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REVIEW

Molecular and cellular evolution of corticogenesis in amniotes

Adrián Cárdenas[1] · Víctor Borrell[1]

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Abstract

The cerebral cortex varies dramatically in size and complexity between amniotes due to differences in neuron number and composition. These differences emerge during embryonic development as a result of variations in neurogenesis, which are thought to recapitulate modifications occurred during evolution that culminated in the human neocortex. Here, we review work from the last few decades leading to our current understanding of the evolution of neurogenesis and size of the cerebral cortex. Focused on specific examples across vertebrate and amniote phylogeny, we discuss developmental mechanisms regulating the emergence, lineage, complexification and fate of cortical germinal layers and progenitor cell types. At the cellular level, we discuss the fundamental impact of basal progenitor cells and the advent of indirect neurogenesis on the increased number and diversity of cortical neurons and layers in mammals, and on cortex folding. Finally, we discuss recent work that unveils genetic and molecular mechanisms underlying this progressive expansion and increased complexity of the amniote cerebral cortex during evolution, with a particular focus on those leading to human-specific features. Whereas new genes important in human brain development emerged the recent hominid lineage, regulation of the patterns and levels of activity of highly conserved signaling pathways are beginning to emerge as mechanisms of central importance in the evolutionary increase in cortical size and complexity across amniotes.

Keywords Mouse · Chick · Robo · Radial Glia · Neurogenesis · Primate

Introduction

Brain size varies dramatically across amniotes, a monophyletic group of vertebrates that appeared in the late Carboniferous, about 320 million years ago, and originated the lineage of extant reptiles, birds and mammals (Fig. 1) [1, 2]. Differences in brain size between amniotes are largely due to the disproportionate expansion of the dorsal telencephalon during evolution [3–5]. In mammals, the dorsal cortex gives rise to the neocortex, which as the name indicates is the newest evolutionary addition to the cerebral cortex. Indeed, the neocortex appeared around 200 million years ago, in the Jurassic period, with the emergence of the stem mammals, and became a defining feature of this clade [6–8]. The neocortex is characterized by its organization in six neuronal layers, with a specific composition of neuron types, intrinsic cortical connectivity and long-range connections. Whereas these features are conserved across mammals [9, 10], the neocortex underwent significant modifications during mammalian evolution in terms of shape, size and organization [3, 11, 12]. The impact of these changes on the cognitive abilities of mammals is thought to be key in the success of their radiation and evolution [13]. Even though the neocortex appeared with the emergence of mammals, some of its components and characteristics precede their common ancestor and are shared with other amniotes [14, 15]. For example, it has a similar embryonic origin in mammals, birds and reptiles, many genes involved in its development are strongly conserved in sequence, patterns of expression and function, and there is significant homology in neuron types and connectivity [5, 14, 16–18].

The extraordinary complexity of the neocortex arises from its similarly complex embryonic development, generating myriads of extraordinarily diverse neurons. The integration of these neurons into exquisitely precise neural circuits underlies perception and behavior, which are particular in different species according to their ecological niche. The process by which neurons are produced is called

✉ Víctor Borrell
vborrell@umh.es

[1] Instituto de Neurociencias, Consejo Superior de Investigaciones Científicas y Universidad Miguel Hernández, 03550 Sant Joan d'Alacant, Alicante, Spain

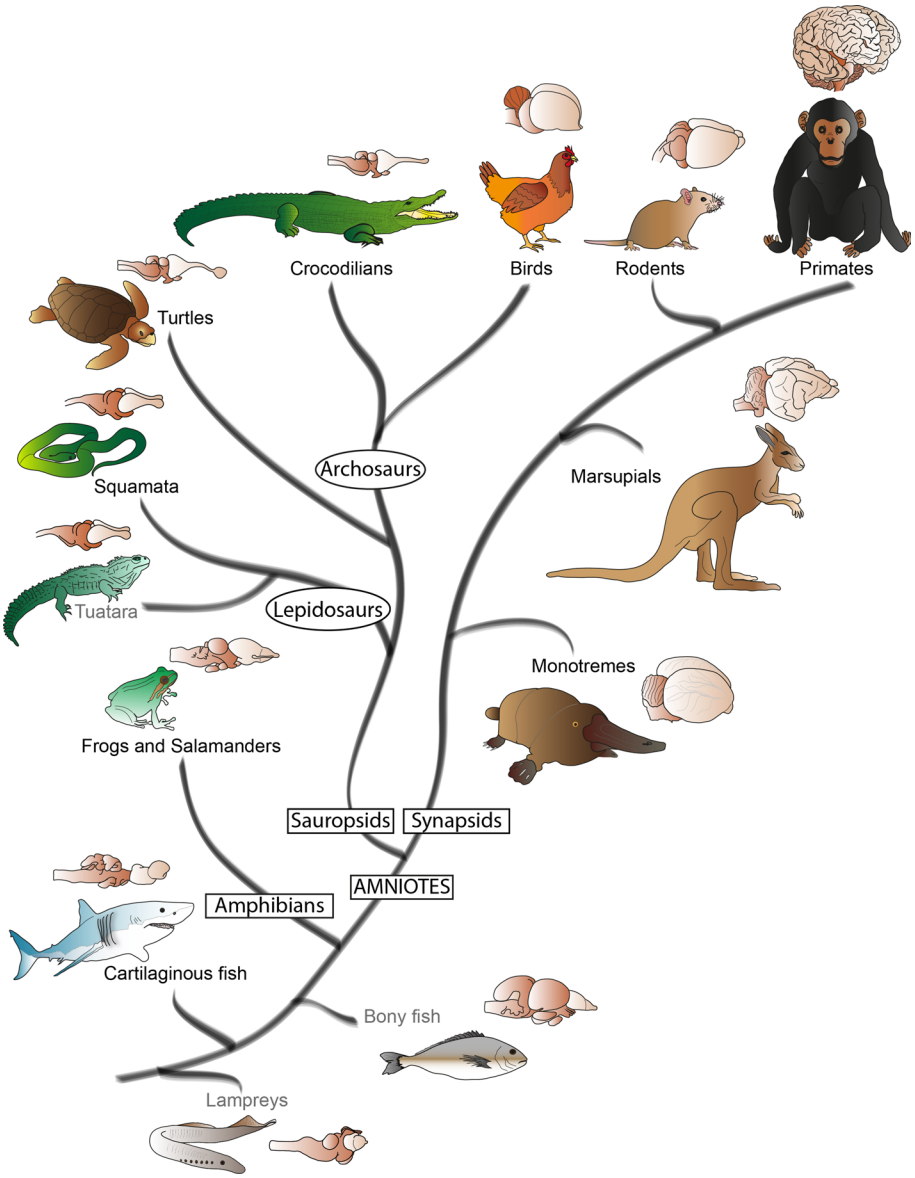

Fig. 1 Vertebrate phylogeny. Simplified phylogenetic tree of vertebrates, illustrating the appearance of representative species and their brains. Drawings are not at scale

neurogenesis, and this must be precisely regulated during cerebral cortex development. Differences in cerebral cortex size and complexity across amniote phylogeny are the result of the evolution of mechanisms regulating its development [3]. The cellular and genetic mechanisms underlying the fabulous evolutionary expansion and complexification of the human cerebral cortex from the stem amniotes remain largely unknown [19]. This review aims to integrate a wide spectrum of points of view on this issue into a coherent framework. First, we summarize comparative studies of pallial organization in representative groups of amniotes, then we discuss principles of cortical neurogenesis with an emphasis on homologies and divergences between amniotes, and finally we conclude focusing on the cellular and molecular events that seemingly allowed the emergence and further expansion of the mammalian neocortex.

Pallial organization and evolution in amniotes

The stem amniote lineage is divided into two distinct clades: sauropsids and synapsids, distinguished by the presence of cranial temporoparietal arches in their skull [20]. All mammals belong to the synapsid lineage (with one cranial arch), whereas reptiles and birds belong to the sauropsid lineage (with two temporal arches, or none). Sauropsids are further subclassified into lepidosaurs (snakes, lizards and tuatara) and archosaurs (birds, crocodilians and turtles) (Fig. 1) [1, 21–24]. Based on gene expression, patterns of cell migration, neural connectivity and cytoarchitecture, the embryonic pallium of amniotes is initially composed of four subdivisions: medial, dorsal,

lateral and ventral [14, 17, 25, 26], which at later stages diverge functionally and structurally [13] (Fig. 2). The medial domain gives rise to the hippocampal formation and the entorhinal cortex [5, 27]. The dorsal part develops into the neocortex in mammals, the dorsal cortex in reptiles, and the Wulst or hyperpallium in birds [5, 28]. The lateral part constitutes the insular cortex, the claustrum/endopiriform complex and the amygdala [25, 29, 30]. And the ventral pallium gives rise to the olfactory bulb and olfactory cortex, among other nuclei, in mammals (Fig. 2) [25, 30, 31].

Reptiles and birds (sauropsids) develop a prominent domain from the lateral and ventral regions, named dorsal ventricular ridge (DVR), which in the mature brain receives sensory information from the thalamus [9, 25, 27, 32]. Some authors have proposed that the combination of the DVR with the dorsal cortex in reptiles, or the Wulst in birds, are homologous to the mammalian neocortex [33, 34]. However, the more broadly accepted view is that the DVR is homologous to the mammalian claustrum and amygdala, whereas the dorsal cortex and the Wulst are homologous to the mammalian neocortex (Fig. 2) [5, 9, 25]. Additional support for this notion comes from recent single-cell transcriptomic analyses comparing mammals and reptiles, which provide evidence for the conservation of the hippocampal region, the homology between DVR and the mammalian ventral pallium, and between the anterior dorsal cortex of reptiles and the mammalian neocortex [17, 27].

The reptile dorsal cortex is a relatively simple structure organized in three layers, with two cell-sparse plexiform layers (inner and outer) surrounding a densely packed neuronal layer [23]. This simple organization is also found in evolutionarily ancestral regions of the mammalian brain, such as the hippocampus and the piriform cortex [9, 10, 29], suggesting that it already existed in the common ancestor of extant reptiles and mammals. The six layers typical of the neocortex appeared only with the emergence of mammals, becoming a defining trait. Neocortical neurons are arranged in highly stereotyped laminar patterns, which vary according to their sensory, motor or associative functions. Cortical neurons within individual layers establish stereotyped connectivity patterns with neurons in other layers [10, 35]. Accordingly, neurons in layers 5 and 6 (deep) project to the thalamus; layer 5 neurons also project to the spinal cord; neurons in layer 4 are the main receivers of thalamic input; and neurons in layers 2 and 3 (superficial) connect different cortical regions. In contrast to the laminar organization of the cortex in mammals and reptiles, projection neurons in the avian hyperpallium display a unique nuclear-like, or semi-layered, organization [13, 36]. Despite this absence

