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REVIEW

Is this a brain which I see before me? Modeling human neural development with pluripotent stem cells

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ABSTRACT

The human brain is arguably the most complex structure among living organisms. However, the specific mechanisms leading to this complexity remain incompletely understood, primarily because of the poor experimental accessibility of the human embryonic brain. Over recent years, technologies based on pluripotent stem cells (PSCs) have been developed to generate neural cells of various types. While the translational potential of PSC technologies for disease modeling and/or cell replacement therapies is usually put forward as a rationale for their utility, they are also opening novel windows for direct observation and experimentation of the basic mechanisms of human brain development. PSC-based studies have revealed that a number of cardinal features of neural ontogenesis are remarkably conserved in human models, which can be studied in a reductionist fashion. They have also revealed species-specific features, which constitute attractive lines of investigation to elucidate the mechanisms underlying the development of the human brain, and its link with evolution.

KEY WORDS: Cerebral cortex, Human brain evolution, Neural development, Neurogenesis, Patterning, Pluripotent stem cell

Introduction

The mechanisms of human brain development have long been attracting researchers' attention, but they have been challenging to explore because of the inherent difficulty of their experimental study. Although it is safe to assume that many aspects of human neural development are similar to those uncovered using animal (and particularly mammalian) models, this remains to be formally demonstrated in many cases. On the other hand, it is clear that human brain development displays species-specific features, in particular to generate the structures thought to be at the core of human-specific cognitive abilities, such as the cerebral cortex (DeFelipe, 2011; Lui et al., 2011). Recent advances in cell modeling using pluripotent stem cells (PSCs), whether of embryonic origin [embryonic stem cells (ESCs)] or induced by reprogramming [induced pluripotent stem cells (iPSCs)] (Belmonte et al., 2009; Smith, 2001), are providing new opportunities for the analysis of brain development using human cells and tissue. In this Review, we will first provide a brief overview of what is thought to distinguish human brain development from that of commonly employed animal models, and then review some of these recent PSC-based studies of neural development, emphasizing basic developmental mechanisms and potential links with evolution.

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Human-specific features of brain development

The brain, and most strikingly the neocortex, has undergone a rapid and considerable increase in relative size during the last few millions of years of hominid (human and great apes) evolution, with a very significant impact on the acquisition of higher functions in the human species (Hill and Walsh, 2005; Lui et al., 2011; Rakic, 2009). This has led to the enlargement of the surface and thickness of the cortex, associated with relative expansion and diversification of certain cortical areas, more profound cortical lateralization, and expansion of the uppermost layers (Amadio and Walsh, 2006; Rakic, 2009). From a more cellular viewpoint, comparison of the cortex from human and non-human primate species has revealed increased number and diversity of human cortical neurons (Bystron et al., 2006; Hill and Walsh, 2005; Nimchinsky et al., 1999; Roth and Dicke, 2005) and distinct patterns of neuronal morphology, such as increased size and density of dendritic spines in some areas (Benavides-Piccione et al., 2002). Many of the species-specific features of the human cortex are thus most likely to be linked to differences in the mechanisms underlying the generation, specification and differentiation of cortical neurons, i.e. cortical neurogenesis (Bystron et al., 2008; Fish et al., 2008; Lui et al., 2011; Taverna et al., 2014), with a direct impact on the number and diversity of cortical neurons (Bystron et al., 2006; Lui et al., 2011; Rakic, 1988, 1995).

Although the basic mechanisms of cortical neurogenesis appear to be well conserved among mammals, a number of divergent features have been identified as well, which are thought to be mainly linked to the properties of cortical progenitors (Fietz et al., 2010; Fish et al., 2008; Hansen et al., 2010; Kriegstein et al., 2006; Lui et al., 2011; Taverna et al., 2014). These include expansion of the amplification period of early neural progenitors, i.e. neuroepithelial (NE) progenitors, as well as protracted periods of generation of neurons from neurogenic progenitors (Rakic, 1995). This capacity to generate neurons for a prolonged period could be linked to species-specific properties intrinsic to neural progenitors, such as differential cell cycle control or tuning of self-renewal versus terminal differentiation (Lukaszewicz et al., 2005), but overall the underlying mechanisms remain unknown (Dehay and Kennedy, 2007). Species differences could also be linked to other types of progenitors, such as basal progenitors (see sections below on cortical development for further details), which are characterized by a higher capacity to undergo multiple rounds of neurogenic divisions and thereby contribute to increased neuronal output (Betizeau et al., 2013; Dehay et al., 2015; Fietz et al., 2010; Garcia-Moreno et al., 2012; Hansen et al., 2010; Kelava et al., 2012; Lui et al., 2011; Reillo and Borrell, 2012; Reillo et al., 2011). In addition, human neurons in general, but human cortical neurons in particular, display much more prolonged patterns of neurite

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outgrowth, dendritic spine maturation and synaptogenesis, which is thought to underlie the neoteny that characterizes human brain development (DeFelipe, 2011; Petanjek et al., 2011).

In summary, human brain (cortical) development does not present obviously 'unique' features compared with other species, including other primates, but does display a number of quantitative differences that are thought to be essential for human brain ontogenesis.

Neural induction from pluripotent stem cells

Brain development starts with neural induction, which is the entry of multipotent ectodermal cells into the neural lineage (Fig. 1A) (Muñoz-Sanjuán and Brivanlou, 2002; Weinstein and Hemmati-brivanlou, 1999). Neural induction is thought to depend mostly on a 'default pathway' triggered by inhibition of suppressive extrinsic cues, in particular TGFβ signaling including via BMP and Nodal (Muñoz-Sanjuán and Brivanlou, 2002; Stern, 2006; Weinstein and Hemmati-brivanlou, 1999). The default model was originally proposed from experiments in amphibian models and its evolutionary conservation had been somewhat controversial (Stern, 2006), but its robust validation in mammalian systems has now emerged. The first hint for the existence of a robust default pathway in mammalian cells came from studies using mouse ESCs, which start to express neural markers within hours of culture in media deprived of any growth factor/morphogen (Smukler et al., 2006; Tropepe et al., 2001). Since then, these observations have been largely confirmed and extended using mouse and human PSCs, and indeed most protocols used today to generate neural cells from human PSCs typically involve culture conditions without morphogens and/or with morphogen inhibitors, in particular those of the Nodal/Activin and BMP pathways (Fig. 1B) (Anderson and Vanderhaeghen, 2014; Bertacchi et al., 2013; Chambers et al., 2009; Espuny-Camacho et al., 2013; Gaspard et al., 2008; Gaspard and Vanderhaeghen, 2010; Hansen et al., 2011; Juliandi et al., 2012; Watanabe et al., 2005; Ying et al., 2003).

PSC-based models of neural induction recapitulate key molecular and cellular features thought to occur *in vivo* (Fig. 1B), and as such have been used to dissect the underlying molecular mechanisms. For example, Kamiya et al. (2011) performed an expression screen during PSC-derived neural induction, which led to the identification of Zfp521, a transcription factor required and sufficient to induce neuroectodermal cells from definitive ectodermal progenitors in both mouse and human models. Similarly, Smad-interacting protein 1 (Sip1; also known as Zeb2) (Chng et al., 2010) and Smad7 (Ozair et al., 2013) were found to act as key intrinsic neural inducers from PSCs, by directly inhibiting the downstream effectors of BMP and Activin signaling. Oct6 (also known as Pou3f1) is another positive inducer of neural fate identified in PSC models, which acts through repression of downstream targets of BMP and Wnt signaling (Zhu et al., 2014).

