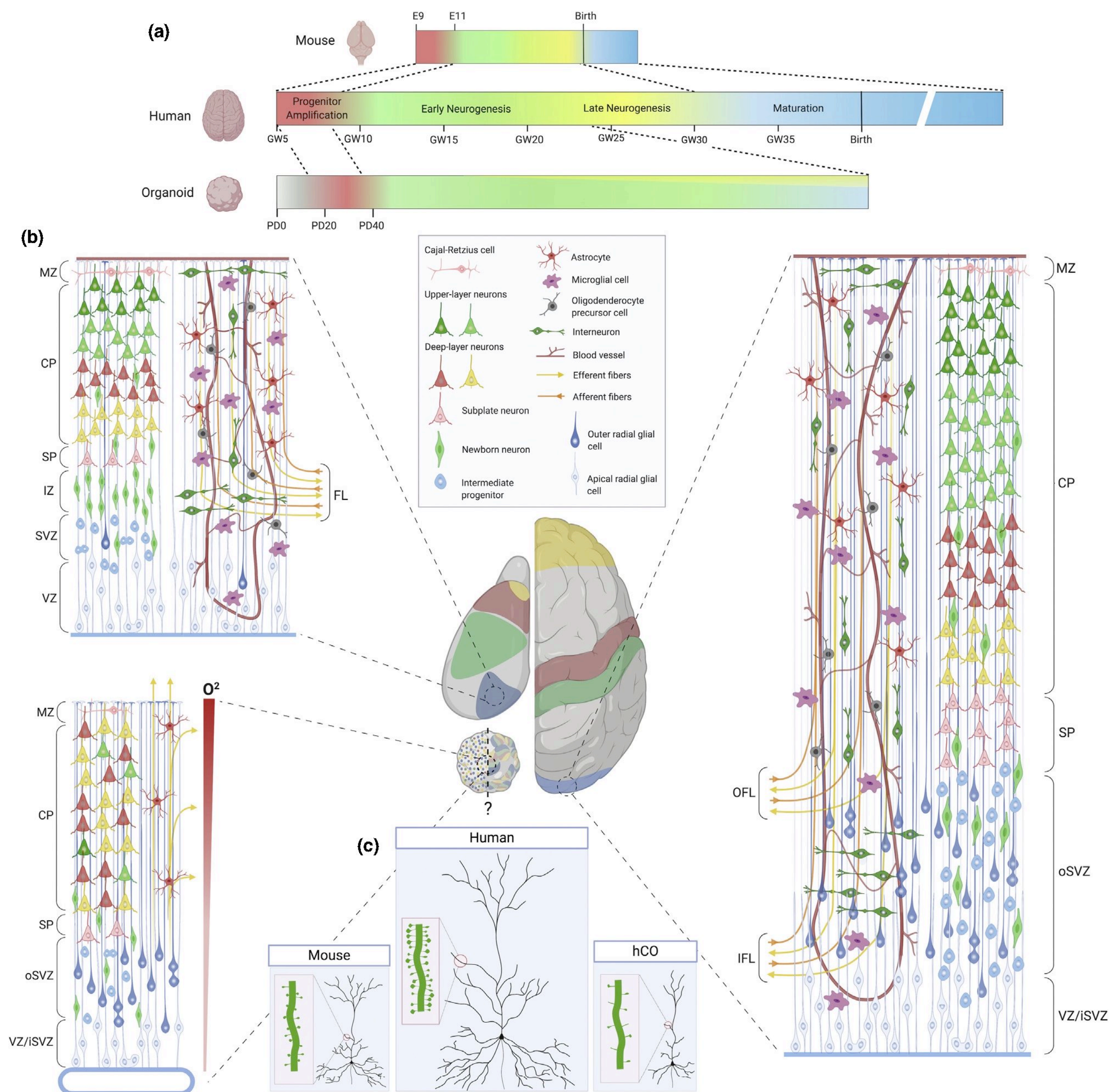

Besides differences in cell proliferation and neurogenic capacity, the human cortex also shows specific patterns of neuronal maturation characterized by neoteny of synaptic spines and the protracted formation of mature neuronal circuits (**Figure 2c**) [69,70]. Remarkably, hESC-derived cortical and GABAergic neurons recapitulate the slow acquisition of mature neuronal excitability and synaptic activity patterns [13,14,60,61,71–73], even following transplantation into the mouse brain, very reminiscent of the human brain situation [13,61,74••]. In particular, hESC-derived cortical cells showed a very slow time dependent dendritic tree arborization and spine formation accompanied by the establishment of functional synapses within the murine host brain [13,61,74••].