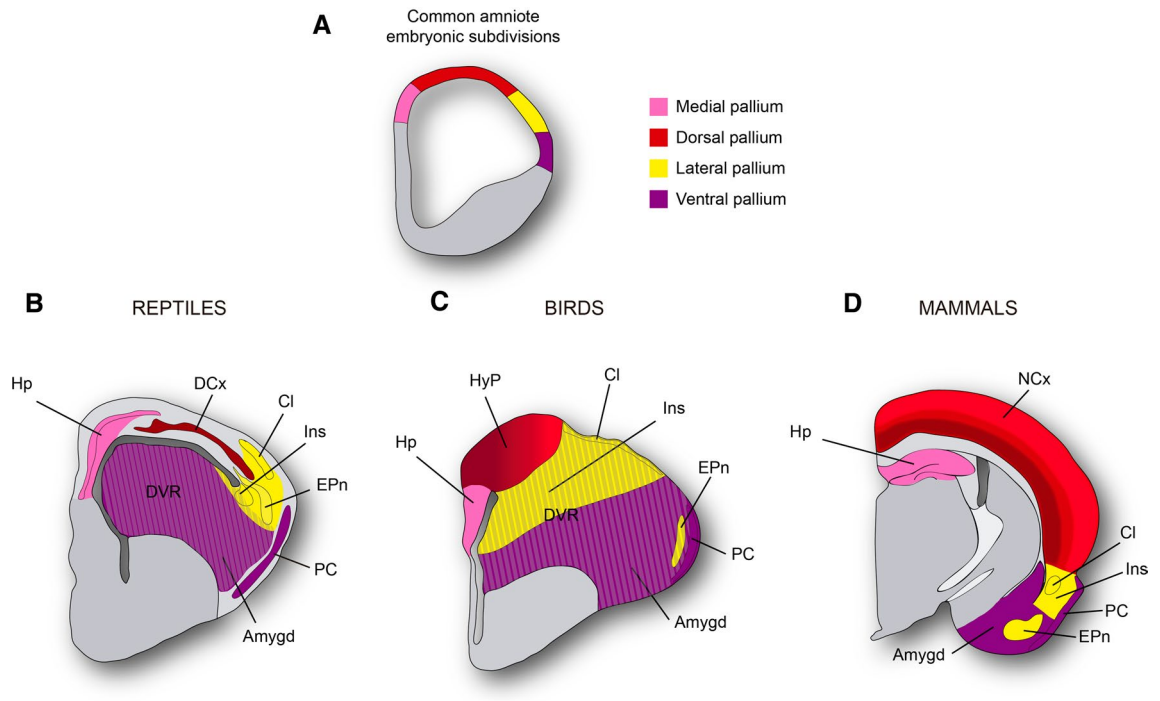

Fig. 2 Schematic drawings of coronal sections through the telencephalon of amniotes indicating the extent of subdivisions. **a** Early embryonic telencephalon of a model amniote. **b–d** Adult telencephalon of reptiles, birds and mammals, as indicated, based on the tetrapartite model [25]. The subpallium is represented in gray and subdivisions of the pallium are color coded as indicated. The striped pattern in the reptile and bird pallium indicates the extension of the DVR. *Hp* hippocampus, *DCx* dorsal cortex, *Cl* claustrum, *Ins* insula, *EPn* endopiriform nucleus, *PC* piriform cortex, *Amygd* amygdala, *DVR* dorsal ventricular ridge, *Hyp* hyperpallium, *NCx* neocortex

of layers in the avian pallium, excitatory neurons establish input and output connections in a manner very similar to the mammalian neocortex [14]. Thus, evolution may have been restrictive at conserving the basic layout of circuits, cell types and embryological territories of the amniote pallium, but not a specific cytoarchitecture [14, 17, 37].

Based on morphology, neurotransmitter expression and connectivity, the classical view is that the reptile and avian pallium only contain neurons homologous to those in the mammalian layers 5 and 6, missing the neuronal populations corresponding to layers 2 and 3. This has suggested that the mammalian neocortex may have evolved from the reptilian three-layered dorsal cortex by acquiring these novel cell types [38, 39]. A recent transcriptomic study challenges this view by revealing that the major signatures of cortical neurons are conserved in turtles, lizards and mice, including expression of some determinants of mammalian upper layer neuron types, such as *Satb2*, already found in regions of the reptile dorsal cortex [17]. However, in reptiles these genes typical of upper layer neurons are co-expressed with deep-layer neuron genes, in contrast to mammals where they are mutually exclusive [17, 40]. This is also the case in the avian Wulst, where differentiated neurons may co-express *Ctip2* and *Satb2*, which in the mammalian neocortex are specifically expressed by lower- and upper layer neurons, respectively [40]. In addition, the axon projection patterns of these neurons in the reptilian and avian dorsal pallium differ from those in the mammalian neocortex: whereas *Sabt2*+ neurons of the mammalian neocortex project to the contralateral hemisphere via the corpus callosum, avian *Ctip2-Sabt2* co-expressing neurons project to the septum [40]. Altogether, these differences strongly suggest that projection neuron types are not really homologous among amniotes, and that making *Ctip2* and *Sabt2* expression mutually exclusive may have been important for the advent of upper neocortical layers in mammals [40]. Accordingly, the genetic signatures of superficial and deep layer cortical neurons may have already existed in the dorsal cortex of amniote ancestors, and the new cortical layers of mammals may have emerged by modifying the genetic programs of ancestral neurons. The emergence of new repressive interactions between previously co-expressed transcription factors, together with the elongation of the neurogenetic period, may have underlined the segregation of superficial and deep layer neurons [17, 41]. It has been also suggested the existence of a conserved circuit motif in which pallial intratelencephalic neurons, which can be viewed as functionally analogous of upper layer neurons, were already present in the common amniote ancestor linking input and output neuronal populations. Later on this population of intratelencephalic neurons would independently diversify and greatly expand in number, as observed in big-brained amniotes, generating a complex integrative circuit composed of upper layer neurons and cortices with associative function in mammals, which served as substrate for the evolution of higher cognitive abilities [14].

During the evolution of mammals, the size and complexity of the neocortex increased disproportionately compared to other brain regions [4, 7] (Fig. 3). Detailed analyses show that this scaling is both a matter of neuron number and of neuron packing and connectivity patterns [42]. Some parrots and songbirds have much smaller brains than some primates do, and yet twice as many neurons, hence a much greater neuronal density [43]. Therefore, the expansion of the cerebral cortex during evolution resulted from an increase in neuronal production combined with changes in neuron type composition and morphology, including the development of more complex dendritic arbors and neuropile [44]. Across mammalian phylogeny, differences in cerebral cortex size are mainly related to surface area rather than thickness [6]. According to the radial unit hypothesis [45], this evolutionary expansion of the cortical surface area must have been achieved by increasing the number of cortical stem and progenitor cells prior to the neurogenic period [6, 7, 44]. In contrast, expansion of the neocortex in the radial axis resulted from increased neurogenesis and the generation of new types of specialized neurons. As mentioned above, the generation of intracortically projecting neurons, clustered into new superficial cortical layers, was a key novelty in the evolution of the mammalian cerebral cortex, and this is thought to have been possible largely thanks to the emergence of new types of progenitor cells [46, 47]. Finally, the remarkable expansion of the mammalian neocortex during evolution was also linked to its folding [48–50]. This is considered a fundamental milestone of mammalian evolution to accommodate the increasing cortical surface area inside a limited skull, with key consequences on its function [3, 6, 7, 51].

Cortical germinal zones in mouse

The mouse neocortex is composed of roughly 80% excitatory neurons and 20% inhibitory neurons (interneurons) [52]. Cortical interneurons are generated in the ganglionic eminences of the subpallium, from where they migrate tangentially for long distances along stereotyped routes to reach the developing cerebral cortex [53, 54]. In contrast, projection neurons are generated locally within the germinal zones of the cerebral cortex, and migrating radially for a short distance toward the cortical surface to form the nascent neuronal layers [55]. Whereas the pallium was subject to intense modification during evolution, the subpallium remained relatively conserved across amniotes [28], where interneurons are produced by homologous sources and display homologous migratory routes and diversity, in mammals, birds and reptiles [17, 56]. Therefore, the evolutionary expansion and increased complexity of the telencephalon during amniote

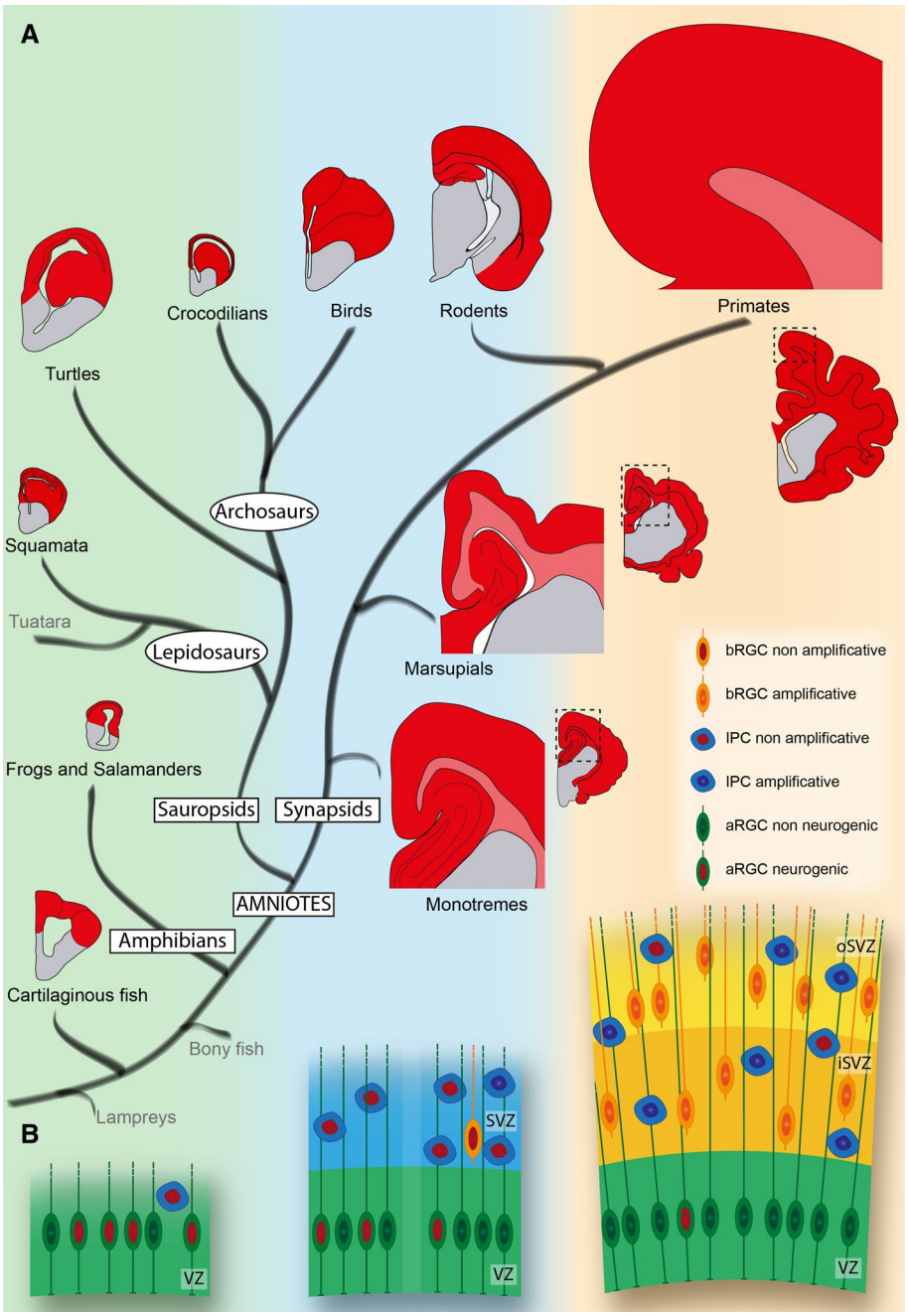

Fig. 3 Brain size and modes of neurogenesis in the pallium of vertebrates. **a** Simplified phylogenetic tree of vertebrates with schematic drawings at scale of the brain of model species in cross section. Differences in brain size between model species are proportional to their relative encephalization quotient (brain-to-body mass ratio) [29, 236]. Red and gray areas correspond to the pallium and subpallium, respectively. For monotremes, marsupials and primates, only a portion of the telencephalon can be shown at scale due to differences in size. Black dashed boxes indicate the brain areas shown in the drawings at scale. **b** Schematic drawings of germinal layers and types of progenitor cells in the embryonic pallium of vertebrates. Background colors group the clades according to their germinal layers: green, only ventricular zone (VZ); blue, VZ + subventricular zone (SVZ); orange, VZ + inner SVZ (iSVZ) + outer SVZ (oSVZ). The relative abundance of progenitor cell types in each group is also represented. *aRGC* apical radial glia cell, *IPC* intermediate progenitor cell, *bRGC* basal radial glia cell

evolution seem to have emerged largely from the formation of new neural progenitor cells, with increased neurogenic potential and producing new projection neuron subtypes in the pallium. Emerging evidence indicates that the innovative features of cortical progenitor cells in mammals held the key for the evolutionary expansion of their cerebral cortex. In the following sections, we summarize the main characteristics of the pallial germinal zones and progenitor cell types that led to the expansion of the mammalian pallium, and compare them with their homologous counterparts in the other groups of amniotes.