PSC models have thus enabled researchers to demonstrate that the default mechanism of neural induction is a highly conserved feature throughout vertebrates, from amphibians to humans, and to start dissecting the underlying molecular mechanisms. PSC models have also started to uncover species-specific features of the transcriptional mechanisms of early neural development: one of the striking differences is the involvement of the transcription factor Pax6 (Zhang et al., 2010). Pax6 is known to be involved in the determination of dorsal forebrain identity after neural tube closure during vertebrate embryogenesis (Stoykova et al., 1996; Stoykova and Gruss, 1994). However, this gene starts to be expressed at much earlier time points in human PSC-derived models, as well as in human embryos (Zhang et al., 2010). Moreover, PAX6 expression in human early neuroectodermal cells is essential to terminate the expression of pluripotent genes and to initiate expression of neural genes such as *SOX1* and *SOX2*. This is in contrast to the situation observed in the mouse, where *Sox1* is the earliest marker gene expressed during neural commitment, while *Pax6* is induced afterwards (Zhang et al., 2010). Furthermore, the distinct splice isoforms of this gene have specific neural-inducing activity in human cells (Zhang et al., 2010). The human-specific Pax6 function appears to involve a defined set of miRNAs that are direct targets of Pax6. The Pax6-miRNA pathway acts to suppress multiple components of BMP signaling and thereby enhances neural differentiation (Bhinge et al., 2014). Human-specific mechanisms for suppression of BMP signaling also involve extrinsic cues. NPTX1, a secreted protein, shows a peak of expression at the very beginning of neural induction, even earlier than Pax6 and Sox1 (Boles et al., 2014). NPTX1 protein directly binds to the Nodal receptor co-factor TDGF1, and can thereby reduce the levels of Nodal and BMP signaling. This neural-inducing function of NPTX1 appears not to be conserved in mouse (Boles et al., 2014).

In summary, PSC studies have revealed that neural induction through BMP/TGFβ suppression is a highly conserved mechanism but there appear to be species-specific differences in the underlying molecular mechanisms, the significance of which will be interesting to study further in the frame of human brain evolution.

Regional specification of the central nervous system

A fundamental aspect of regional patterning of the vertebrate central nervous system is that the most anterior/rostral fate constitutes the 'primitive' identity, i.e. the first regional identity to be established (Levine and Brivanlou, 2007; Stern, 2001; Wilson and Houart, 2004). Following rostral neural fate acquisition, the rostrocaudal and dorsoventral axes within the neural tube are determined and refined by positional cues supplied by several organizing centers located within and outside of the neural tube, allowing the emergence of all brain regions and domains (Fig. 1C, and discussed further below).

As *in vivo*, neural progenitors derived from PSCs *in vitro* first acquire rostral identity by default (Chambers et al., 2009; Gaspard et al., 2008; Kawasaki et al., 2000; Li et al., 2009; Pera et al., 2004; Vallier et al., 2004; Watanabe et al., 2005), and this primitive identity can be converted to more caudal fates by various cues such as retinoic acid (RA), Wnts or FGFs (Elkabetz et al., 2008; Kirkeby et al., 2012; Wichterle et al., 2002; Zeng et al., 2010), thereby mimicking the *in vivo* situation (Wilson and Houart, 2004) (Fig. 1D). Inhibition of caudalizing Wnt signaling by small molecules effectively enhances the production of forebrain cells (Kadoshima et al., 2013; Watanabe et al., 2005). Conversely, when a GSK3β inhibitor, which activates canonical Wnt signaling, is applied during neural differentiation from PSCs, neural progenitors of different rostrocaudal identity are induced in a dose-dependent manner – higher levels of inhibitor induce cells with more caudal identity, such as the midbrain (Kirkeby et al., 2012). FGF8, which is secreted from the isthmic organizer located in the midbrain-hindbrain boundary *in vivo* (Allodi and Hedlund, 2014; Stern, 2001), is a potent inducer of midbrain precursors from PSCs (Allodi and Hedlund, 2014; Elkabetz et al., 2008; Kriks et al., 2011; Perrier et al., 2004; Soldner et al., 2009; Yan et al., 2005). These precursors are in turn terminally differentiated into dopaminergic neurons under the effect of other signals (Fig. 1D).

Similarly, spinal cord identity can be induced through the combinatorial addition of the 'caudal morphogens' RA, Wnts and FGFs (Amoroso et al., 2013; Boulting et al., 2011; Dimos et al.,

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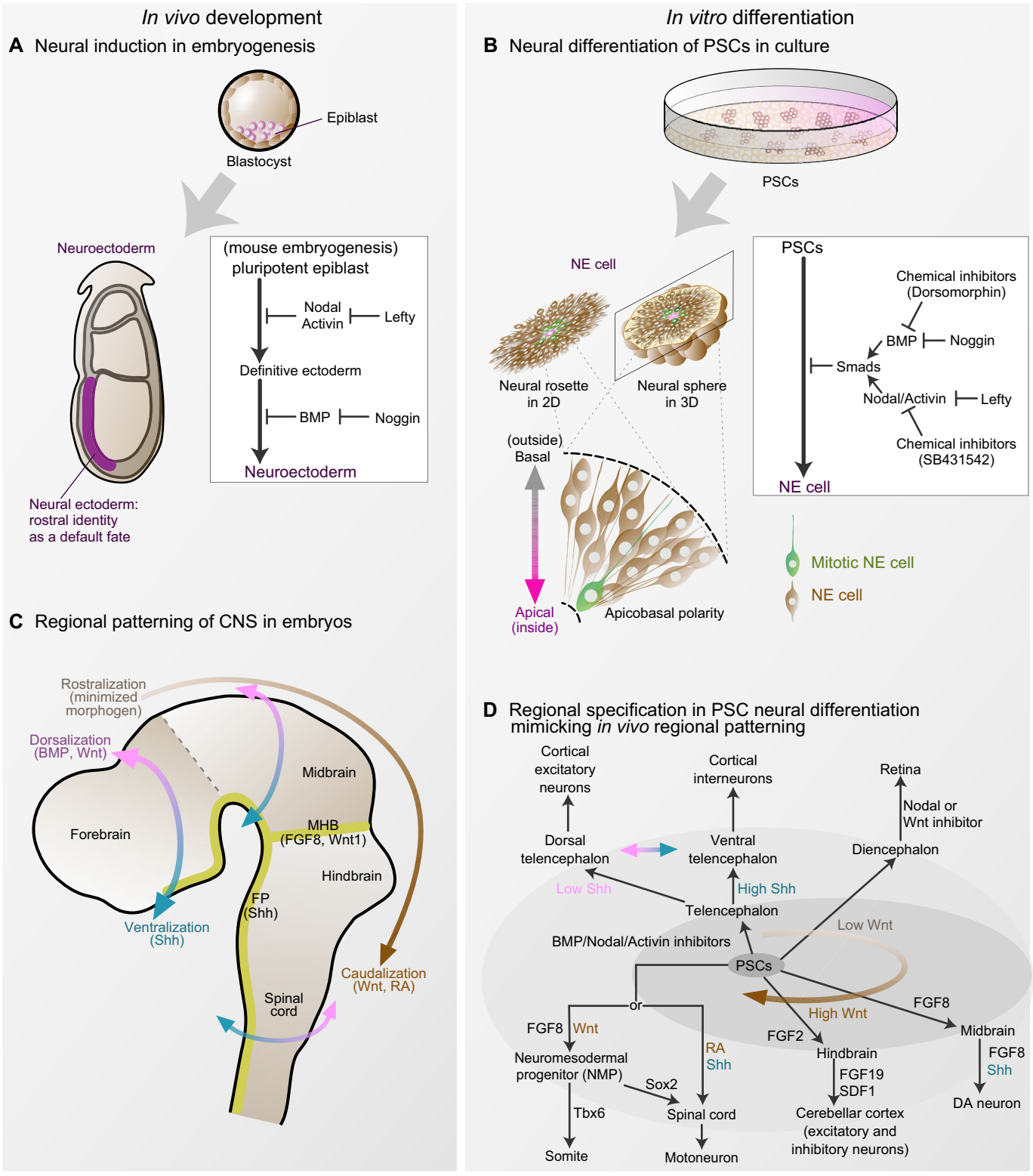

