

Review

Shaping the brain: The emergence of cortical structure and folding

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SUMMARY

The cerebral cortex—the brain's covering and largest region—has increased in size and complexity in humans and supports higher cognitive functions such as language and abstract thinking. There is a growing understanding of the human cerebral cortex, including the diversity and number of cell types that it contains, as well as of the developmental mechanisms that shape cortical structure and organization. In this review, we discuss recent progress in our understanding of molecular and cellular processes, as well as mechanical forces, that regulate the folding of the cerebral cortex. Advances in human genetics, coupled with experimental modeling in gyrencephalic species, have provided insights into the central role of cortical progenitors in the gyrification and evolutionary expansion of the cerebral cortex. These studies are essential for understanding the emergence of structural and functional organization during cortical development and the pathogenesis of neurodevelopmental disorders associated with cortical malformations.

INTRODUCTION

The cerebral cortex constitutes the majority of the human brain by mass, and the neocortex—the newest evolutionary addition to the cerebral cortex—has expanded extraordinarily in humans when compared with our phylogenetically closest relatives, the great apes.^{1,2} Recent progress in describing the human neocortex has revealed unique features relating to the number and diversity of cell types.^{3–8} However, our understanding of how the neocortex has acquired its shape and organization remains incomplete. Some of the remarkable human cognitive abilities that resulted from the increases in size and complexity of the neocortex are disrupted in patients with cortical malformations, neurodevelopmental disorders characterized by abnormal cortical structure and/or organization, and understanding the process of neocortical formation may give us insights into these disorders.^{9–12}

The presence of folds in the neocortex is a hallmark in many mammals, including humans. Neocortical folding is characterized by patterns of fissures called sulci and ridges called gyri, hence the name gyrencephalic for describing species with a folded cortex. The thickness of the neocortex varies across the folds: the gray matter (where neuronal cell bodies reside) is thickest at the peaks of gyri and thinnest at the deepest points of sulci. Therefore, areas corresponding to gyri and sulci can be inferred from the variation in thickness.^{13,14} Cortical folding is thought to provide compaction of the neocortical structure, allowing a larger surface area to fit in a smaller volume. In humans, primary gyri and sulci are the first convolutions to emerge during development, are the largest folds, and display consistent locations

across individuals.¹⁴ By contrast, secondary and tertiary folds are formed later in development and vary to some degree in their locations and orientations.^{15–18} Importantly, patterns of cortical folding may have consequences for functional connectivity. For instance, neurons separated by a gyrus are more likely to be functionally connected than those separated by a sulcus.^{19–21}

Not all mammals have a gyrencephalic neocortex. Common animal models in neuroscience research such as mice and rats are lissencephalic (i.e., their cortices are smooth). Cortical gyrification was first proposed to have emerged from common lissencephalic ancestors as neocortical size increased.²² However, broad comparative analysis of mammalian brain anatomy suggests differently. Cortical gyrification is observed even in Monotremes and Marsupials,²³ far removed from the common ancestor of mice and gyrencephalic species, and within Rodentia, members with larger brains such as capybaras show gyrification. Thus, in rodents, and particularly in mice and rats, lissencephaly more likely represents a derived trait, likely selected for through demands for a smaller head or less energetically expensive cortex.²⁴ A gyrencephalic cortex was very likely present in the ancestral animal of all living mammals today, suggesting that cortical folding was a core feature of the early neocortex.^{25,26} In gyrencephalic species with only primary folds, such as ferrets, the sulcation pattern is essentially invariant between animals.²⁷ Although mice do not develop cortical gyration, they remain useful model organisms to explore aspects of neocortical development, even related to cortical folding, because of the high degree of conservation in mechanisms of neocortical development across all mammals. Recently, understanding cortical folding has benefited from the study of cortical



folding disorders in human individuals,²⁸ and the more recent development of genetic manipulation in gyrencephalic models like ferrets has led to a new era of exploring the biology of cortical folding.^{29–31}

In this review, we explore and discuss advances in our understanding of the development of cortical folding as a central process underlying the emergence of shape in the human brain. We provide a comprehensive review on the role that cortical progenitors play in the formation of cortical folding, with interdisciplinary consideration of studies of the biophysics of this process. Our attention is also focused on comparative analyses across species that have provided key insights into the cellular mechanisms underlying cortical folding. We review signaling pathways that regulate the dynamics and maintenance of cortical progenitors and concentrate on specific examples for which extensive studies of animal modeling as well as human genetics converge to inform us about their function in cortical gyrification. We discuss recent data establishing a key role for Sonic Hedgehog (Shh) signaling in cortical folding, based on experiments in ferret models and genetic studies of patients with cortical malformations. Next, we highlight findings of evolutionary mechanisms that illustrate the importance of human-specific genetic changes in regulating the behavior of cortical progenitors in the developing cortex and how these evolutionary specializations may have impacted cortical size and complexity. Finally, we provide a perspective on recent studies that point toward emerging cellular mechanisms shaping the development of neocortical gyrification.