The neuroepithelium emerges from the dorsal half of the telencephalic vesicles and derives into the primordium of the cerebral cortex at mid-stages of embryogenesis [50, 55]. This is a monolayer of neuroepithelial cells that span the entire thickness of the telencephalic wall, and that present typical features of epithelial cells. They are highly polarized along the apical–basal axis, with their basal plasma membrane resting on the basal lamina and their apical membrane facing the lumen of the neural tube. Neuroepithelial cells undergo interkinetic nuclear migration (INM), whereby the cell nucleus moves along the cell's apical–basal axis in

coordination with the phases of the cell cycle, conferring its typical appearance of pseudostratification [3, 55, 57]. At the onset of corticogenesis, neuroepithelial cells undergo symmetric proliferative divisions, expanding their population and, consequently, the surface area and thickness of this germinal zone in the cerebral cortex [55]. Because these neuroepithelial cells are the founder progenitor cells of the pallium, their initial expansion before neurogenesis determines the number of neurogenic progenitors and, ultimately, the final amount of cortical neurons. Thus, the evolutionary expansion of the cortical surface area is initially dependent on the size of the neuroepithelium [47, 54].

At the onset of neurogenesis, around embryonic day E10.5 in mouse [58], neuroepithelial cells start losing some of their epithelial features, like tight junctions, and begin to acquire glial features like the expression of paired-box transcription factor 6 (Pax6), brain lipid-binding protein and vimentin, thus transforming into apical radial glia cells (aRGCs; classically known simply as radial glia cells). In spite of their differences with neuroepithelial cells, aRGCs still retain some epithelial features including expression of nestin and several adherens junction proteins, marked apical–basal polarity with processes contacting both sides of the developing cortex, INM and division at the apical surface [59]. However, the cell body of aRGCs now resides in the ventricular zone (VZ) and they have greater fate restriction, becoming the primary neural progenitor cells that produce, directly or indirectly, all cortical projection neurons [3, 58–60].

The apical–basal polarity of aRGCs and symmetry of inheritance of cellular components upon cell division are key features that define their type of division and, seemingly, the identity of their daughter cells [57, 59]. Accordingly, aRGCs may undergo four main types of division: (1) symmetric proliferative, generating aRGCs, (2) asymmetric neurogenic, producing one aRGC and a neuron, (3) asymmetric proliferative, generating one aRGC and a different type of progenitor, (4) symmetric neurogenic, producing two neurons [57]. The probability of occurrence of each type of division varies during development [3]. In addition to aRGCs, the VZ contains other types of progenitor cells at low abundance, namely apical intermediate progenitors (aIPs) and subapical progenitors (SAPs) [3, 61], but their impact on the development of the cerebral cortex remains poorly understood.

At the onset of corticogenesis, aRGCs undergo symmetric proliferative divisions. Soon, aRGCs begin undergoing asymmetric proliferative divisions to produce intermediate progenitor cells (IPCs). Upon their birth at the apical surface, IPCs undergo a brief transitional phase in which they retain the apical process inherited form the mother aRGC while downregulating Pax6 and increasing the expression of the T-box transcription factor 2 (Tbr2), which becomes their specific marker [62–64]. But soon newly generated IPCs migrate to the basal side of the VZ, where they coalesce forming a secondary germinal layer: the subventricular zone (SVZ) [3, 55]. Once in the SVZ, IPCs acquire a multipolar morphology and lose most of the defining features of their mother aRGCs, including astroglial markers, adherens junction proteins, apical–basal polarity and INM [7, 65, 66]. In the mouse cerebral cortex, IPCs are the main type of basal progenitor cell, representing up to 90%. Most IPCs divide only once, symmetrically, to generate two neurons, thus amplifying neuron production [65, 67–69]. Occasionally, IPCs also undergo proliferative divisions, thus increasing in number before producing neurons. This expansion of IPCs further amplifies the neurogenic potential of their mother aRGC [70, 71]. In summary, studies of the rodent cerebral cortex show that neurogenesis is mainly a two-step process: first, aRGCs divide at the VZ to produce IPCs and, second, IPCs divide in the SVZ to produce neurons for all cortical layers [37, 47, 68, 72]. For these reasons, the generation of IPCs and their coalescence into an SVZ are considered as a fundamental milestone in evolution for the expansion of the cerebral cortex and the emergence of the mammalian neocortex.

There are dramatic differences in size of the neocortex between mammals. Gyrencephalic species like carnivores and primates, including human, have a very large and folded neocortex. One of the most important evolutionary innovations in this regard is the remarkable expansion of the SVZ by the massive accumulation of basal progenitor cells, and its splitting into inner and outer SVZ (ISVZ and OSVZ, respectively) [73–75]. These basal progenitors include IPCs and, importantly, basal Radial Glial Cells (bRGCs; also known as intermediate radial glia, or outer radial glia—oRG) [74–77]. bRGCs have also been identified in rodents with a smooth cortex (mouse, rat), albeit at much lower abundances than in gyrencephalic species [78, 79]. bRGCs display two characteristics essential for the eventual expansion and folding of the gyrencephalic neocortex: a large capacity for self-amplification before generating neurons, and the extension of a basal process to the cortex pial surface [74–77]. This basal process drives the tangential dispersion of radially migrating neurons, leading to the expansion in surface area and folding of the cerebral cortex. For a comprehensive review on cortical folding determinants, please see ref. [51].

Cortical germinal zones in amniotes

Differences among amniotes in the size of the mature cerebral cortex, and in the number and types of cortical neurons, result from variations in neural progenitor cells during development [3, 7, 47, 72].

Primary germinal zones

Among mammals, the cerebral cortex expands mostly in surface area rather than in thickness, eventually leading to the formation of folds [3, 6, 7]. The radial unit hypothesis proposed that the expansion in surface area is determined by the number neuroepithelial and aRGC progenitors before the onset of neurogenesis [45]. Accordingly, a delay in the onset of neurogenesis in primates compared to rodents results in an increased number of primary germinal cells, leading eventually to an exponential increase in neuron production [45]. Similarly, neurogenesis in songbirds and parrots, with big brains, is developmentally delayed compared to chickens, with a smaller brain [80], suggesting that this is a mechanism for telencephalic expansion also in birds. Hence, extension of the pre-neurogenic period and delay of neurogenesis onset may have been a conserved strategy during amniote evolution underlying the expansion of cortical surface area [6, 7].

Identified as germinal cells clustered in a ventricular germinal zone that undergo mitosis in the apical surface, with apical–basal polarity and that express Pax6, aRGCs are found in nearly all vertebrates, including sharks [81], bony fish [82], amphibians [56], reptiles [47, 83], birds [40] and mammals [59, 62]. In fact, aRGCs seem to have originated very early in evolution, as they may exist also in lampreys, an evolutionary ancient form of jawless fish [81, 84, 85]. Given that the lineage of all cortical excitatory neurons originates in aRGCs [3], the size of the aRGC population at the onset of neurogenesis determines the final number of cortical neurons and the size of the mature cerebral cortex.

Once the number of aRGCs at the onset of neurogenesis is defined, the final number of cortical neurons produced depends on the type and total number of neurogenic divisions occurring, which depend on the length of the neurogenic period and the length of the cell cycle [46]. As discussed next, these parameters are highly variable between species, resulting in distinct neurogenic strategies and cortical phenotypes [7]. With regard to the neurogenic period, its total duration determines the number of cell cycles and cell divisions, such that a longer period in principle increases the total amount of neurons produced [7, 86]. However, the length of the cell cycle may also vary significantly, critically conditioning the total number of cell divisions that may occur during a given neurogenic period. These parameters have been studied in multiple species across amniote phylogeny, offering insights into their impact on neurogenesis and cortex size. In reptiles and birds (sauropsids), the period of neurogenesis terminates much earlier than embryogenesis. For instance, embryogenesis in chicken (*Gallus gallus*) lasts 20 days [87] while cortical neurogenesis extends between days 4 and 9–10 [88]. In Gecko (*Paroedura pictus*), the embryo develops during 60–80 days and neurogenesis takes place between days 7 and 23 [83]. This is in contrast to mammals, where the neurogenic period extends for a much greater part of embryogenesis, which suggests that this is a trait selected during sauropsid evolution [24]. A brief period of neurogenesis as in sauropsids limits the total rounds of neurogenic cell division and, hence, the final number of neurons produced [83].

The length of the cell cycle also varies dramatically across species, as well as across stages of embryonic development [86]. In mouse, the cell cycle lengthens gradually as neurogenesis progresses, from 8 h at early stages to 18 h at the end [89]. This lengthening of the cell cycle is associated with the gradual developmental switch in the modes of cell division, from proliferative to neurogenic [46, 86, 90]. Accordingly, the experimental modification of cell cycle length alters the fate of the resulting daughter cells and the neurogenic output [91]. In primates, with the greatest neurogenic output, the dynamics of cell cycle length during cortex development is more complex due to a midway period of progenitor amplification. In the first part of cortical neurogenesis, the cell cycle lengthens from 23 to 54 h by mid-gestation, during the formation of layer IV. At this point, immediately prior to the beginning of neurogenesis for layer II/III, the cell cycle shortens to 27 h, coincident with an increase in proliferative divisions and a reduction in neurogenesis [76, 92]. This novel mechanism greatly amplifies the number of basal progenitors (bRGCs and IPCs), resulting in the formation of the massive OSVZ typical of primates prior to the production of supragranular neurons [76]. A similar process exists in ferret, where aRGCs transiently change their mode of division to massively produce bRGCs and form the OSVZ during a brief period of corticogenesis [93]. In ferret, there is also a transition phase of cell cycle shortening as in primates, again coincident with the onset of neurogenesis for supragranular layers [12].

Birds and reptiles seem to follow other strategies for brain expansion regarding cell cycle length. The brain of chicken is bigger than of quails (*Colinus virginianus*), and yet the duration of the cell cycle is similar at the onset of neurogenesis. However, during the period of progenitor amplification prior to neurogenesis, the cell cycle length in quail (24 h) nearly doubles that in chicken (14 h), resulting in much lower production of neurogenic progenitor cells [94]. With a larger initial pool of neurogenic progenitors, neuron production is then much greater in chicken than in quails, resulting in a bigger brain. The same mechanism seems to explain also the differences in brain size between parakeets (*Melopsittacus undulatus*) and quails [80]. In turtle, the surface of the VZ does not expand significantly during development, hence containing a small amount of aRGCs prior to neurogenesis, which likely reflects a limited amplification of aRGCs before the onset of neurogenesis, as in quail [95]. This low amplification may be due to a very low rate of

proliferation at early stages, as described in the Madagascar ground gecko (*Paroedura pictus*), where this results from a dramatic lengthening of the cell cycle (50 h, compared to 8–18 h in mouse). In the case of gecko, the limiting effect of having a small pool of neurogenic progenitors is combined with a short neurogenic period, severely reducing the number of neurogenic divisions and of neurons produced, finally resulting in a very small dorsal cortex [83]. Therefore, regulation of the abundance of primary progenitor cells, the duration of the neurogenic period and the cell cycle length are critical determinants of brain size during development and in evolution.

Secondary germinal zones

Whereas aRGCs are highly conserved across phylogeny, IPCs are much more divergent [3, 39, 72, 95, 96]. This secondary type of progenitor cell populating the secondary germinal zone SVZ is characterized by having a multipolar morphology, expressing Tbr2 and undergoing basal mitosis, away from the ventricular surface [62, 70]. In mice, IPCs most frequently undergo symmetric terminal division, producing two neurons [65, 68, 97]. Indirect neurogenesis, where neurons are produced by aRGCs via IPCs, is the main source of neurons and of neuronal diversity in the mammalian cerebral cortex [68, 72]. Due to the amplificative nature of IPCs as secondary progenitor cells, this mode increases neuron production when compared to direct neurogenesis and allows sparing aRGCs from self-consuming neurogenic divisions, hence further extending neurogenesis [98]. Despite the importance of the SVZ for the development, expansion and increased complexity of the mammalian neocortex, the evolutionary origin of this layer is still under debate. It is thought that a distinct SVZ emerged during the transition sauropsids–synapsids (Fig. 1), contributing to the expansion of a reptilian-like three-layered cortex into the mammalian six-layered neocortex [47, 96]. This notion is supported by the identification of several features typical of the SVZ in clades much older than mammals:

Cartilaginous fish

Cartilaginous fish (chondrichthyans) is one of the most basal extant monophyletic group of vertebrates, sibling to all other jawed vertebrates (gnathostomes), including bony fish and tetrapods. Because of their evolutionarily ancient origin, as a basal radiation of gnathostomes, they are strategically unique for evolutionary studies [29, 81, 99]. In the developing dorsal pallium of the cat shark (*Scyliorhinus canicula*), Tbr2+ cells have been reported to group in a distinct SVZ and undergo basal mitoses [81]. Although the reported amount of these progenitor cells is significantly smaller than in mammals, the appearance of these new progenitors may have been necessary to develop the evaginated and expanded pallium of sharks, as opposed to lampreys [81]. This suggests that the basic features of the SVZ and the mechanisms for its development may have already existed in the ancestor common to all jawed vertebrates [81].