Fig. 1. Modeling neural induction and regional patterning. (A) Neural induction is considered a 'default' pathway of differentiation, and occurs mainly through inhibition of BMP/Nodal signaling *in vivo*, resulting in neural cells with rostral identity. (B) This is mimicked *in vitro* during neural differentiation from PSCs, where rostral neural plate-like cells also display landmark features of NE cells such as apicobasal polarity. (C) The regional identities within the central nervous system (CNS) are determined along the rostrocaudal and dorsoventral axes through the action of morphogens derived from various organizing centers (yellow). FP, floor plate; MHB, midbrain-hindbrain boundary. (D) This process can be mimicked *in vitro* through the combinatorial use of the same morphogens in culture medium.

2008; Li et al., 2005; Maury et al., 2015; Takazawa et al., 2012; Wichterle et al., 2002), again similar to the mechanisms operating *in vivo* (Liu et al., 2001; Nordström et al., 2002; Nordström et al., 2006). Interestingly, recent lineage-tracing experiments in amniote models have suggested a more complex picture: spinal cord precursors share ontogenic origin with the paraxial mesoderm and

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appear to be derived from a common class of transient precursors called neuromesodermal progenitors (NMPs) (Brown and Storey, 2000; Gouti et al., 2015; Tzouanacou et al., 2009), which can be specified into neural or mesodermal fates (Chapman et al., 1996; Chapman and Papaioannou, 1998; Takemoto et al., 2011). This *in vivo* developmental scheme can be observed in PSC-derived models, where NMP-like cells can be derived from PSCs using Wnt and FGF ligands (Gouti et al., 2014; Turner et al., 2014). Collectively, these data indicate that PSCs can either undergo a differentiation pathway to the spinal cord through caudalization of precursors for more rostral brain regions, or can be specified into NMP-derived spinal cord precursors (Fig. 1D): it will be interesting to explore how they relate to events occurring *in vivo* in various species.

Throughout the neural tube, different concentrations of morphogens such as Sonic hedgehog (Shh), which is mainly derived from the most ventral part of the neural tube called the floor plate, induce the specific expression of transcription factors in successive discrete domains along the dorsoventral axis. These general mechanisms also apply to PSC-derived progenitors of spinal cord, hindbrain, midbrain or forebrain identity (Fig. 1C,D). The acquisition of specific regional identity has one important consequence, whether *in vitro* or *in vivo*, which is to confer a specific range of competence to the progenitors in terms of their terminal differentiation into specific types of neurons or glial cells. Importantly, the neural progenitors that are regionally specified from PSCs also acquire equivalent competence, enabling the generation of neurons of diverse regional identity. Through this general paradigm of recapitulating regional patterning through the combinatorial use of extrinsic cues, a very diverse set of neurons and glial cells has been reported (Fig. 1D).

In most of these systems, neurons are generated from conventional monoadherent cultures or embryoid bodies, both of which mostly lack any three-dimensional (3D) organization. This indicates that the neuronal specification programs can at least partly be executed without much spatial information or cytoarchitecture. On the other hand, over the last few years other systems of differentiation have emerged that have revealed that PSC-derived differentiating cells can self-organize into defined 3D structures that strikingly resemble human brain subregions (Lancaster and Knoblich, 2014; Sasai, 2013; Sasai et al., 2012). In the next sections we will review data relevant to the generation of cell diversity and/or cytoarchitecture, focusing on three paradigmatic examples: the cerebral cortex, the cerebellum and the retina. For other types of neural cells or regions, and for translational perspectives of PSC-derived neural cells, the reader is referred to excellent recent reviews (Allodi and Hedlund, 2014; Davis-Dusenbery et al., 2014; Gage and Temple, 2013; Gouti et al., 2015; Heilker et al., 2014; Parent and Anderson, 2015; Paşca et al., 2014; Peljto and Wichterle, 2011; Southwell et al., 2014; Tabar and Studer, 2014; Yu et al., 2014).

The cerebral cortex

Corticogenesis from PSCs is an interesting case study, as it is the brain structure that probably displays the highest level of complexity, both in terms of cell diversity and connectivity, raising the question of the extent to which it can be reproduced in simplistic settings of PSC differentiation. The mammalian cerebral cortex consists of six layers of excitatory and inhibitory neurons, the former being generated by radial glial progenitors (RGs) in the ventricular zone (VZ) of the dorsal telencephalon (Kriegstein and Alvarez-Buylla, 2009; Taverna et al., 2014), whereas the latter are produced in the ventral telencephalon and subsequently migrate to the dorsal telencephalon (Fig. 2A) (DeFelipe et al., 2013; Greig et al., 2013; Sur and Rubenstein, 2005). Cortical RGs generate neurons directly or indirectly through the transit amplification of progenitors [such as basal progenitors (BPs)] in the subventricular zone (SVZ) (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004) (Fig. 2A). A prominent characteristic of cortical neurogenesis is that laminar fate specification is tightly linked to neuron birthdate, as early-born neurons settle in deep layers of the cortical plate (CP), whereas late-born neurons populate the upper