Similarly, excitatory sensory neurons differentiated from human stem cells showed delayed neuronal maturation and expression of genes involved in dendrite and synapse development

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when compared to counterparts derived from chimpanzee or bonobo [75]. The molecular network that control neoteny in human start to be understood.

Among them, the protein of SRGAP2C, a human specific gene duplicated from the SRGAP2 gene, antagonizes the ancestral SRGAP2 function. By doing so, it prolongs the maturation of cortical projection neurons spines. Remarkably, expression of SRGAP2C in mouse neurons delayed spine maturation and increased spine density, mimicking human-specific patterns for neuronal maturation and spine morphology [76,77].

These data suggest that cell intrinsic properties control and balance species-specific rate of proliferation, timing of neurogenesis and time-dependent neuronal maturation in the brain.

Organoid models to study brain development and diseases

During CNS development, neural progenitors are specified and patterned along the anterior-posterior (A-P) axis, resulting in the regionalization of the forebrain, the midbrain, the hindbrain and the spinal cord. The current model proposes that progenitors adopt a rostral identity and that some acquire progressively caudal fates through the combined action of posteriorizing signals such as BMP, WNT, FGF2 and retinoic acid [78,79].

Organoids can model different brain regions such as those with ventral telencephalic (hSS/hMGEO) [80••,81••,82••], diencephalic and thalamic (hThOs) [83••], midbrain (hMLOs) [84], cerebellar [85], and spinal cord [86,87] identities. Interestingly, the specification of ventral and caudal SNC identities from human pluripotent stem cells (hPSC) is achieved by recapitulating *in vivo* developmental cues for A-P and D-V patterning (**Figure 3a**). Moreover, brain region-specific organoids recapitulated some important *in vivo* features such as neuronal migration and establishment of some connectivity patterns. For instance, fusion of human cortical and ventral telencephalic organoids allow migration of GABAergic interneurons from MGE-like structures towards cortical organoids (hCS-hSS/hCO-hMGEO) [80••,81••,82••] (**Figure 3b**). Interestingly, fused dorsal and ventral forebrain organoids unraveled altered interneuron migration patterns in Timothy syndrome [81••].

Single-cell transcriptomic analysis unequivocally confirmed thalamic cellular identities within hThOs and the subsequent fusion of cortical and thalamic human organoids (hCOs-hThOs) showed reciprocal axon connectivity between hThOs and hCOs. Thalamic afferents have been shown to shape cortical area subdivisions, such as the barrel field in the somatosensory cortex at late stages of mouse cortical development, and to fine-tune progenitor cell proliferation and consequently layer cortical neuronal output [88–91]. Further, thalamo-cortical afferents innervate the cortex at earlier stages of development in primates when compared to mouse counterparts [92]. Thus, hCOs-hThOs fusion might represent a more faithful model to study human cortico-thalamic circuitry and related disorders *in vitro* [83••] (**Figure 3c**).

Transcriptomic and cytoarchitectural analysis of human midbrain-like organoids (hMLOs) revealed subregions similar to the developing midbrain [84,93], including MASH1+ and OTX2

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+ VZ progenitors, intermediate zone NURR1+ postmitotic cells, and a marginal zone containing tyrosine hydroxylase (TH) midbrain dopaminergic neurons (mDA) (**Figure 3a**). Importantly, TH+ neurons exhibited biochemical and electrophysiological properties of mature mDA neurons, expressed functional DA receptors and produced neuromelanin-like granules [84]. A recent study showed that hMLOs from isogenic hPSC lines carrying LRRK2 mutations from Parkinson disease's patients (PD) recapitulated cardinal features of the disease, including gene expression profiles and a-synuclein aggregates [94].

Novel 3D models to generate human cerebellar organoids showed expression of hindbrain regional markers and robust differentiation towards cerebellar specific neuronal subtypes including Purkinje cells (PCs) and granular cells (GCs) with the formation of a continuous cerebellar plate with dorso-ventral polarity and a rhombic-like structure, similar to the embryonic cerebellum [85,95] (**Figure 3a**). A recent study comparing hPSC-PCs and mouse PCs revealed the existence of shared developmental gene expression patterns as well as human-specific genes [96•]. Following this model, spinocerebellar ataxia 6 (SCA6) patient-derived organoids showed accumulation of Cav2.1 calcium subunit channel and enhanced Purkinje cell degeneration in the absence of thyroid hormone (T3) [95]. However, the current model only recapitulates early stages of cerebellar development and therefore a future challenge remains to establish long-term culture systems to recapitulate late cerebellar developmental stages.