Amphibians

One key point for understanding the evolution of the cerebral cortex is the anamniote-amniote transition. The colonization of land by tetrapods is one of the most remarkable evolutionary events, which required a number of critical adaptations including the formation of the amnion and other fetal membranes. This membranous structure around developing embryos protects them from drying out in the non-aquatic atmosphere [100]. Anamniotes do not have this innovative protection and cannot escape laying eggs in water. Amphibians are the only group of vertebrate tetrapods that are anamniotes, sharing features with amniotes and other anamniotes like fish [29], so they are critical models for comparative studies. In amphibians, the embryonic pallium is divided into the same four regions as in amniotes (Fig. 2) [29, 101], with the dorsal pallium considered the cortex homolog [29], but with no signs of lamination in adult animals [101]. The developing embryonic pallium of the clawed frog (*Xenopus laevis*) displays a small amount of mitotic progenitors in abventricular (non-ventricular) position, a feature reminiscent of IPCs, but these do not express Tbr2 [101, 102]. Vice-versa, although Tbr2 is expressed in ventricular and non-ventricular cells of the *Xenopus* embryo pallium, these are neither mitotically active nor express proliferation markers [101]. Hence, in amphibians Tbr2 expression is not sufficient for IPC formation, but it may be related to the molecular specification program of glutamatergic neurons.

Sauropsids

The telencephalon experienced its greatest expansion in size and complexity in amniotes, and the classical view is that this was intensified with the emergence of the SVZ at the point of divergence between synapsids and sauropsids. For these reasons, most efforts have focused on understanding the evolution of the SVZ in this group of vertebrates, where we find a full palette of evolutionary solutions.

Lepidosaurs (snakes and lizards): Several model species of the superorder *lepidosauria* are extensively studied to understand the evolution of vertebrate brains, in particular the emergence of those traits underlying the expansion of the cerebral cortex. One characteristic feature of these animals is the absence of abventricular mitoses in the developing pallium, both in lizards like the green anole (*Anolis carolinensis*) and gecko (*Paroedura pictus*), and in snakes (*Lamprophis fuliginosus*) [72, 83, 85]. Similar to amphibians, the

pallium of gecko and anole embryos contains Tbr2-expressing cells, within the VZ or basal to it, that do not express progenitor markers but rather neuronal markers [83, 85]. As in amphibians, this demonstrates that lepidosaurs do not form an SVZ during embryonic development of the telencephalon, and again suggests the involvement of Tbr2 in defining the glutamatergic lineage.

Archosaurs (turtles, crocodiles and birds): turtles have been classically considered as a stem amniote, the common ancestor of mammals and reptiles [103, 104]. However, recent genomic analyses place turtles as the sibling group of archosaurs (crocodiles and birds) rather than as primitive reptiles (Fig. 1) [21, 28, 105]. Studies in the semiaquatic red-eared turtle (*Trachemys scripta elegans*) show a small number of abventricular mitoses distributed across the developing cortex, which are neither organized in a distinct SVZ nor increase in abundance during cortical development as neurogenesis progresses, unlike in mammals [95]. Subsequent studies report the presence of Tbr2-expressing proliferative cells (PCNA+) in the developing dorsal cortex and DVR of this turtle, which cluster forming a distinguishable SVZ in the DVR but not in the dorsal cortex [85, 103]. The low abundance of abventricular mitoses and the absence of a distinct SVZ in the developing dorsal cortex of turtles has been confirmed independently in *Emys orbicularis* [106] and in the Chinese softshell turtle (*Pelodiscus sinensis*) [102]. The absence of basal mitoses in the dorsal cortex of lizards, and their presence in turtles but without forming a distinct SVZ (only suggested to exist, rudimentarily, in the most lateral part [95]), prompted examining the development of crocodilians, considered the extant group closest to birds (Fig. 1) [28]. Basal mitoses are also absent from the embryonic pallium of alligators, existing only in their subpallium [107].

Birds are the non-mammalian order closest to mammals in terms of brain size and in displaying comparable behavioral and cognitive higher-order skills, thus both represent the pinnacle of vertebrate brain evolution. Accordingly, the comparative study of the development and expansion of the avian pallium is fundamental to understand the evolutionary expansion of the cerebral cortex in amniotes. The avian pallium is essentially composed by the Wulst (or hyperpallium) and the DVR [108–110]. Early studies in chicken demonstrated the existence of a distinct SVZ in the DVR and subpallium during late stages of neurogenesis, but not in the hyperpallium (homolog to mammalian neocortex). Only a small number of abventricular mitoses take place in the lateral pallium [85, 106, 107], where they occur in a decreasing lateral-medial gradient [107]. Later studies in chicken and dove showed that some of the basal mitotic cells are positive for Tbr2 [85], confirming the existence of a defined SVZ with IPCs in the chicken hyperpallium and mesopallium, and in the dove forebrain. However, the scarcity of basal progenitors in the Wulst of basal avian orders such as *Galliformes* (chicken and relatives) maintains alive the debate about the presence of a *bona fide* SVZ [13]. In contrast, the presence of an SVZ in orders of birds that emerged more recently, like *Passeriformes* (parakeets) and *Psittaciformes* (songbirds), is more obvious and less questioned and probably related to the greater complexification of their brains [80].

In mammals, there are two main types of basal progenitor cells in the SVZ: IPCs (Tbr2+) and bRGCs (Pax6+). A recent study in the chick dorsal pallium identified a band of basal cells containing two different cell populations: one mitotic and expressing Pax6 or Sox2, resembling bRGCs; the other expressing Tbr2 but non-mitotic, hence non-IPCs but possibly committed to the neuronal glutamatergic lineage [102]. Experimental manipulations expanding the pool of progenitor cells successfully increased the population of Tbr2+ cells, but these remained non-mitotic (non-IPCs). Altogether, this suggested a lack of conservation of proliferative activity in Tbr2 cells across amniotes [102]. In contrast, our most recent investigations also in the chick dorsal pallium demonstrate a well-defined SVZ populated by mitotically active progenitors including, but not restricted to, Tbr2+ mitoses [72], in agreement with previous results by other groups [85, 106, 107]. Basal mitoses negative for Tbr2 might be positive for Pax6, as previously reported [102]. We also found a lateral-to-medial gradient of basal mitoses abundance in the SVZ of the hyperpallium, which become negligible in the medial part [72], again coincident with previous studies [85, 107]. In addition, and contrary to Nomura and colleagues, our genetic manipulations of progenitor cells increased four-fold the abundance of Tbr2+ mitoses [72]. These results strongly support the existence of a rudimentary SVZ in the chick hyperpallium and the evolutionary conservation of Tbr2 as a marker of the glutamatergic cell lineage, eventually including IPCs (Fig. 3) [3, 62].

The main conclusion from these comparative studies is that the regulatory mechanisms to develop an SVZ proper appeared long before the emergence of mammals. Some of these traits appeared before the emergence of amniotes, but their presence separately would not be sufficient to drive the expansion and development of a six-layered cortex as in mammals. Only the convergence of all these characteristics in high order birds and mammals was sufficient to form a true SVZ. Subsequently, the addition of other critical factors such as specific proliferative kinetics and length of the neurogenic period were key for the expansion and complexification of the mammalian neocortex.

Synapsids (mammals)

Mammals are the only living group of the synapsid radiation [111]. Mammals and reptiles diverged from their last common amniote ancestor 310 millions of years ago, and extant mammals emerged about 170–180 million years ago,

in the Early or Middle Jurassic [2, 8, 29, 112], when the modern neocortex appeared [8, 113, 114]. Modern mammals are subdivided into monotremes (Prototheria), marsupials (Metatheria) and placentals (Eutheria), the later splitting from early marsupials about 150–125 millions of years ago [2, 29, 113, 115] (Fig. 1). All these lineages develop a *bona fide* neocortex characterized by its radial expansion in six layers and the generation of multiple functional areas [114]. However, the large diversity of neocortical phenotypes among different groups of mammals, with a wide range of morphological, cellular and molecular properties, has made it difficult to understand the evolutionary history of the neocortex [116]. The most obvious difference among mammals is the size and degree of folding of the neocortex, which are much greater in gyrencephalic ("folded brain") species, like ferret and primates, than in lissencephalic ("smooth brain") species, like mouse and rat. This increased complexity also allows the development of new functional cortical areas and, concurrently, the emergence of new higher order behaviors [44, 117]. Despite this diversity among mammalian neocortices, there are some shared commonalities reflecting a common starting point during development and conserved cellular and genetic mechanisms for cortical growth [29].

The evolutionary origin of the six-layered mammalian neocortex remains under debate, but a widely accepted point of view is that it evolved from an ancient structure resembling the three-layered dorsal cortex of reptiles [29]. This organization is present in two conserved regions surrounding the neocortex: the hippocampus (archicortex) and the olfactory cortex (paleocortex) [15]. Two alternative views propose that the neocortex may be a modification of a highly expanded olfactory cortex or that it evolved as a multimodal interface in the olfactory–hippocampal axis for behavioral navigation, two neural systems essential in the initial phases of evolution [8, 9, 112, 114, 118]. In any case, there is consensus in that complex neocortical traits, such as six layers and gyrencephaly, evolved from simpler, three-layered, lissencephalic cortices, where changes in developmental programs increased the number of layers and neuronal diversity during evolution [17, 29, 39].

Monotremes: this is the earliest/oldest branch of the mammalian radiation, which includes platypuses and echidnas. Monotremes present several primitive features including egg-laying, reptile-like cloaca and sex-determining chromosomes similar to birds and reptiles. Interestingly, whereas the neocortex of the platypus is lissencephalic, in echidnas it is gyrencephalic [119], supporting the notion that cortex folding is an ancestral trait of mammalian evolution [49, 115, 116].

As expected, both platypus and echidna develop a six-layered neocortex, but this lacks particular cortical areas typical in eutherians and it also fails to form a corpus callosum. The development of the monotreme neocortex involves abundant basal mitoses forming an SVZ remarkably large but, again, not quite as distinct as in eutherians [29, 120]. These differences may represent traits of the primitive mammalian neocortex. This further reinforces the notion that the formation of an SVZ was a necessary step in evolution for the generation of six layers and the expansion of the cerebral cortex into the neocortex [24, 29, 120]. Of particular interest in neocortical evolution is the diversification of commissures, such as the formation of the corpus callosum, which is in fact considered a milestone for the evolution in complexity of the six-layered neocortex, improving interhemispheric connections [121].

Marsupials: this group is mostly established in South America with opossums and opossum-like, and in Australia and New Guinea with possums and species adapted to a wide range of niches, including kangaroos, koalas, wallabies and the Tasmanian devil [29, 122, 123]. Several of the most remarkable differences between marsupials and eutherians is the lack of placenta, short gestational periods and the birth of very altricial young, which continue developing in the marsupium [29, 123]. The brains of marsupials vary dramatically in size, shape, folding and encephalization [123], but all of them display a six-layered neocortex [96, 124]. They are characterized by an enlarged anterior commissure but no corpus callosum, a more protracted period of cortical development and, similar to monotremes, by the lack of a clear differentiation of the motor cortex from the somatosensory cortex, thus combining evolutionary ancient and novel features [96, 123]. With regard to the development of the SVZ, there is some discrepancy also in this case. Analyses in opossum (*Monodelphis domestica*) and the tammar wallaby (*Macropus eugenii*) by Molnar and colleagues show that these species possess a clearly distinct SVZ with mitotic figures during cortical development [96]. However, the number of superficial and deep-layer neurons per cortical column is smaller in these marsupials than in placental mammals, even though the former have a very protracted period of cortical development. This may be due to the relatively late emergence of the SVZ during cortical development in marsupials, limiting its contribution to the final numbers of neurons [96]. An independent study by Mallamaci and colleagues reported the absence of a distinct SVZ in opossum during cortical neurogenesis, where this basal mitotic compartment only forms at the very end of neurogenesis [124]. Tbr2 expressing cells were found clustered between the VZ and IZ throughout the neurogenic period, but showed that these are not IPCs but a transient population of post-mitotic cells. Importantly, they did find that in spite of the absence of an SVZ, the molecular diversity and inside-out pattern of neurogenesis are largely similar between marsupials and eutherians. These results indicate that most neocortical neurons in these marsupials are generated by direct neurogenesis from aRGCs and suggest that the absence of IPCs may be the

main reason for the reduced cortical thickness and neuronal density in marsupials [124]. Other authors have also suggested that marsupials do not have an SVZ in the neocortex, but only in the subpallium [107]. However, a more recent study shows that the tammar wallaby displays a proliferative SVZ in the pallium during the entire period of neurogenesis, containing Tbr2-positive IPCs that undergo basal mitoses, and similarly to eutherians it shows that this layer becomes thicker at late stages of neurogenesis, when upper cortical neurons are produced [125]. Intriguingly, bRGCs are also present in the tammar wallaby neocortex, identified as Pax6-positive basal mitotic cells with RGC morphology. In fact, at peak stages of neurogenesis IPCs are progressively replaced by bRGCs [125].