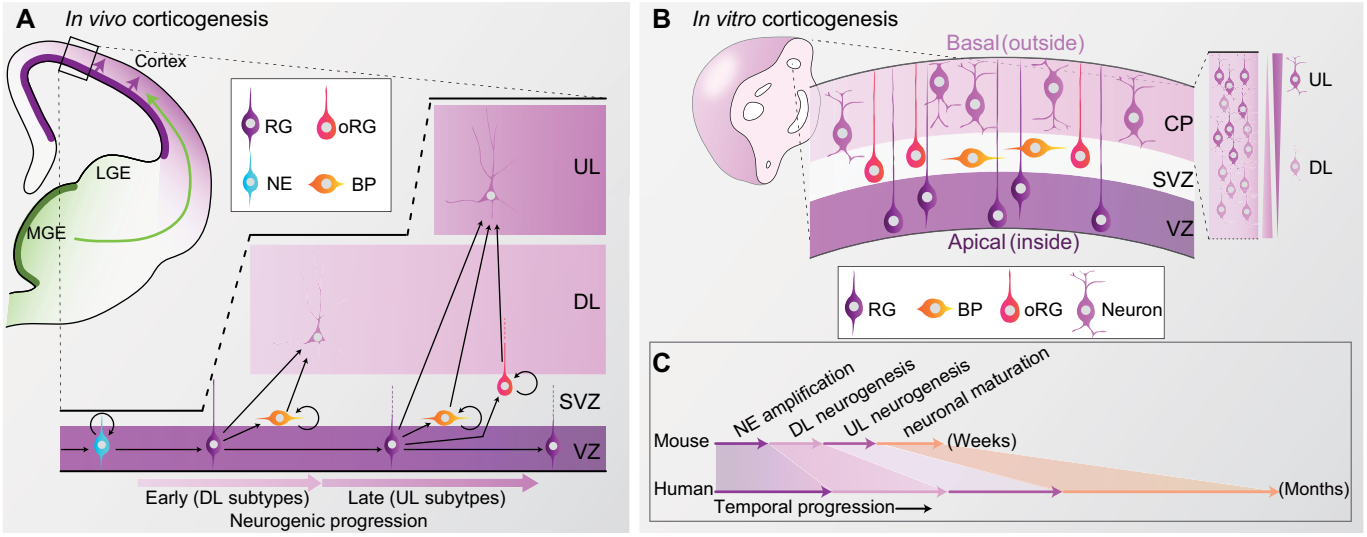

Fig. 2. Modeling spatial and temporal patterns of corticogenesis. (A) Pyramidal neurons of the cerebral cortex are produced in the dorsal telencephalon, whereas cortical interneurons are generated from the ventral telencephalon. In the dorsal telencephalon, the neuroepithelial cells (NE) first expand and then convert to radial glial progenitors (RG) to initiate neuron production, either directly or through transit progenitors in the subventricular zone (SVZ), including basal progenitors (BP) and outer radial glial progenitors (oRG). This will result in the sequential generation of early deep layer (DL) and late upper layer (UL) neurons. (B,C) These temporal and spatial patterns are reproduced during corticogenesis from PSCs (B), and display protracted timing in human cells, as *in vivo* (C). CP, cortical plate; VZ, ventricular zone; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence.

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layers (Fig. 2A). Cortical RGs are thought to be mostly multipotent and to undergo a sequential shift in their competence to generate different laminar neuronal fates (Eckler et al., 2015; Frantz and McConnell, 1996; Gao et al., 2014; Greig et al., 2013; Guo et al., 2013; McConnell and Kaznowski, 1991; McConnell, 1988, 1991; Price and Thurlow, 1988; Reid et al., 1995; Walsh and Cepko, 1988; but see Franco et al., 2012), and such properties are conserved when cortical progenitors are cultured *in vitro* (Shen et al., 2006).

Remarkably, similarly complex temporal patterns of pyramidal neurogenesis have been observed during human and mouse PSC-derived corticogenesis, in which PSC-derived cortical-like progenitors sequentially generate pyramidal neurons with identities corresponding to all six layers (Eiraku et al., 2008; Gaspard et al., 2008) (Fig. 2B,C). This pattern has been reported using a wide range of different culture conditions and media (Eiraku et al., 2008; Espuny-Camacho et al., 2013; Gaspard et al., 2008; Hansen et al., 2011; Lancaster et al., 2013; Shi et al., 2012; van de Leemput et al., 2014). This suggests that a highly robust temporal pattern of generation of neuronal diversity is imprinted in cortical progenitors, perhaps already acquired in dorsal telencephalic progenitors of non-mammalian ancestors (Suzuki and Hirata, 2013, 2014; Suzuki et al., 2012).

On the other hand, cortical inhibitory neurons can also be generated by PSCs *in vitro*, but through a different pathway – consistent with their distinct embryonic origin. During embryogenesis, these cells are not generated from the same niche as pyramidal neurons, but rather from two ventral telencephalic regions, namely the medial (MGE) and caudal (CGE) ganglionic eminences (DeFelipe et al., 2013; Sur and Rubenstein, 2005; Wilson and Rubenstein, 2000). Recapitulating *in vivo* development, the specification of ventral telencephalic cells from both human and mouse PSCs requires inductive input from the Shh morphogen, alone or together with inhibition of the dorsal morphogen Wnt (Danjo et al., 2011; Germain et al., 2013; Li et al., 2009; Liu et al., 2013; Maroof et al., 2010, 2013; Nicholas et al., 2013; Watanabe et al., 2005). The concentration and timing of exposure to Shh lead to different types of ventral progenitors and hence to distinct subtypes of neurons, including the hypothalamic/striatal projection neurons and cortical/striatal interneurons (Danjo et al., 2011; Germain et al., 2013; Ma et al., 2012), which is thus reminiscent of the time dependence of Shh signaling in *in vivo* contexts (Briscoe and Thérond, 2013).

These data demonstrate that all main types of cortical neurons can be generated *in vitro*, following precise, lineage-specific temporal patterns, and depending on the levels of Shh signaling during early steps of PSC neural differentiation. These progenitors will then generate specific types of neurons, i.e. dorsal progenitors will generate primarily glutamatergic pyramidal neurons, whereas ventral cells will generate mostly GABAergic neurons. Human and mouse PSCs do not differ in this respect, as *in vivo* (Hansen et al., 2013), although some intriguing differences are commonly observed between mouse and human PSC-derived models. For instance, mouse cortical progenitors are best differentiated from mouse ESCs in the presence of a chemical inhibitor of Shh signaling (Gaspard et al., 2008), whereas in otherwise similar culture conditions human PSCs convert efficiently to cortical precursors without Shh inhibition (Espuny-Camacho et al., 2013). Intriguingly, the species-specific requirement of Shh inhibition is similarly observed in the cerebellar models as well (Muguruma et al., 2015, 2010; Muguruma and Sasai, 2012; and see below). The origin of these differences remains unclear: whether they are related to differences in the levels of Shh signals or to differential sensitivity to Shh, or to other signals, will be interesting to explore further, as it could also be related to differences found *in vivo*, such as the relative sizes of the cerebral or cerebellar cortices.

Another important lesson learned from modeling corticogenesis *in vitro* is that some key components of cortical cytoarchitecture appear to emerge through a surprisingly high degree of self-organization of cortical cells. Even in monoadherent culture conditions, PSC-derived NE cells form organized aggregates called neural rosettes, in which the cell apical sides cluster with each other at the core of the rosette and the basal sides orient to its periphery (Elkabetz et al., 2008; Li et al., 2005) (Fig. 1B). Moreover, when mouse or human PSCs are cultured as balls of cells differentiating into a cortical lineage in a 3D space, they can develop into organized structures that display a polarized multilayer organization, with radial glia-like progenitors occupying the deepest layers of the balls, intermediate or basal progenitors in the middle, and postmitotic neurons accumulating at their periphery, following an organization that is highly reminiscent of a nascent cortical primordium from VZ to CP (Fig. 2B) (Eiraku et al., 2008; Mariani et al., 2012; Nasu et al., 2012). More refined 3D models have been developed to follow corticogenesis for long (several months) periods of time, leading to cortical-like structures containing specific germinative zones containing diverse types of apical or basal progenitors, and even sometimes CP-like structures, displaying layer-like domains that contain early- and late-born neurons (Kadoshima et al., 2013; Lancaster et al., 2013). Thus, these *in vitro* cultures partially recapitulate the inside-out pattern of corticogenesis typically found *in vivo* (Fig. 2B).