Figure 3. State of the art of human organoid models across CNS brain areas. (a) Organoid models patterned towards different CNS regional identities along the anterior-posterior (A-P) and dorsal-ventral (D-V) axis are represented. Forebrain, midbrain, hindbrain and spinal cord organoids are specified through the combined action of caudalizing, dorsalizing or ventralizing morphogens, such as fibroblast growth factor 2/8 (FGF2, FGF8), Sonic hedgehog (Shh), bone morphogenetic protein (BMP), Wnt and insulin, as indicated by the gradient lines. Progenitor and neuronal-specific subtype markers are indicated for each organoid type. Ventricle-like regions (VZ) are also represented. (b,c) Schemes show fused hCS-hSS/hCO-hMGEO [80^^,81^^,82^^] (b) and hCO-hThO [83^^] (c) organoids, a novel approach to study cell migration and neural circuit formation. In (b) arrows indicate the migratory direction of the cINs generated in hSS/hMGEOs. In (c) arrows indicate cortico-thalamic (yellow) and thalamo-cortical (orange) neural projections. hCS/hCO: human cortical spheroid/human cortical organoid; hThO: human thalamic organoid; hMLOs: human midbrain-like organoid; hSS/hMGEO: human subpallium spheroid/human medial ganglionic eminence organoid; VZ: ventricular zone, SVZ: subventricular zone; CP: cortical plate; IZ: intermediate zone; MZ: marginal zone; PCPZ: Purkinje Cell progenitor zone; TH: Tyrosine hydroxylase; PV: Parvalbumin; Calb: Calbindin; VGLUT2: Vesicular glutamate transporter-2.

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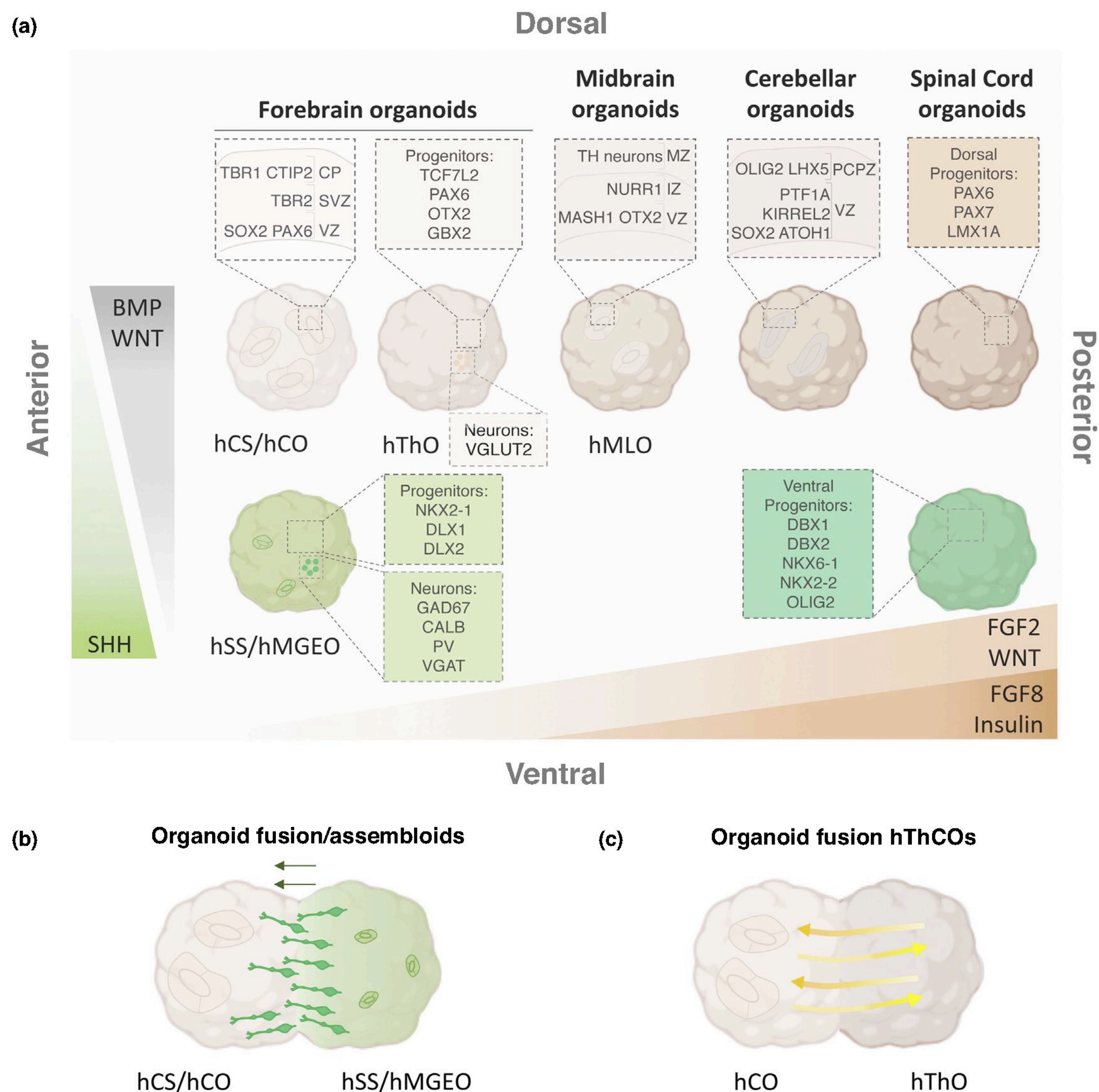