The above findings support the idea that bRGCs were present in the common ancestor of mammals, before the metatherian–eutherian divergence, which would have developed a large gyrencephalic cortex. Then, bRGCs would have been secondarily lost (completely or partially) in lissencephalic species [3, 116]. Similarly, the degree of neocorticalization may have been defined by the relative abundance of neurogenic progenitor cells and their cell cycle kinetics [125]. Therefore, differences between opossum and tammar wallaby in SVZ development and presence of bRGCs may explain the development of a smooth neocortex in the former and of a folded neocortex in the latter [50, 125]. The lack of an active neurogenic SVZ in opossums would explain their smooth cerebral cortex, whereas the development of the proliferative SVZ populated with bRGCs in the tammar wallaby supports the importance of acquiring these evolutionary traits for the emergence of gyrencephaly.

Placentals: eutherians are divided into four superorders, each one with a variety of species adapted to a wide range of niches, and comprising 90% of extant mammals: *Xenarthra* (armadillos, sloths…), *Afrotheria* (tenrecs, elephants), *Laurasiatheria* (shrews, bats, cats, whales) and *Euarchontoglires* (rodents, rabbits, primates) [29, 113, 126]. The complexity, shape and size of the neocortex vary significantly between species [3], with many features seemingly retained from a common ancestor and others acquired or lost [113]. Some of the main traits that emerged with placentals were the distinction of motor and premotor cortices, formation of a corpus callosum as a main interhemispheric connection, and acquisition of new associative areas. The latter has been proposed to result from the lesser temporal restriction of developmental periods [20, 113, 127]. The emergence of these features was accompanied by a radiation of new neuron subtypes, mostly reflected in the evolution of supragranular layers [127], but in some cases also by more unusual type of specializations like the loss of secondary visual and auditory areas as in the small shrew [20].

Recent analyses conclude that the stem eutherian was small and insectivorous, similar to early marsupials or opossums, with a relatively small neocortex relative to body size [20, 29, 115]. A major evolutionary challenge for mammals was increasing the size of the cerebral cortex, where the SVZ seems to have played a central role. As already mentioned, in placental mammals the majority of cortical neurons are produced by indirect neurogenesis via IPCs in the SVZ [3, 65, 67–69]. In those with a small and smooth cortex, most IPCs divide only once to produce two neurons, only occasionally undergoing 1–3 rounds of self-amplification before the terminal neurogenic division [65, 68, 69, 128]. Because indirect neurogenesis involves progenitor cell amplification, it allows significantly increasing total neuron production, and as such is a fundamental process for the developmental expansion of the mammalian cerebral cortex [3, 47]. Contrary to marsupials, reptiles and other examples mentioned above, in placental mammals the expression of Tbr2 in progenitor cells is a defining feature of IPCs, which in fact is critical for the differentiation of IPCs from aRGCs by seemingly repressing the expression of Pax6 [62, 71, 78, 129].

Initial studies on the fate of SVZ-derived neurons suggested that these produce only late-born neurons fated to upper layers (supragranular), whereas early-born, deep layer neurons would be produced by VZ progenitors [130]. However, it is now clear that IPCs produce excitatory neurons for all cortical layers, while maintaining the inside-out relationship between birth date and laminar fate [68, 70, 128, 131, 132]. Accordingly, the loss of Tbr2, which profoundly affects the abundance of basal mitoses, disrupts the generation of both lower and upper layer neurons, the last most severely affected possibly due to a premature depletion of progenitor cells [71, 131, 132]. This suggests that an evolutionary increase in Tbr2 expression may have been important for the emergence of IPCs and the development of an SVZ, thereafter enhancing neuron production. This may have been particularly critical for the emergence of upper layer neurons, as found in mammals, which relies on a highly increased IPC production at late stages of development [63, 70, 76, 133]. In addition to IPCs, the SVZ contains bRGCs in varying amounts depending on the species: low in marsupials and small rodents with a smooth cortex, very large in gyrencephalic cortices [12, 75, 79, 134]. Multiple studies demonstrate the direct role of bRGCs in determining the surface area size and degree of folding of the neocortex across mammals [75, 135–140] (reviewed in [51, 55, 86]).

Gyrencephalic species are characterized by their folded neocortex, where folds (outward bending) and fissures (inward bending) form in stereotyped patterns [6, 50, 51]. The degree of neocortical folding bears some relationship with brain size, where animals with a large brain have a folded neocortex, like humans and other primates, and those with relatively smaller brains have a smooth cortex [51]. However, brain expansion and cortex folding did not

evolve in a single lineage. On the contrary, lissencephalic and gyrencephalic species exist in all superorders of mammals. For example, most members of the rodent lineage are lissencephalic, but the capybara is gyrencephalic; many primates are gyrencephalic, but most new-world monkeys are almost lissencephalic [50, 116]. Two main models explain the diversity of neocortex conformations in the different lineages of mammals: gyrencephaly emerged multiple times and independently in mammals by convergent evolution; or gyrencephaly appeared in the stem mammal and it followed parallel evolution in different clades [50, 116]. The second option seems more parsimonious according to current knowledge, because developmental and genetic mechanisms underlying cortex folding are very well conserved across mammalian clades, and their phylogenic divergence was very recent. Moreover, a secondary loss of gyrencephaly would be genetically and developmentally much more economical than the independent emergence of folding strategies in multiple clades [55, 116, 141].

The development of gyrated neocortices requires the generation of large amounts of neurons and their tangential dispersion during radial migration [50, 51, 142]. This correlates with a significant expansion of the SVZ, which becomes subdivided into inner and outer SVZ (ISVZ and OSVZ, respectively) abundantly populated by IPCs and bRGCs [12, 74–77, 143]. Analyses in ferrets demonstrate that the OSVZ emerges during embryogenesis by the seeding of a large number of bRGCs produced from aRGCs during a brief period of development, which subsequently self-amplify to expand this new germinal layer [93]. The cell body of bRGCs is located at the SVZ (ISVZ or OSVZ), where they divide, typically express the transcription factor Pax6 and extend a basal process contacting the pial surface, while lacking an apical process [74, 75, 77]. Variations on these typical bRGC features have been found in primates and carnivores (macaque, marmoset and ferret), including multiple morphotypes and co-expression of Pax6 with Tbr2 (30–50% of bRGCs) [12, 61, 75, 76].

The critical role of bRGCs in neocortical expansion and folding is twofold: (1) increase neuron production, together with IPCs; (2) tangential dispersion along the cortical surface of radially migrating neurons [51]. The dramatic increase in neocortical neuron numbers between mouse and human leads to a 1000-fold increase in surface area but only a 2-fold increase in thickness [6]. This suggests that the acquisition of cortical folds during amniote evolution resulted from a biased increase in bRGCs over IPCs (Fig. 3) [3, 55]. This notion is well supported by the observations that bRGCs are only 3–5% of neocortical progenitor cells in the lissencephalic mouse or the near-lissencephalic marmoset, 15–20% in the moderately folded ferret and sheep cortex, and up to 75% in the highly folded macaque and human neocortex [61, 74, 76, 78, 134, 144]. Moreover, experimental manipulations of bRGC abundance greatly affect the development of neocortical folds and, thus, the shape and size of the cerebral cortex, in mice and ferret [75, 135, 138].

In summary, we propose a model of neocortical evolution where the expansion and folding of the cerebral cortex were gradually selected and shaped, culminating in the human neocortex. According to our model, this process involved several steps, each adding a new level of complexity. The generation of intermediate progenitor cells (IPCs) seems to have been one of the earliest events in this evolution, leading to a significant increase in neurogenesis. IPCs were progressively organized around a secondary germinal layer, the SVZ, which may have improved their neurogenic efficiency. In parallel, the length of the neurogenic period and the number of cell cycles by neurogenic progenitor cells in the cerebral cortex increased significantly, further augmenting total neuron production. At this point, the cerebral cortex of stem mammals was ready to produce large amounts of neurons with novel identities and functionalities, which generated new cortical layers (upper layers). The increased neuronal productivity by IPCs was then followed by the formation of bRGCs, which further increased neuron production. The high amplificative capacity of bRGCs led them to quickly increase in number and, together with IPCs, eventually split the SVZ into ISVZ and OSVZ. The additional advantage of bRGCs over IPCs, favoring the tangential dispersion of neocortical neurons, was also strongly selected and cortex folding emerged. Finally, some species continued to select the increase in numbers of IPCs and bRGCs, augmenting cortex size and folding like old-world primates, while others selected a secondary loss or reduction of bRGCs and cortex folding, regressing to a small and smooth neocortex like most rodents.

Modes of neurogenesis and cerebral cortex development

Modes of neurogenesis in mouse: direct versus indirect

There are two main modes in which cortical excitatory neurons are produced from aRGCs: directly, and indirectly via IPCs. In direct neurogenesis, each neurogenic division from an aRGC is generally asymmetric, producing one neuron and one aRGC [65, 72, 117]. In indirect neurogenesis, usually IPCs divide symmetrically to produce two neurons [65, 68, 69, 72], doubling the number of neurons generated from each aRGC division [37]. Hence, regulation of the mode of division during development is essential to determine the final number of neurons [7].

Since the discovery of aRGCs and IPCs as the main progenitor cell types generating cortical neurons [60, 145–147], many efforts have been made to determine the exact contribution of each mode of neurogenesis to the development of the cerebral cortex. These studies were pioneered by Malatesta and colleagues, who used in vitro cultures to determine that at mid-cortical neurogenesis half of the progeny of aRGCs are neurons [145]. Later, Noctor and colleagues performed time-lapse imaging of individual aRGCs from rat cortical slices and reported that 80% of them undergo asymmetric division at late developmental stages (Noctor 2001). The vast majority of these divisions were asymmetric neurogenic, generating one neuron and one aRGC, while only 7% were asymmetric proliferative, generating one aRGC and one IPC, and symmetric amplificative divisions (producing two aRGCs) were a minority. Follow-up studies focused on IPCs reported that 90% of them undergo symmetric terminal division (generating two neurons) and only 10% generate again IPCs [65, 69]. Later, Noctor and colleagues performed similar experiments to determine variations in the behavior of progenitor cells at different embryonic stages in rat. They found that early on (E13–E15) the majority of aRGCs divided symmetrically to produce two daughter aRGCs, while only a minority divided asymmetrically giving rise to one neuron or one IPC. At later stages, the proportion of asymmetric divisions increased, coincident with the accumulation of IPCs in the SVZ [64]. Coetaneous studies by the Huttner lab used *Tis21*-GFP mice to identify the nature and dynamics of progenitor cells undergoing neurogenic divisions. They reported the presence of basally dividing GFP+ cells (presumptive IPCs) from the very onset of neurogenesis, and that the vast majority of these mitoses were symmetric neurogenic, producing two neurons. They also found a proportion of *Tis21*-GFP positive cells dividing apically, all of which were asymmetric divisions [68]. These observations were confirmed and extended later on by crossing the *Tis21*-GFP mouse line with a *Tubb3*-GFP line, the latter expressing GFP specifically in newborn neurons [67]. These analyses found that, at the onset of cortical neurogenesis, only a minority of *Tis21*+ apical progenitors give rise directly to neurons, whereas IPCs produce neurons from the very onset of neurogenesis and by symmetric division [67]. Kowalczyk and colleagues further confirmed the presence of dividing IPCs in the developing mouse neocortex from the very onset of neurogenesis. They found Tbr2+ progenitors dividing in the SVZ already at E10.5, which increased progressively throughout the neurogenic period until E16.5. Importantly, the fraction of neurogenic divisions corresponding to IPCs (indirect neurogenesis) was always much higher than that of aRGCs (direct neurogenesis). The high ratio of indirect versus direct neurogenesis, combined with the greater neurogenic output of IPC divisions, and their presence since the onset of neurogenesis, resulted in IPCs producing the majority of cortical neurons for all layers. Moreover, IPC abundance was found to increase from E10.5 to E14.5, suggesting the existence of a subpopulation of IPCs that self-amplify before generating neurons, which enhances neuronal production even further [70].