Importantly, PSC-derived corticogenesis is now being used to understand species-specific features of human corticogenesis. Indeed, the cortex has evolved rapidly in size and complexity in the hominid lineages, which is likely to be caused by quantitative and qualitative divergence in patterns of cortical neurogenesis (Fietz et al., 2010; Fish et al., 2008; Hansen et al., 2010; Kriegstein et al., 2006; Lui et al., 2011). One important difference is related to timing: cortical neurogenesis in human is characterized by an extended period of initial amplification of NE precursors followed by a much protracted period of neurogenesis, thereby allowing the generation of higher numbers of neurons (Fig. 2C) (Rakic, 1995). Remarkably, the direct comparison of human with mouse PSC corticogenesis reveals that human PSC-derived corticogenesis presents temporal specificities that are strikingly reminiscent of human-specific features of cortical development. Human PSC-derived cortical progenitors start to generate neurons after a much longer period of about 4 weeks, instead of 6-8 days in the mouse. Similarly, the generation of distinct types of cortical neurons is also much protracted, requiring about 1 week in the mouse (Gaspard et al., 2008) but several months starting from human ESCs (Fig. 2C) (Eiraku et al., 2008; Espuny-Camacho et al., 2013; Gaspard et al., 2008; Kadoshima et al., 2013; Shi et al., 2012).

Another distinctive feature proposed to link the development and evolution of human corticogenesis is the diversity of progenitors (Fig. 2A,B). Several types of progenitors, most strikingly the so-called outer/basal radial glia progenitors (oRGs), were first described in the human and ferret cortex, and since then found to be much more prevalent in species characterized by a folded and enlarged cerebral cortex (Dehay et al., 2015; Lui et al., 2011; Taverna et al., 2014). These progenitors are typically located further away from the VZ in a specialized niche called the outer SVZ (Fig. 2A). Interestingly, such cells have been observed during corticogenesis in human (Lancaster et al., 2013; Shi et al., 2012) but not in mouse PSC models: it will be interesting to test whether

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specific alterations of the properties of these cells in *in vitro* models could have an impact on the size or shape of the cortical-like tissue generated, or on the number of neurons generated.

Finally, human PSC models of corticogenesis have started to enable dissection of the molecular mechanisms potentially underlying human cortical development, taking advantage of iPSC models derived from patients displaying specific brain alterations of genetic origin. These include forms of primary microcephaly caused by point mutations in *CDK5RAP2* (Lancaster et al., 2013), for which iPSC models recapitulated the neurogenic defects potentially relevant to the pathogenesis of the disease. iPSC models were also used to study the mechanisms underlying more complex genetic disorders, such as copy number variants (CNVs), which are difficult to recapitulate with animal models. Modeling from patients presenting 15q11.2 microdeletions associated with schizophrenia and autism revealed specific defects in early neural development, in particular the apicobasal polarity of NE cells (Yoon et al., 2014). These could be directly linked to a specific gene found in the interval of the microdeletion, *CYFIP1*, thereby potentially linking the pathogenesis of this CNV to early defects in neural development, specifically the control of neural cytoskeletal dynamics through the WAVE complex (Yoon et al., 2014).

In summary, despite its complexity, many features of corticogenesis can be mimicked using PSC modeling. Perhaps the most remarkable observation in this context is that key aspects of spatial and temporal patterning are recapitulated without much experimental manipulation, indicating that they are, to a large extent, intrinsic to the cortical lineage.

The cerebellum

The mammalian cerebellum consists of the cerebellar cortex and the deep cerebellar nuclei, both of which emerge from the dorsal part of the hindbrain. The cerebellar cortex displays a highly stereotyped structure with three distinct layers: the molecular layer, the Purkinje cell layer and the granule cell layer (Fig. 3A) (Butts et al., 2014; Martinez et al., 2013; Millen and Gleeson, 2008). The major cell types in the cerebellum originate from two spatially distinct populations of neural progenitors (Fig. 3A). The GABAergic neurons, such as Purkinje cells and the basket cells, are generated from Ptf1a-expressing progenitors in the cerebellar VZ (Hoshino et al., 2005). By contrast, the excitatory neurons, including granule cells, are generated from the Math1 (Atoh1)-positive rhombic lip, which is located in the dorsal tip of the hindbrain (MacHold and Fishell, 2005). Dorsoventral patterning of the hindbrain is regulated by counteracting morphogen signals, Shh and BMP, which are produced by the ventral floor plate and the dorsal roof plate, respectively. The dorsally generated granule cells migrate ventrally and eventually settle to construct the granule cell layer beneath the Purkinje cell layer. These processes of cerebellar development were identified essentially from the studies in animal models and direct observation in human is largely missing.

This highly complex pattern of cell specification has been partially recapitulated in mouse and human PSC-derived systems (Salero and Hatten, 2007; Su et al., 2006; Erceg et al., 2010). In these studies, PSC monoadherent cultures treated with chemically defined media containing a cocktail of morphogens can convert into granule cell precursors and Purkinje cells. When PSC-derived granule precursors are transplanted in the neonatal mouse cerebellum, they can migrate and acquire characteristic morphology (Erceg et al., 2010; Salero and Hatten, 2007; Su et al., 2006).

A more complete and complex 3D model of early cerebellar development was recently described with mouse and human PSCs (Muguruma et al., 2015, 2010) (Fig. 3B). These models enable stepwise progression of cerebellar patterning, closely mimicking the *in vivo* situation, by sequential application of defined combinations of morphogen signals, such as FGF2, FGF19 and SDF1 (Cxcl12) (Fig. 1D). Under these conditions, an initially unpatterned neural aggregate derived from PSCs shapes into a NE sheet, consisting of a rhombic lip-like region at the edge of the sheet, with the rest of the sheet displaying an identity reminiscent of the cerebellar field (Fig. 3B). Remarkably, this sheet displays an apicobasally layered arrangement of multiple cerebellar cell types, including progenitors similar to the cerebellar VZ, an intermediate layer containing precursors of Purkinje neurons, and an outermost layer occupied by derivatives of the rhombic lip, which have the characteristics of granule cell precursors. The granule-like cells observed at the latest stage of the human 3D model still express precursor marker genes, but if co-cultured with primary granule cells isolated from postnatal mice they take on their characteristic T-shaped morphology and