Several protocols to generate spinal organoids have been described and include FGF2 and Wnt activators in the neural induction media to caudalize neural identities [86,87] (**Figure 3a**). Further, treatment with Shh activators increases the number of ventralized progenitors such as motor neuron (MN) progenitors, whereas BMP4 dorsalizes spinal organoids towards the generation of interneurons [97,98].

Thus, improvement of human 3D cultures will help understanding how different neuronal identities arise along the neural tube and could set the basis for engineering new models to study neurological disorders affecting distinct brain areas.

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Are organoid models faithfully recapitulating brain development?

Single cell analysis of individual organoids have shown high reproducibility [99•] and a broad representation of cell-types present in the fetal brain, which matured following reproducible pseudo-time pathways [64,73,81••,100]. However, other studies have described a poor representation of subtype identities, such as oRGs, IPs, SATB2+ callosal neurons and layer I Cajal Retzius cells in cortical organoids, when compared to primary fetal cortex [53•]. Moreover, they show rudimentary separation of the cortical plate (CP) into distinct layers [101] (**Figure 2b**). These discrepancies may reflect limitations of the current culture conditions, as metabolic stress was detected in organoids [53•] and lack of vascularization triggers hypoxia followed by ER-stress and cell fate defects [102], as previously shown in mice [103]. Technical improvements such as growing organoids in bioreactors [99•] or culturing organoid slices in an air-liquid interface, may promote IP generation and increase CP thickness by boosting neuron maturation and axonal outgrowth [53•,104•,105]. Moreover, human organoids grown in the air-liquid interface showed enhanced axonal outgrowth and innervation of mouse spinal cord explants [104•]. *In vivo* transplantation of organoids or dissociated organoid cells into the mouse brain allows their vascularization from the host and reduced expression of stress-related genes when compared to *in vitro* samples [53•,106]. Therefore the *in vivo* environment may provide cues, metabolites and oxygenation to normalize metabolic stress, consecutively improving subtype specificity and maturation [53•]. Recently, a model for human brain organoids supplemented with vascular cells has been generated [107•]. The presence of endothelial vascular cells enhanced the functional maturation of organoids, and as such, could represent a more physiological model to study brain development and disease *in vitro* [107•]. Innovative brain organoid models including CNS-barrier [108,109•] and choroid plexus with cerebrospinal fluid-like production [109•] will be instrumental to study the blood-brain-barrier in health and disease as well as its permeability to possible therapeutic molecules [110].

Concluding remarks and perspectives

The fast-paced field of organoid research has come a long way in improving reproducibility and characterizing cellular diversity in cortical organoids. Current protocols are promising systems to study human-specific cortical development but further optimizations of culture conditions are required to improve the diversity of subtype-identities, reduce metabolic stress and recapitulate *in vivo* patterns of neuronal migration and maturation. Future prospects of cortical organoid models should apply new technologies to reproduce specific patterns of neural connectivity to fully address neuronal identity and function in purely *in vitro* set ups. Further, organoid models could represent an ideal tool to unravel human and primate-specific differences in cortical evolution and for the study of human brain diseases and its associated-molecular pathways.

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Conflict of interest statement

Nothing declared.

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