In spite of the multiple reports mentioned above supporting the predominance of indirect over direct neurogenesis throughout neocortex development since early stages, recent studies employing novel experimental approaches challenge this conclusion. Jabaudon and colleagues performed a series of analyses where mouse embryos of different developmental stages were electroporated in the neocortex with GFP plasmids and immediately after received a chronic administration of BrdU. According to this paradigm, GFP+/BrdU- neurons would be borne by direct neurogenesis. They found that ~ 40% of neurons were born by direct neurogenesis at early stages (E12.5–E13.5), which suddenly decreased to 10% in the following 2 days (E15.5) [148]. The authors related these results with the long-held concept that deep layer neurons are mainly produced by apical progenitors, whereas upper layer neurons are produced indirectly via basal IPCs, coincident with the expansion of the SVZ [149]. Systematic analyses by videomicroscopy of aRGC divisions along the neurogenic developmental stages, and across cortical areas, should shed light into this issue and help settle this ongoing controversy.

We have recently used multiple approaches to investigate the extent of direct and indirect neurogenesis in the mouse rostral neocortex, adjacent to the olfactory bulb, at early stages of development (E12.5) [72]. First, we injected a single pulse of BrdU to label cycling progenitor cells in S-phase, and when BrdU+ cells were in mitosis (3 h later) we transfected aRGCs with GFP-encoding plasmids by in utero electroporation. Because IPCs do not undergo apical mitosis, they are not targeted by electroporation. Finally, we analyzed the dosage of BrdU labeling in GFP+ neurons of the postnatal cortex. In this paradigm, GFP+ neurons containing a full dose of BrdU labeling are produced directly by aRGCs, whereas lower BrdU doses generally indicate further dilution from proliferative cell divisions prior to neurogenesis. As a second approach, we used a low-titer stock of GFP-encoding retrovirus to label individual aRGCs at clonal dilution in developing embryos in utero. Third, we used videomicroscopy to monitor the progeny of individual aRGCs in neocortical slices in culture. Fourth, we analyzed the presence of cells in VZ that expressed markers of newborn neurons; these are expected with direct neurogenesis, because neurons born from the apical mitoses of aRGCs must cross the VZ en route to their final location in the cortical plate. All these analyses systematically and robustly showed that direct neurogenesis occurs in less than 5% of total apical mitoses in the developing neocortex at early stages of development. Identical analyses in the olfactory

bulb at the same stage revealed high levels of direct neurogenesis (20%), validating these approaches and the results in the neocortex [72].

A number of studies from several authors, including our own, appear contradictory with regard to the frequency of direct versus indirect neurogenesis during mouse neocortical development [64, 67–70, 72, 128, 131]. It seems reasonable that at the onset of neurogenesis, when the number of IPCs is still low, some aRGCs may generate a number of neurons seeding the preplate, even if the overall trend is toward self-amplification [3, 70, 148]. Taken together, the data available today indicate that at the beginning of neurogenesis aRGCs undergo mostly symmetric proliferative divisions to expand their pool, with a small amount of direct neurogenesis. As corticogenesis progresses, there is a gradual switch in the type of aRGC divisions towards asymmetric, generating neurons or IPCs. In mouse, IPCs soon become the main neurogenic progenitor cells, giving rise to most cortical projection neurons. Although IPCs contribute to generate neurons for all cortical layers, their gradual accumulation in the SVZ results in a greater generation of upper layer neurons [47].

Direct neurogenesis during amniote evolution

The acquisition of indirect neurogenesis is considered a key milestone for the expansion of the neocortex during evolution [3, 47]. As already mentioned, the emergence of IPCs and their coalescence into the SVZ allowed a remarkable increase in neuronal production and was seemingly essential for the formation of the six neocortical layers [68, 72, 95, 96, 106]. Hence, evolution and expansion of the amniote cerebral cortex involved a switch from direct to indirect neurogenesis. This switch required two fundamental changes: (1) the abundant generation of IPCs grouped into the SVZ; and (2) the repression of direct neurogenesis from aRGCs [3, 72].

Reptiles: As already mentioned, IPCs (or any type of basal progenitor) are virtually absent in the pallium of reptiles [83, 85, 107], so all neurons must be generated by direct neurogenesis from aRGCs [72, 83]. The limited productivity of this mode of neurogenesis is further reduced by the very long cell cycles of cortical progenitors in reptiles, such that the total number of cell cycles within their neurogenic period is quite small [83]. This combination results in a very low production of cortical neurons and, therefore, in the formation of a relatively small and thin, three-layered dorsal cortex (Fig. 4). We have recently demonstrated that the experimental manipulation of the balance between direct and indirect neurogenesis in a variety of amniotes, including reptiles and birds, leads to the emergence of mammalian features. Repression of direct neurogenesis in snake cortices leads to the de novo emergence of basal progenitors, which align in a proto-SVZ [72]. This further suggests that direct neurogenesis is a default state in amniotes, susceptible to repression leading to indirect neurogenesis.

Birds: In the chicken Wulst, there is a rudimentary SVZ containing a relatively small number of basal mitoses [72, 107], some of which are Tbr2-positive, typical of IPCs [72, 85, 106]. This suggests that in the embryonic avian pallium there is a partial restriction of the direct neurogenic program, resulting in a modest increase on the amount of basal progenitors and, eventually, leading to the expansion of the avian pallium as compared to reptiles. Similar differences also distinguish lower from higher avian orders (with larger and comparatively more complex brains), with the latter producing more basal progenitors [80]. As in the case of reptiles, the experimental inhibition of direct neurogenesis promotes the generation of more basal progenitors, and this augmented indirect neurogenesis leads to an increase in neuron production [72]. In contrast, experimental favoring of direct neurogenesis reduces dramatically the production of basal progenitors, particularly those positive for Tbr2. These experiments demonstrate that the shift in neurogenic modes, from direct to indirect, was likely one of the main evolutionary mechanisms leading to the increase in neuron production and expansion of the avian pallium [72].

Mammals: Evolution of the mammalian neocortex involved a complete inversion of the modes of neurogenesis compared to its ancestors. Direct neurogenesis was largely suppressed, only generating 10–20% of projection neurons [70], and IPCs became the main source of neurons, settling in a clearly defined SVZ (Fig. 4) [70, 72, 150]. This change led to a dramatic increase in neuronal production and to an unprecedented expansion of the neocortex [47]. The evolutionary emergence of indirect neurogenesis as a two-step process for producing neurons, and its importance on the amplification of cell numbers in the mammalian cerebral cortex (intermediate progenitor hypothesis), was first proposed by Kriegstein and colleagues [47, 95]. Even though IPCs produce neurons for all layers [70, 150], their accumulation in an expanding SVZ at mid-neurogenic stages is essential to generate the massive numbers of upper layer cortical neurons, typical of mammals and critical for the formation of the six-layered neocortex [40, 95, 130, 149]. This event is particularly noticeable in gyrencephalic species, in which indirect neurogenesis produced in the highly populated OSVZ leads to a massive expansion of upper layers [41, 55]. Importantly, the expansion of the SVZ and the remarkable increase in upper layer neuron abundance, observed across mammalian phylogeny, has no equivalent in other vertebrates (Fig. 4) [41, 47].

The balance between direct and indirect neurogenesis seems to be key also in defining subtypes of neurons. We have experimentally demonstrated that forcing direct neurogenesis in the mouse neocortex leads to the overproduction of *bona fide* deep-layer neurons at expense of upper

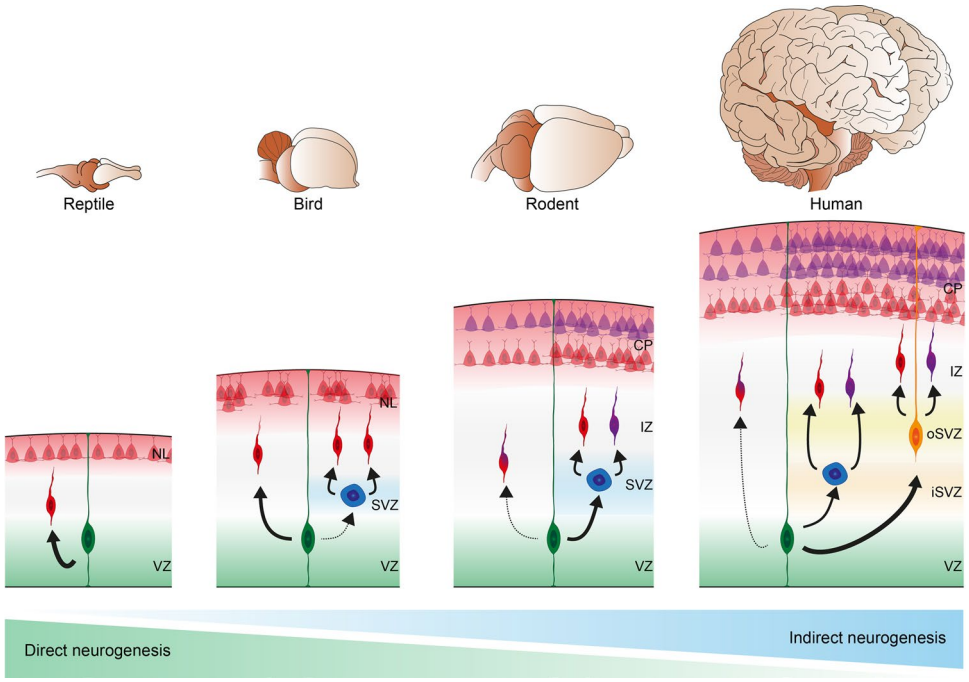

Fig. 4 Modes of neurogenesis and neuron number in the pallium of amniotes. Schematic drawings of the adult brains of representative reptile, bird, rodent and human, and schemas of their embryonic pallium depicting the germinal layers, types of neurogenic progenitor cells and mode of neurogenesis. Reptiles only have ventricular zone (VZ) and apical radial glial cells (aRGCS, green), so all cortical neurons are produced by direct neurogenesis. Neurons (red) resemble those in deep layers of the mammalian neocortex. Birds show the first signs of a subventricular zone (SVZ), populated by intermediate progenitor cells (IPCs, blue), and an incipient shift towards indirect neurogenesis, leading to the generation of a greater amount of neurons. In mammals, IPCs and indirect neurogenesis become much more predominant, and new types of neurons are produced to establish superficial layers (violet). Neuron production is increased even further in gyrencephalic mammals like humans, by greatly expanding the SVZ into two distinct germinal layers: inner SVZ (iSVZ) and outer SVZ (oSVZ). These secondary germinal layers accumulate an exceptional abundance of basal progenitor cells: IPCs and basal radial glia cells (bRGCs, orange). Newborn neurons migrate through the intermediate zone (IZ, gray) to reach their final destination in the neuronal layer (NL) or cortical plate (CP) (red). Arrow thickness represents the relative frequency of each mode of neurogenesis

layer neurons [72]. Thus, suppression of direct neurogenesis promotes indirect neurogenesis and increases the final number of neurons, but it also critically determines the generation of upper layer neuron types [72]. Direct neurogenesis is very abundant in the mouse olfactory bulb, hippocampus and spinal cord, all phylogenetically ancient brain structures. In these, neurogenesis also takes place during much shorter developmental periods than in the neocortex. For example in the olfactory bulb, most glutamatergic projection neurons (mitral cells) are produced in only 2 days (E11–E13), in contrast to the 6 days in the neocortex [72, 151]. Thus, direct neurogenesis predominates in brain regions where speed in neuron production is preferred over abundance [72, 152]. The occurrence of this neurogenic mechanism in mammals seems to be an ancient trait already existing in stem amniotes and conserved during evolution [83], which was eventually modified into the more efficient indirect neurogenesis during the Jurassic period, when big and complex cerebral cortices became advantageous for the emerging mammalian clade [72].