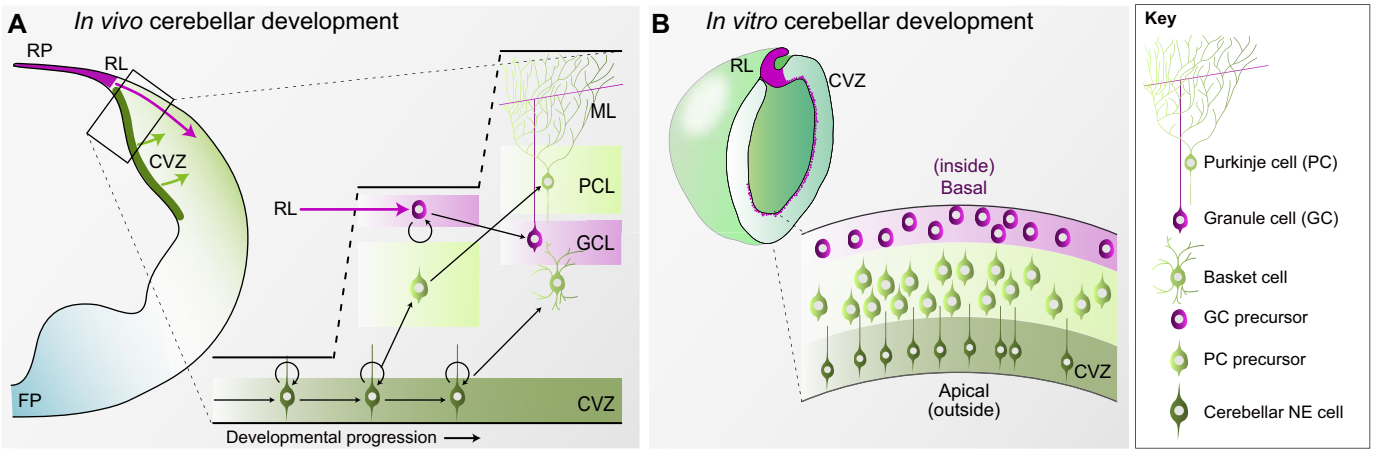

Fig. 3. Modeling spatial and temporal patterns of cerebellar development. (A) The cerebellar cell types are derived from the rhombic lip (RL) and the cerebellar ventricular zone (CVZ). The excitatory cell types, such as the granule cells (GC), are derived from the RL, whereas the inhibitory cell types, such as Purkinje cells (PC) and basket cells, are produced in the CVZ. ML, molecular layer; PCL, Purkinje cell layer; GCL, granule cell layer. (B) Differentiation from PSCs similarly leads to the generation of a cerebellar anlage that contains spatially distributed neurons generated from distinct niches. FP, floor plate; RP, roof plate.

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express marker genes of postmitotic granule neurons. The two main extrinsic cues that appear to be strictly required for the emergence of this highly ordered spatial patterning are FGF19 and SDF1. These were identified among many other cues tested, and their mechanisms of action have not been fully uncovered: given their dramatic morphogenetic effects *in vitro* it will be interesting to explore or revisit their role in animal models *in vivo*. In any case, the fascinating aspect of this model is the ability to generate completely different neuronal subtypes, which come from distinct neurogenic niches *in vivo* and potentially also in this *in vitro* system (although this remains to be established), and which then integrate with each other to contribute to a complex structure.

Aside from their differential dependence on Shh signaling discussed above, the mouse and human models of cerebellar morphogenesis display additional differences (Muguruma et al., 2015, 2010; Muguruma and Sasai, 2012): the temporal progression of differentiation and functional maturation of cerebellar cell types takes two to three times longer with human cells and the final structure generated is much larger in size than with mouse cells (Fig. 2C). In addition, Purkinje cells derived from human PSCs display a larger soma and more complex dendritic arbors than their mouse counterparts, providing evidence that such species-specific aspects of neuronal patterning are at least in part intrinsic to the neurons.

The retina

The retina develops as part of the central nervous system from the diencephalic neuroepithelium. The morphogenetic events leading to a functional eye are highly conserved among vertebrates (Bazin-Lopez et al., 2015; Cepko, 2014; Dyer and Cepko, 2001; Fuhrmann, 2010; Graw, 2010) (Fig. 4A). The lateral part of the diencephalic NE sheet evaginates and gives rise to the optic vesicle (Fig. 4A, step i). The distal and proximal regions of the optic vesicle are specified into the neurosensory retina (NR) and retinal pigment epithelium (RPE), respectively (Fig. 4A, step ii). The distal tip of the optic vesicle then invaginates such that the future NR and RPE become closely apposed to each other (Fig. 4A, steps iii and iv). These dynamic morphogenetic events of the NE sheet are tightly coupled with the coordinated movements of surrounding placodal ectoderm. It has been thought that the close interaction of the two ectodermal layers is essential for retinal morphogenesis (Bazin-Lopez et al., 2015; Fuhrmann, 2010; Hyer et al., 2003; Smith et al., 2009). Within the NR, neural progenitors then generate the various types of retinal neuronal and glial cells following a defined temporal pattern, eventually yielding the layered adult retina (Bassett and Wallace, 2012; Cepko, 2014).

These sequential events of dynamic retinal morphogenesis are strikingly recapitulated in the mouse and human PSC-based 3D models, through a self-organizing process that does not appear to involve interactions with non-neural tissues (Fig. 4B) (Ader and Tanaka, 2014; Eiraku et al., 2011; Kuwahara et al., 2015; Nakano et al., 2012; Sasai et al., 2012). Initial retinal regional specification is achieved using culture with morphogen inhibitors such as Nodal for mouse PSCs (Eiraku et al., 2011) and Wnt inhibitor for human PSCs, in the presence of basement membrane matrix components (Matrigel) (Nakano et al., 2012). The culture medium itself is sufficient to induce retinal fate, but the presence of Matrigel is necessary for constructing continuous sheets of retinal epithelium (Eiraku et al., 2011; Nakano et al., 2012). The PSC-derived aggregates then spontaneously develop optic vesicle-like structures, which then invaginate to form cup-shaped structures, as *in vivo* (Fig. 4B) (Eiraku et al., 2011).

The accessibility of this *in vitro* system has enabled a more in-depth investigation of the physical dynamics underlying optic cup formation (Eiraku et al., 2011; Nakano et al., 2012; Sasai, 2013). The invaginating NR region initially demonstrates a relaxation of constriction on the apical (inner) surface, which makes the tissue permissive for subsequent folding. The 'hinge' region, located between the NR and the RPE, was observed to be the most dynamic, being strongly constricted to allow tissue invagination leading to optic cup formation. Subsequently, the apical side of the NR gradually expands more than the basal side to complete invagination. Once the NR field is specified, NE cells start neurogenesis following a temporal pattern that mimics the *in vivo* situation, enabling the sequential generation of various retinal cell populations organized in a layer-like structure, which resembles the early neonatal eye of rodents (Eiraku et al., 2011; Nakano et al., 2012; Osakada et al., 2009) (Fig. 4B). Another aspect of the self-organizing property of retinal development from human PSCs was demonstrated more recently (Kuwahara et al., 2015), in which a specific stem cell niche called the retinal ciliary margin (RCM) (Agathocleous and Harris, 2009),which is located at the boundary of the NR and RPE, could be generated *in vitro* and displayed the potential to produce retinal progenitors, which in turn generate various retinal subtypes including photoreceptors (Fig. 4C).

In summary, although some earlier *in vivo* studies had indicated that the tissue interaction between the placodal ectoderm and neural retina is important for normal optic cup morphogenesis (Bazin-Lopez et al., 2015; Fuhrmann, 2010; Hyer et al., 2003; Smith et al., 2009), PSC-derived complex choreography can be achieved in the absence of interaction with non-retinal tissues, confirming the 'retina-autonomous' nature of its development (Fuhrmann, 2010; Martinez-Morales and Wittbrodt, 2009), and suggesting that eye formation is mainly the result of the self-organizing properties of retinal cells.

Late aspects of brain development: neuronal maturation, neurite outgrowth and guidance, and synaptogenesis

The final steps of brain development essentially consist of key aspects of neuronal maturation and patterning, including neurite outgrowth, acquisition of electrical excitability, and eventually circuit formation through synaptogenesis. Their study using PSCs has revealed interesting features of human neuronal development, in particular regarding their maturation kinetics, which suggest that neurons mature along their own, species-specific 'clock'. Indeed, human neurons display much more prolonged patterns of morphological and electrophysiological maturation than their mouse counterparts (Espuny-Camacho et al., 2013; Kriks et al., 2011; Maroof et al., 2013; Nicholas et al., 2013; Shi et al., 2012; Studer et al., 2015; Takazawa et al., 2012). For instance, whereas mouse cortical neurons typically mature in 2-4 weeks *in vitro* (Gaspard et al., 2008), *in vitro* derived human cortical neurons exhibit immature profiles of gene expression and excitability for several weeks. Extended cultivation periods reaching to several months are needed to observe more mature patterns of action potentials and signs of significant synaptic activity, which even after such long periods still appear immature compared with their mouse counterparts (Espuny-Camacho et al., 2013; Mariani et al., 2012; Nicholas et al., 2013; Shi et al., 2012). Even more strikingly, although transplantation of the cortical cells generated by mouse PSCs into the postnatal mouse cortex leads to fully mature pyramidal neurons after a few weeks (Fig. 5A,B) (Gaspard et al., 2008), the corresponding human PSC-derived excitatory and inhibitory cells still develop at their own pace upon

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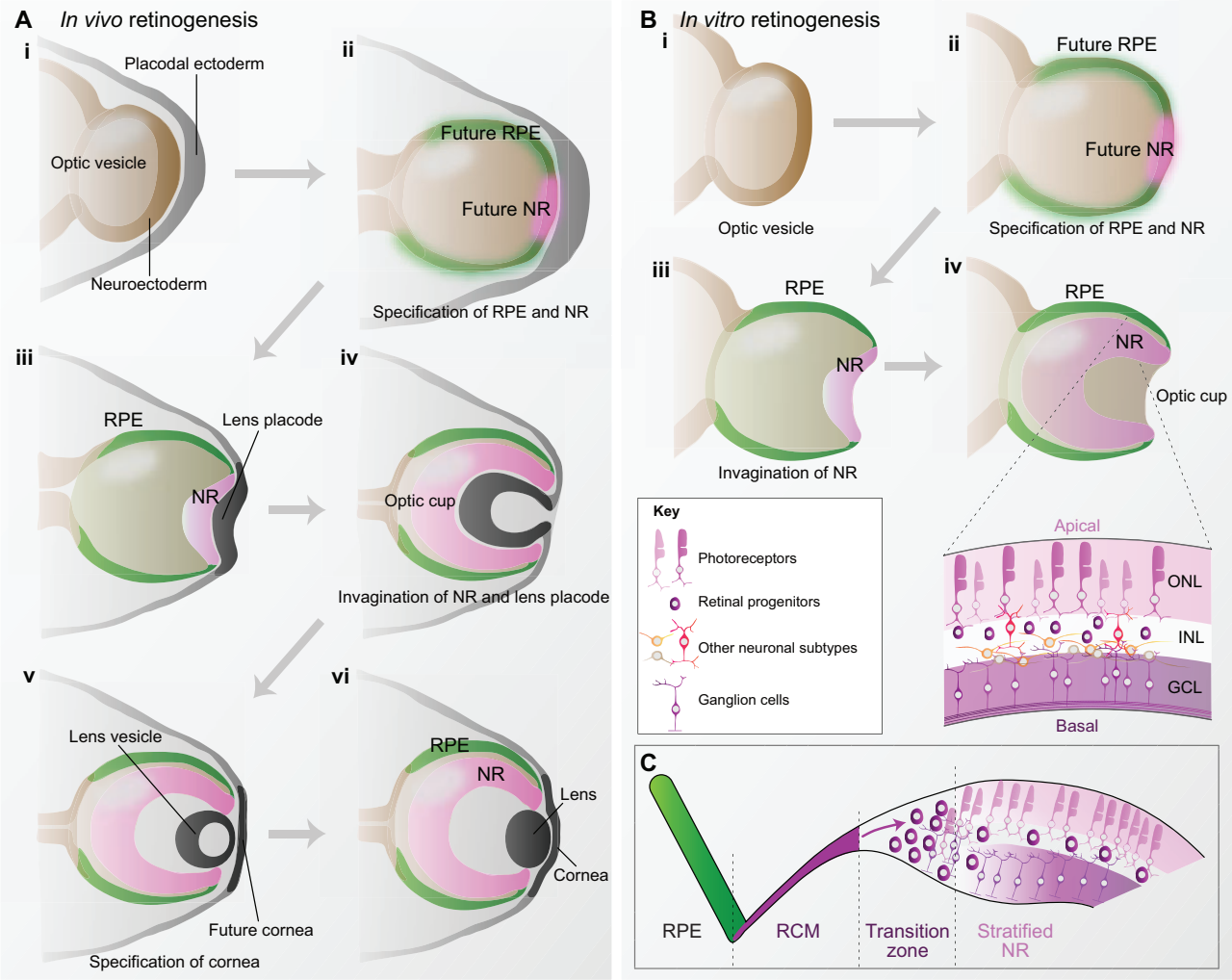

Fig. 4. Modeling spatial and temporal patterns of retinogenesis. (A) The main tissue interactions that are observed in retinal morphogenesis *in vivo*. The lateral part of the diencephalic neuroectoderm, which is covered by the placodal ectoderm, protrudes to form the optic vesicle (step i). The distal and proximal regions of neuroectoderm are specified to neural retina (NR) and retinal pigment epithelium (RPE), respectively (step ii). The distal tip of the NR invaginates together with the surrounding ectodermal tissue, the lens placode (step iii). The tissue invagination is accomplished to form a transient structure called the optic cup (step iv). The lens placode detaches from the rest of the ectodermal layer to form the lens vesicle. The ectodermal sheet covering the lens vesicle is specified as the future cornea (step v), leading to final development of the eye (step vi). (B) The morphogenesis of neuroectodermal retina is recapitulated in a PSC-derived *in vitro* model without interactions with the other tissues. Optic vesicle-like buddings are generated from PSC-derived NE cell aggregates (step i). Then, the distal and proximal regions of the vesicle are specified into NR and RPE, respectively (step ii). The distal tip of the NR invaginates to form an optic cup-like structure (steps iii and iv). The mature NR exhibits a retina-like laminar architecture with photoreceptors occupying the most apical layer [outer nuclear layer (ONL)], dividing progenitors and interneurons located in an inner nuclear layer (INL), and retinal ganglion cells in the most basal layer resembling the ganglion cell layer (GCL). (C) 3D retinal development from PSCs can also lead to the generation of a special stem cell niche, termed the retinal ciliary margin (RCM), located between the RPE and NR. The RCM further supplies retinal progenitors that proliferate and differentiate into various retinal cell types.

transplantation into mouse, and only develop axons, dendrites and functional synapses several months after transplantation (Fig. 5C) (Espuny-Camacho et al., 2013; Kirkeby et al., 2012; Maroof et al., 2013; Nicholas et al., 2013).

This pattern of protracted maturation is strikingly reminiscent of the situation in the developing human brain, where neurons take months and sometimes years to reach maturation; this might underlie some of the relative neoteny that characterizes human brain maturation, particularly in specific cortical areas (DeFelipe, 2011; Petanjek et al., 2011). Overall, these data point to cell-intrinsic mechanisms that control a 'clock' of neuronal maturation and connectivity. Taken together with the species-specific pace of neurogenesis, PSC-based models may provide attractive experimental systems with which to approach this fascinating unsolved biological issue, i.e. the nature and evolution of species-specific 'clock(s)' of neuronal development.