Progenitor cells and neuron fate restriction

During the development of the mammalian cerebral cortex, a series of glutamatergic neuron subtypes are sequentially generated following an inside-out pattern, with neurons produced early occupying deep layers, and neurons generated later occupying superficial layers [153–155]. The distinct neuronal populations are specified during embryogenesis by a network of transcriptional regulators relatively well conserved along evolution [14]. Neuron subtypes allocated in each layer share specific morphological characteristics, gene expression, neural connectivity and function [156]. How a single set of progenitor cells produces this phenomenal diversity of cortical neuron types is a fundamental question that remains poorly understood [52, 149]. Two main hypotheses are classically considered: (1) predetermined fate restriction model, by which a series of progenitor cell pools are pre-committed to each generate a subset of projection neuron classes. (2) Progressive competence restriction, where a homogeneous

pool of progenitor cells is subject to a progressive limitation in the diversity of neuron classes that can be generated as corticogenesis progresses.

McConnell and colleagues first proposed, and provided evidence supporting, the temporal restriction hypothesis for cortical neurogenesis [157–159]. Later studies suggested that neuronal fate commitment is intrinsically determined, where distinct populations of progenitors give rise to restricted and different subtypes of cortical neurons [130, 160]. This was later contradicted by results showing that subpopulations of aRGCs produce neurons for all layers [161, 162]. In contrast, the temporal restriction hypothesis by McConnell has been further supported by later studies. Fate mapping experiments [163], in vitro culture of multipotent progenitors [164] and Flash-Tag labeling experiments [165] demonstrate that the potential of progenitor cells to generate diverse subtypes of neurons is a cell-autonomous mechanism and becomes restricted over the neurogenic period. It has also been suggested that the full diversity of cortical neurons may be best explained by a combination of both hypotheses (Fig. 4) [166]. Recent studies embrace a mixed model, where progenitor cells are somehow partly predetermined but the definitive cell fate is subject to the influence of extrinsic factors [46, 50, 75, 165, 167–173].

The emergence of different progenitor cell types during corticogenesis has also been related to the production of distinct subtypes of projection neurons. IPCs generate up to 80% of cortical projection neurons for all layers of the mouse cortex, in agreement with the temporal fate restriction model [64, 70, 128, 131]. However, due to the late accumulation of IPCs in the SVZ, their contribution is particularly essential to produce upper layers, especially in the evolutionary expansion of the SVZ [47]. Our own results demonstrate that the acquisition of indirect neurogenesis via IPCs is crucial to generate upper layer neurons and that forcing direct neurogenesis (bypassing IPCs) increases the production of deep layer neurons at the expense of upper layer neurons [72].

The layer specificity of neurons in the mammalian neocortex has been proposed to be maintained in the avian hyperpallium. In contrast to the temporal restriction of the mammalian neocortex, however, the avian pallium is thought to present a spatiotemporal restriction in neuronal subtype generation. Accordingly, progenitors in the medial hyperpallium are committed to generate deep layer-like neurons in early stages of neurogenesis, whereas upper layer-like neurons are produced in the lateral hyperpallium at late stages [174]. This model argues in favor of a predetermined fate restriction model, but more recent studies have challenged these results and conclusions, showing evidence that progenitor cells in chick and gecko pallium are in fact multipotent [40].

Molecular regulation of cortical evolution

Understanding the development and evolution of cortical diversity involves understanding the genetic basis of cellular events that underlie the appearance of novel phenotypes and, therefore, it requires understanding the origin and evolution of genes [51, 175]. The largest changes in gene content and organization during evolution are at the level of chromosomes, and indeed the number of chromosomes is highly variable among amniotes. Reptiles and birds have up to 10 pairs of macrochromosomes and a variable number of microchromosomes [176]. Mammalian karyotypes also vary between the three main lineages: monotremes are more similar to reptiles, with eight large and many small chromosomes. Marsupials have several large chromosomes with a high level of conservation. Eutherians are quite diverse, with extensive chromosomal rearrangements [176]. Chromosomal rearrangements likely had profound consequences on gene regulation and function during amniote evolution. Importantly, conserved chromosomal regions are enriched in genes critical for development, particularly of the central nervous system [176].

Major mechanisms of evolutionary change are the addition or removal of genes, modification of levels or patterns of gene expression, and alterations in the coding and non-coding sequence of genes [177, 178]. Understanding how new genes originated and evolved is critical to understand the origin and evolution of biological diversity [175]. Several processes contribute to the formation of new genes, including gene duplication, modifications of pre-existing genes, and formation of new genes from previous non-coding DNA [175, 178]. The de novo generation of a new gene forming a non-coding region, without a functional ancestral gene, is a rare event [179], but considered an essential phenomenon in early steps of evolution [180]. In contrast, the evolution of new genes from ancestral ones is considered the most viable option to generate diversity [181]. Phenotypic diversity among animals extensively involves new combinations and modifications of pre-existing molecular characters. The acquisition of new functions for, or new regulation of, ancestral genes is known as co-option, and this is thought to be critical for evolutionary changes in developmental and metabolic systems [180].

Early studies comparing humans and non-human primates already postulated that changes in the coding region of genes are insufficient to explain the phenotypic differences between both and suggested that an important part of the phenotypic evolution must be explained by modifications in the non-coding genome. Indeed, changes in gene regulatory sequences have been shown to play an important role in evolutionary complexity [182]. One important example of the importance of these regulatory regions

during evolution of the cerebral cortex is human accelerated regions (HARs). Genomic comparisons between mouse, rat, chimpanzee and human revealed the presence of these short, evolutionarily conserved sequences that have, otherwise, a high rate of divergence specifically in the human genome [183–185]. Not surprisingly, HARs are mainly located in non-coding regions proximal to genes enriched for neuronal processes [55, 182, 185]. Several HARs have been shown to interact with promoters of dosage-sensitive neurodevelopmental genes, such as transcription factors, strongly suggesting the importance of HARs in the regulation of neurodevelopmental processes [186]. The modulation of expression of these dosage-sensitive genes by epigenetic modifications has been demonstrated as essential during amniote brain development and evolution [129, 187].

The discovery of genetic differences between species is essential to understand the basis of cortical evolution and development. Particularly interesting is the identification of those differences that distinguish humans form primates and that allowed the fabulous expansion and emergence of intellectual capabilities of the human neocortex. Genomic sequence comparisons between species are useful to identify portions of genome that may have contributed to developmental and evolutionary divergences. These, together with the analyses of epigenomic marks to identify changes in gene regulatory function, have opened new avenues for the study of human evolution [182]. Although a mounting number of studies contribute to clarify the molecular mechanisms of brain development, little is known about their conservation/divergence and importance during brain evolution. In the following sections, we present some of the main genetic factors regulating cellular mechanisms that were key in brain evolution.

Regulation of radial glia cell proliferation and differentiation in amniotes

Multiple molecular pathways have been identified that regulate the balance between aRGCs proliferation and differentiation, but few have been tested through amniote phylogeny. The transcription factor Pax6 is characteristic of aRGCs and essential for their differentiation, initiating the lineage transition towards glutamatergic projection neurons [62, 146, 188]. These roles are highly conserved among amniotes [187]. In addition, in the mouse neocortex Pax6 promotes the proliferation or differentiation of aRGCs in a stage dependent manner, whereas in the chick pallium a high dose of Pax6 promotes their differentiation independently from the neurogenic stage [189–193]. This demonstrates the conservation of the proliferative role in both mouse and chick pallium, but a species-specific divergence in the differentiation role [187]. Therefore, the fine-tuning of Pax6 expression in a spatial–temporal fashion and dosage-dependent manner is essential to coordinate the proliferation and fate of aRGCs across amniotes.

Sonic hedgehog (Shh) is a secreted protein that acts as a morphogen in early stages of development of various tissues and organs and is essential in the specification of the dorsal forebrain territory [194, 195], a function conserved in a wide range of species [195]. Shh also controls the cell cycle kinetics of aRGCs and their transition into IPCs, being essential for the proliferation and survival of aRGCs in the mouse neocortex [194, 196]. Similarly, Shh controls the mode of division also in progenitor cells of the chicken spinal cord, promoting amplificative divisions and preventing the switch towards neurogenesis [197]. Therefore, the role of Shh regulating the mode of division of progenitor cells is conserved in the central nervous system of different amniotes.

Another essential mechanism controlling the expansion and differentiation of progenitor cells is the regulation of the cell cycle [86]. The cell cycle of progenitor cells in the mouse neocortex progressively lengthens as development progresses. This is due particularly to the lengthening of the G1 phase and linked to the progressive increase in the frequency of neurogenic divisions [58, 133]. The cell cycle length is regulated by the modulation of cyclin/cdk (cyclin dependent kinase) complexes, specifically cdk4/cyclinD1 [91], cyclinD1 or cyclinE1 [198]. Overexpression of cdk4/cyclinD1 or cyclinD1/E1 in the mouse neocortex shortens G1 phase, inhibiting neurogenesis and promoting proliferation, whereas repression of these cell cycle genes has the opposite effects [91, 198]. These results show that lengthening of G1 is necessary and sufficient to switch from proliferative to neurogenic divisions. The regulatory effect of the complex cdk4/cyclinD1 is conserved in gyrencephalic species, as shown in ferrets where overexpression of this complex promotes an expansion of progenitor cells and a delay in neurogenesis [135]. Altogether, this demonstrates that the regulation of cell cycle progression and length by different cyclins and Cdks is a conserved mechanism in mammals to control the amplification of progenitor cells and their switch to neurogenic divisions during cortical development.

Notch signaling is one of the best known conserved molecular pathways regulating cell fate across phylogeny [199–201], including *Xenopus*, zebrafish, chicken and mouse [199]. The family of Notch receptors (Notch1–4 in mammals) is transmembrane proteins activated upon binding to their canonical ligands Delta-like (Dll1, 3 and 4) and Jagged (Jag1 and 2). These are also transmembrane proteins, so Notch binding to its ligands requires cell–cell contact. Upon ligand binding and receptor activation, Notch is cleaved and an intracellular domain (NICD) is released, which translocates to the nucleus where it forms a complex with several other proteins to promote transcription of target genes [201, 202]. The best characterized Notch target

genes are the family of *Hes* and *Hey* genes. These encode for basic-helix-loop-helix (bHLH) proteins that repress the expression of proneural factors such as Ascl1 and neurogenins, thus preventing neuronal differentiation [199, 200, 203]. The classical view of the Notch pathway argues that stochastic variation of Dll levels activates Notch signaling differentially in neighboring cells [202]. Cells with high activation of Notch express *Hes* genes, which repress *Dll1* and proneural genes, and remain as aRGCs. On the contrary, cells with low Notch activation do not repress expression of *Dll* nor proneural genes, initiating neural differentiation [204]. This cross-repressive interaction, where cells committed to differentiate inhibit the differentiation of their neighbor cells, is known as lateral inhibition [202, 205]. However, this classical and static view of the Notch pathway was dramatically challenged by the discovery that expression levels of *Hes* genes change very dynamically in neural progenitors, in an oscillatory manner. The delicate balance of these cyclic oscillations is eventually broken giving rise to lateral inhibition between neighboring cells [202, 206]. Even more strikingly, it has been recently demonstrated that Notch activity has different temporal dynamics depending on the specific ligand bound, and this has key consequences on cell fate [207]. Dll1 activates Notch1 in a pulsating fashion, which promotes *Hes1* expression and promotes myogenesis in chick neural crest, whereas Dll4 activates Notch1 in a sustained manner, driving *Hey1* and inhibiting myogenesis [207]. Similarly, binding of Dll or Jag to Notch determines the mode of tumor angiogenesis [208].

Levels of Notch activity determine the rate of neural differentiation across amniotes. However, it remains unclear if the molecular mechanisms involved are identical, because the patterns of Notch activation in the embryonic pallium differ between clades: mosaic in mammals (mouse) and archosaurs (turtle and chick), but homogeneous in lepidosaurs (gecko) [83]. We have recently shown that the expression levels of Notch ligands across amniotes are regulated by Robo signaling [72]. Robo receptors and their ligands (Slits) are classical regulators of axon guidance in the developing nervous system, with very high conservation in phylogeny [209–211]. Slit-Robo signaling had also been previously related to the regulation of neurogenesis in fruit flies and mice [63, 212, 213]. Specifically, we had demonstrated that low levels of *Robo1/2* expression in mouse cortical aRGCs favor their self-renewal, whereas the complete absence of Robo leads to an excess of IPCs. This modulation of aRGC lineage by Robo receptors is mediated through the transcriptional activation of *Hes1* [63]. In a recent study, we have demonstrated that the modulation of Notch ligand expression by Robo receptors is also a key mechanism defining whether aRGCs undergo direct or indirect neurogenesis and that this is conserved across amniotes [72]. Through the analysis of cortical progenitor cells in species representative of amniote diversity (snake, chick, mouse and human), we demonstrated that the expression level of Robo in aRGCs of the embryonic cortex was attenuated during amniote evolution, whereas expression of Dll1 increased, and that these changes were critical in changing the balance between direct to indirect neurogenesis [72]. In the dorsal cortex of snakes, the medial hyperpallium of chick, and the mammalian olfactory bulb, hippocampus and spinal cord (brain structures phylogenetically older than the neocortex), expression levels of Robo are high and Dll1 is low; in contrast, expression of Robo is low and Dll1 high in the chick lateral hyperpallium and the mammalian neocortex. These endogenous expression levels correlate with the respective frequency of direct neurogenesis, maximum in the snake dorsal cortex (with null indirect neurogenesis in the absence of IPCs) and minimum in the mammalian neocortex (with residual direct neurogenesis) [72]. Experimental manipulations demonstrated that these reciprocal levels of Robo/Dll1 expression are necessary and sufficient to determine the rates of direct and indirect neurogenesis. Accordingly, low Robo and high Dll1 are sufficient for the emergence of some of the goldstandard features of expansion and complexification of the mammalian neocortex: the appearance of IPCs grouped in an SVZ, and the generation of upper layer neurons [72], recapitulating some of the most important events of amniote evolution. At the molecular level, high levels of Robo reduce Dll1 expression and increase Jag1/2, which induces direct neurogenesis. Thus, the mode of neurogenesis seems to be defined by the type of ligand activating Notch, which may modify the dynamics of Notch activation without overall changes in activity levels [72, 207]. This is the first known molecular pathway with a conserved role in determining the mode of cortical neurogenesis along amniote evolution.

Emergence and regulation of IPCs and bRGCs along amniote evolution

The crosstalk between Robo receptors and Notch ligands established the ancestral, rate-limiting mode of direct neurogenesis, and its attenuation later in amniote evolution promoted the emergence of IPCs [72]. On the other hand, the transcription factor Tbr2 is essential for IPC specification and proliferation and regulates the differentiation of cortical layers in mouse neocortex [71, 131, 132]. As mentioned above, multiple evidences point to the importance of Tbr2 in the evolutionary emergence of IPCs and expansion of the mammalian neocortex [85]. For example in ferret, inhibition of Tbr2 reduces IPC and bRGC abundance, altering the formation of upper layer neurons and cortex folding [138]. This is consistent with previous reports on the role of Tbr2 in mouse neocortex [71], suggesting that the roles of Tbr2 on IPC specification and neuronal differentiation are conserved

in mammals. However, there is limited direct evidence on conserved roles in amniotes. While we have shown that blockade of direct neurogenesis leads to increased Tbr2+ IPCs in the chick dorsal pallium [72], Nomura and colleagues have also shown that the manipulation of specific cell cycle proteins may increase the number of Tbr2+ cells in the chick pallium without increasing the abundance of IPCs [102].

Whereas the emergence of IPCs and their coalescence into the SVZ are milestones of mammalian neocortex evolution [95], the generation and amplification of bRGCs are crucial for the further expansion and then folding of this neocortex [51, 76]. Little is known about the potential conservation of mechanisms underlying the emergence of bRGCs during evolution. The transcription factor Pax6, typical of aRGCs, is also the marker protein for bRGCs in the iSVZ and oSVZ of gyrencephalic species [75, 76]. The sustained expression of Pax6 in mouse aRGCs is sufficient to promote the generation of bRGCs and enhance upper layer neuron production, though not to induce cortex folding [189]. The nuclear protein Trnp1 (TMF regulated nuclear protein 1) is another key regulator of neocortical expansion and folding [136]. It is expressed in a subset of aRGCs but not in IPCs, and developmentally downregulated in mouse. Overexpression of *Trnp1* in mouse neocortex promotes aRGCs self-renewal, while its early loss-of-function promotes the generation of IPCs and bRGCs, with eventual folding of the mouse neocortex [136]. In the gyrencephalic ferret, temporal changes in expression levels of *Trnp1* and *Cadherin1* delimit the brief period critical for the massive generation of bRGCs and initiation of the oSVZ. In a role conserved from mouse, increased *Trnp1* expression inhibits the generation of more bRGCs and closes this critical period [93]. Another recent study shows that in mouse bRGCs are relatively abundant in the medial neocortex, as identified by the expression of Hopx as in human embryos [214] and that the expression levels of Hopx regulate the abundance of bRGCs in mouse [215].

Very little is known about the presence of bRGCs in non-mammalian species, as they are considered an evolutionary innovation for the expansion and folding of the neocortex and are nearly absent in mouse [51, 54, 78, 134]. Nomura and colleagues observed in the chick dorsal pallium the presence of basal progenitors displaying some of the defining features of mammalian bRGCs: mitosis at basal position, expression of Pax6 and Sox2, and display of radial processes that extend toward the apical and basal sides [55, 102]. These observations suggest that basal radial glia-like cells may have emerged in ancestral amniotes and then have undergone secondary loss in many of the non-mammalian lineages [102].

What makes us humans?

Understanding what makes the human brain unique and different form our closest relatives is currently a very active field of research. Despite the success of genetic approaches, we are only beginning to identify and understand the genetic changes occurred in evolution that define the human brain as it is and, therefore, that were essential for our evolution, particularly our neocortex (for recent and detailed reviews on genetic signatures of human cortex evolution, see [19, 51, 55]).

As mentioned above, much attention has been recently placed on changes in gene regulatory elements as a main force for phenotypic evolution, particularly the so-called Human Accelerated Regions (HARs) [216]. Among these regions of very active sequence change in the recent human lineage, investigations on the function of HARE5 are of particular interest [217]. This is a regulatory enhancer of *Frizzled8*, a conserved receptor of the Wnt pathway essential for brain development and progenitor cell proliferation. Substitution of the endogenous enhancer in mouse by the human HARE5 dramatically increases the rate of neural stem cell proliferation in the neocortex, accelerating the cell cycle and expanding the pool of progenitor cells, ultimately increasing cortical surface area. These effects are not elicited by the chimpanzee version of HARE5, demonstrating the relevance of the human HARE5 and the transcriptional regulation of the Wnt pathway for the recent evolution of the human neocortex [218]. Moreover, mutations in HARs that interact with promoters of dosage-sensitive neurodevelopmental genes confer risk of developing autism spectrum disorders [186]. Non-coding RNAs provide another level of gene expression regulation. Many primate-specific miRNAs are expressed in cortical progenitor cells, targeting proteins that regulate the cell cycle and neurogenesis [219].

Aside from regulatory regions, mounting evidence supports the importance of changes in the coding sequence of neural genes, or the emergence of new genes, as driver mechanisms of human brain evolution [177]. Gene duplication, creating paralogs from ancestral genes, is one key mechanism [175]. The Slit-Robo Rho GTPase-activating protein 2 (srGAP2) underwent two human-specific partial duplications, leading to the formation of SRGAP2B and SRGAP2C. SRGAP2C is expressed more prominently than SRGAP2B in germinal layers and the cortical plate [220], similar to srGAP2 in mouse [221]. In the mouse neocortex, srGAP2 regulates neuronal migration as well as neurite initiation and branching [221]. Increased mouse srGAP2 expression causes excessive branching of cortical neurons, which impairs their radial migration [221]. However, overexpression of human SRGAP2C in mouse exerts

the contrary effect, promoting neuron migration [220]. In addition, SRGAP2C blocks the function of ancestral srGAP2 on spine maturation, increasing spine density and dendritic complexity [220]. Therefore, the emergence of SRGAP2C and its expression in human pyramidal neurons led to an increase in their synaptic inputs, with potentially critical implications in cognition, learning and memory [220, 222].

Another gene generated by partial duplication and important for human brain development is ARHGAP11B. This arose from ARHGAP11A, an Rho guanosine triphosphatase-activating protein (RhoGAP), after the divergence of the human and chimpanzee lineages, which lost its RhoGAP activity upon a truncation in the GAP domain [137, 223]. ARHGAP11B is highly expressed in aRGCs and bRGCs of the embryonic human cortex, but is virtually absent in cortical neurons. Overexpression of ARHGAP11B in the mouse neocortex drives the formation of self-renewing basal progenitors, especially bRGCs, resulting in thickening of the SVZ and, eventually, folding of the neocortex [137]. Interestingly, the ancestral form of ARHGAP11B, with RhoGAP activity, does not amplify bRGCs. Therefore, the mutations produced in the GAP domain during human evolution would be responsible for the generation of bRGCs and, ultimately, the expansion of the human neocortex [223]. Overexpression of ARHGAP11B in an already gyrencephalic cortex, such as the ferret, further amplifies the pool of bRGCs, lengthening the neurogenic period and increasing neuron production, particularly for upper layers, expanding the neocortex even more [224].

Recently, three human paralogs of NOTCH2 derived from partial duplication have been identified and reported to be expressed in cortical aRGCs and bRGCs: NOTCH2NLA, NOTCH2NLB and NOTCH2NLC [225, 226]. Overexpression of various NOTCH2NL forms in human embryonic stem cells and cerebral organoids causes the amplification of progenitor cells and the corresponding delay in neurogenesis [225, 226]. Conversely, inhibition of NOTCH2NL in human cerebral organoids causes premature neuronal differentiation [225]. NOTCH2NL seems to overactivate the Notch pathway, thus enhancing aRGC self-renewal, expanding the pool of progenitor cells and delaying neurogenesis, which may ultimately be translated in greater neuron production, as proposed by the radial unit hypothesis [6, 225, 226]. The examples of SRGAP2C, ARHGAP11B and NOTCH2NL demonstrate the importance of human-specific gene duplications and modifications in the evolutionary expansion of the human neocortex [19, 51].

Small changes in the coding sequence of genes may also have critical implications in the development of human-specific features, as is the case of FOXP2 and ASPM. Forkhead box P2 encodes a transcription factor highly expressed in the developing and adult neocortex [227]. Although the protein is extremely conserved among mammals [228] and there are orthologues in all groups of amniotes [229], FOXP2 acquired two amino-acid changes in the recent human linage, after the divergence from chimpanzee, that had important functional consequences [228]. Individuals containing a point mutation in FOXP2 have difficulties in the expression and reception of language [230], so the inactivation of a single copy has a profound impact in speech development [231]. Similar inactivating mutations in mice or the avian FoxP2 orthologue also cause alterations in vocalization and song production, supporting the importance of this protein for the development and function of neuronal circuits involving motor skills and vocal behaviors, with a high level of complexity in humans [231].

Abnormal spindle-like primary microcephaly (ASPM) also underwent a strong positive selection for amino acid changes during evolution [232]. This is the most common gene altered in autosomal recessive human microcephaly [233]. Its loss in the developing mouse causes mitotic aberrations in progenitor cells, leading to a reduction in neuron number, thickness of neuronal layers, altered layering, and a decrease in brain volume, although brain architecture is relatively normal [234]. In ferret, the absence of Aspm causes a premature depletion of aRGCs and their progression into bRGCs, suggesting that ASPM may be important regulating the timing of aRGCs to bRGCs transition and, hence, supporting the idea that expansion of the pre-neurogenic VZ during human evolution was essential to expand the cerebral cortex [235].

In summary, the combination of multiple genetic modifications during evolution seems to have contributed to the acquisition of key and specific features of human cortex development, size and complexity. The above examples illustrate the positive pressure to introduce changes in the sequence of protein-coding genes and in their regulatory elements leading to the expansion and complexification of the neocortex. These modifications led to a significant divergence from our closest non-human primate relatives, namely an increased pool of apical progenitors, the generation and amplification of basal progenitors, and the complexification of neuron types and circuits relevant for high-order functions.

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