As the disruption of synapse formation or function is thought to be at the core of several types of human brain diseases, synaptogenesis and synaptic activity have been studied with patient-derived iPSCs in the context of disease modeling (Dolmetsch and Geschwind, 2011; Sandoe and Eggan, 2013). This includes modeling of neuropsychiatric conditions related to *DISC1* mutations (Wen et al., 2014), Rett syndrome (Marchetto et al., 2010), Timothy syndrome (Paşca et al., 2011), sporadic schizophrenia (Brennand et al., 2011) and Phelan–McDermid (PMD) syndrome (Shcheglovitov et al., 2013). These studies

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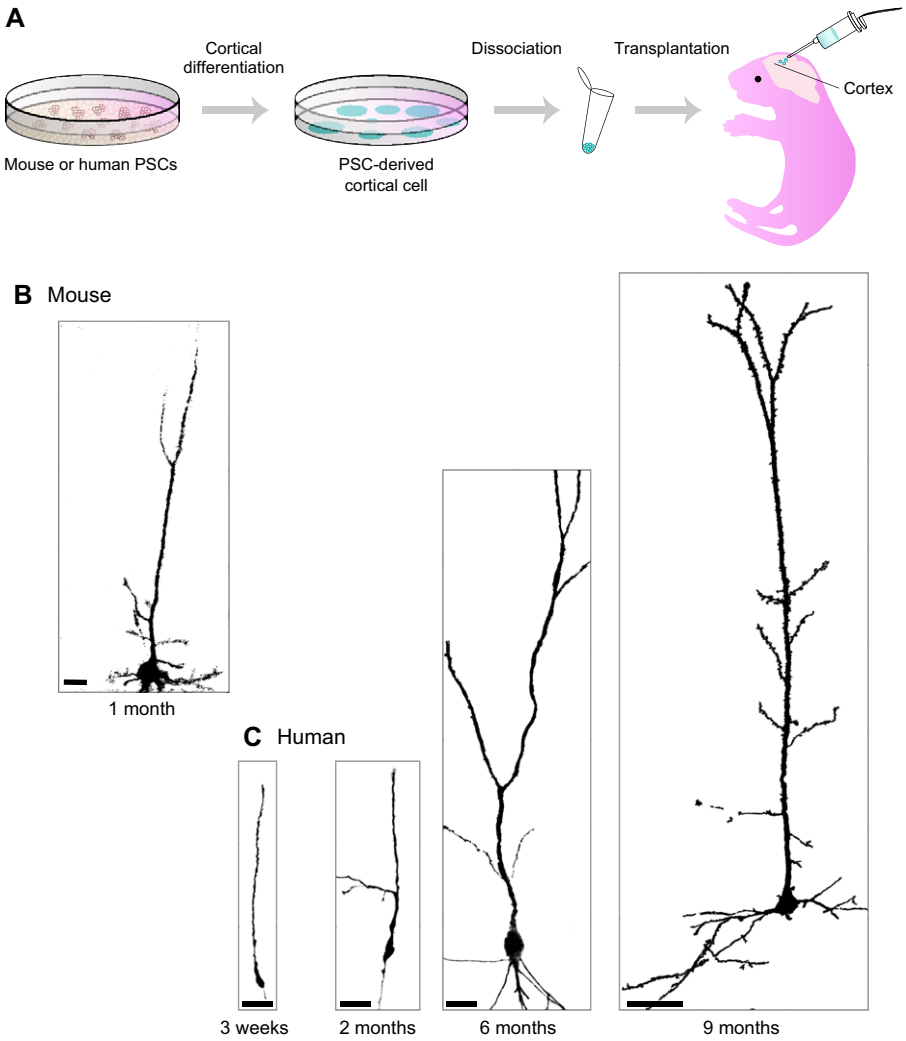

Fig. 5. Modeling neuronal maturation using xenotransplantation. Cortical pyramidal neurons derived from mouse or human PSCs display species-specific maturation following transplantation in the mouse neonatal cortex (A). Whereas a mature-like pattern can be observed 1 month post-transplantation with mouse ESC-derived cortical cells (B), human cells reach a similar stage 9 months post-transplantation (C). Scale bars: 20 µm. Images are adapted from Espuny-Camacho et al. (2013) and Gaspard et al. (2008).

demonstrate the possibility of using PSC-based *in vitro* models for studying synaptic development in the human, although much work will be necessary to relate *in vitro* findings to *in vivo* situations. In this context, two lines of investigations should probably be developed further to assess the connectivity patterns of human neurons in a more physiologically relevant way. On the one hand, it will be interesting to use 3D models of corticogenesis, or other brain structures, to dissect precise patterns of synaptic connectivity (Kadoshima et al., 2013; Lancaster et al., 2013). On the other hand, the transplantation of human PSC-derived cells into recipient mice, which enables human neuron development and connectivity to be followed for up to one year (Espuny-Camacho et al., 2013), could constitute a promising tool to study the impact of genes or environment on human neuron connectivity in an *in vivo* context that is experimentally tractable.

Conclusions, challenges and future perspectives

While it has become trivial to discuss the potential of PSC technology for translational purposes, such as disease modeling and cell therapy, their potential usefulness as a tool in basic developmental biology had remained less clear. From the data discussed here, PSC-derived models of neural development have the potential to do much more than just extend the findings previously obtained in animal models to the human setting. In particular, PSC models have revealed, in sometimes striking ways, the extent to which cell diversity and spatial and temporal patterning can be robustly recapitulated in simplistic *in vitro* settings, emphasizing the importance of intrinsic self-organization during development. Even though this could be considered trivial (after all, the embryo itself is obviously capable of self-organization), the versatility and accessibility of PSC models makes them ideal to study the underlying mechanisms, in particular for those structures that are specific to organisms developing *in utero*. This should constitute a useful source of inspiration to design new experiments *in vitro*, as well in *in vivo* models, to understand the underlying mechanisms.

Another striking observation is that PSC models recapitulate faithfully the timing of developmental events, and in a species-specific way. PSCs thereby constitute promising tools to uncover and dissect the links between development and evolution, not only by comparing human and mouse PSC-derived systems, but also by implementing reprogramming of somatic cells of other mammalian species in which experimental manipulation in embryos is difficult, such as non-human primates (Wunderlich et al., 2014). This might shed further light on the evolutionary conservation and divergence of various other aspects of brain development, such as the gyration patterns of the cerebral cortex that have been independently acquired in multiple mammalian lineages (Lui et al., 2011).

From a molecular perspective, a number of genes have been identified recently, mostly through transcriptomics/genomics approaches, that may be involved in human-specific features of brain

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development (Johnson et al., 2009; Lambert et al., 2011; Pollard et al., 2006; Prabhakar et al., 2006), some of which have been validated functionally (Charrier et al., 2012; Florio et al., 2015; Johnson et al., 2015; Lui et al., 2014). It is likely that human PSC neural development models will constitute the ideal experimental setup to determine the mechanisms of action of such genes, and to identify others.

In conclusion, PSC-derived models have emerged as a highly valuable tool for use and study by developmental neurobiologists, in combination with animal models and human genetics, and have great potential to advance our understanding of the developmental bases of human evolution and disease.

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Competing interests

The authors declare no competing or financial interests.

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