CORTICAL DEVELOPMENT AND NEURAL PROGENITOR CELLS

As corticogenesis begins, neuroepithelial cells (NECs) give rise to cortical progenitor cells known as radial glial cells (RGCs) that are responsible for generating diverse populations of cortical projection neurons (Figure 1). Apical RGCs (aRGCs), whose cell somata reside in the ventricular zone (VZ), initially undergo symmetric divisions to amplify the pool of progenitor cells.^{32–34} aRGCs extend long processes that span from the ventricular surface apically to the pial surface basally^{34,35} (Figure 1). As the neurogenic period of cortical development begins, aRGCs also begin to divide asymmetrically to produce a daughter neuron and another aRGC, which is known as a neurogenic and self-renewal division.³² Furthermore, aRGCs can produce nonpolar, division-limited intermediate progenitor cells (IPCs), which upon division will generate two daughter neurons, hence amplifying the output of neurons produced during neurogenesis^{32,36–38} (Figure 1). The vast majority of IPCs undergo their mitotic division in the subventricular zone (SVZ), although these progenitors can be found across germinal zones (VZ and SVZ), which determines their classification as apical or basal IPCs, respectively.^{38–41,43} Basal IPCs (bIPCs) can exhibit proliferative, self-renewal capacities in primates, in contrast to mouse IPCs that generally undergo self-consuming divisions.⁴³ As corticogenesis proceeds, aRGCs generate a second class of radial glial progenitors called basal RGCs (bRGCs; also known as outer RGCs) that reside basally within the SVZ, delaminate from the apical belt of adherens junctions attached to the ventricular surface, and retain their polarity typically by extending a basal pro-

cess connected to the basal surface of the developing cortex, although they can display remarkable diversity of morphological subtypes^{39,43–45} (Figure 1).

The SVZ can be anatomically subdivided into an inner and an outer layer (iSVZ and oSVZ, respectively): the iSVZ contains randomly organized cells, whereas the oSVZ shows radially organized cells and is the main location of bRGCs.^{43–46} The inner fiber layer constitutes the anatomical landmark separating the iSVZ and oSVZ and emerges around embryonic day 72 in the macaque developing cortex (roughly corresponding to the beginning of the second trimester of gestation in humans).⁴⁶ A growing body of literature has documented that numerous successive rounds of proliferative divisions of bRGCs (in some cases, summing up to 6 division rounds) and to a lower degree of bIPCs, ensure the progressive accumulation of neurons during cortical development. These findings have formed the basis for the view that the high proliferative capacity of bRGCs may underlie the expansion of oSVZ and the increased cortical neuron numbers in primates, achieving its highest levels in humans.^{43–50}

Nascent, immature neurons in the developing cortex utilize the radial glial processes from RGCs that extend to the basal pial surface—adhering to them through cell adhesion mechanisms—to migrate radially through an intermediate zone (IZ) to finally reach the developing cortical plate (CP), where they will mature and form synaptic connections to begin elaborating developing cortical circuits (Figure 1). Thus, radial glial processes (also known as RG fibers) form cytoarchitectonic scaffolds that are essential for radial migration of neurons. The CP appears by splitting a primordial basal zone that was above the early VZ before neurogenesis, known as the pre-plate (PP), separating it into a cell-rich subplate (SP) of early pioneer neurons below the CP and the marginal zone (MZ) above the CP. The MZ terminates at the pial surface. Although early models suggested that nascent neurons may migrate in narrow columns called “radial units,”⁵¹ analysis of excitatory neuron clones derived from single or few RGCs shows a more conical distribution in mice, widening as daughter neurons migrate further from the VZ. In ferrets, clonal marking and live imaging analyses of cortical slice cultures show even more prominent tangential dispersion, suggesting that migrating neurons use the radial glial scaffold as guides in general but do not rigidly adhere to specific RG fibers, allowing them to disperse more horizontally in the developing CP of gyrencephalic species.^{40,52,53} Together with the RG fibers displaying a fanned array in sites of developing folds,⁴⁰ these data provide key insights into the mechanisms for increased surface area in the gyrencephalic cortex (discussed in the next section).

After they are generated from the early neuroepithelium, RGCs undergo multiple rounds of symmetric division to increase the pool of progenitors and later asymmetric divisions to both generate a daughter neuron and self-renew the progenitor cell.^{32,54,55} However, as neurogenesis proceeds, individual RGCs will themselves exit the cell cycle to produce terminally differentiated daughter cells. The timing of the switch from symmetric to asymmetric (i.e., expanding the number of progenitors vs. producing neurons), as well as the timing of cell cycle exit for aRGCs and bRGCs, dictates their neurogenic potential. RGCs that proliferate for shorter times produce fewer daughter cells.

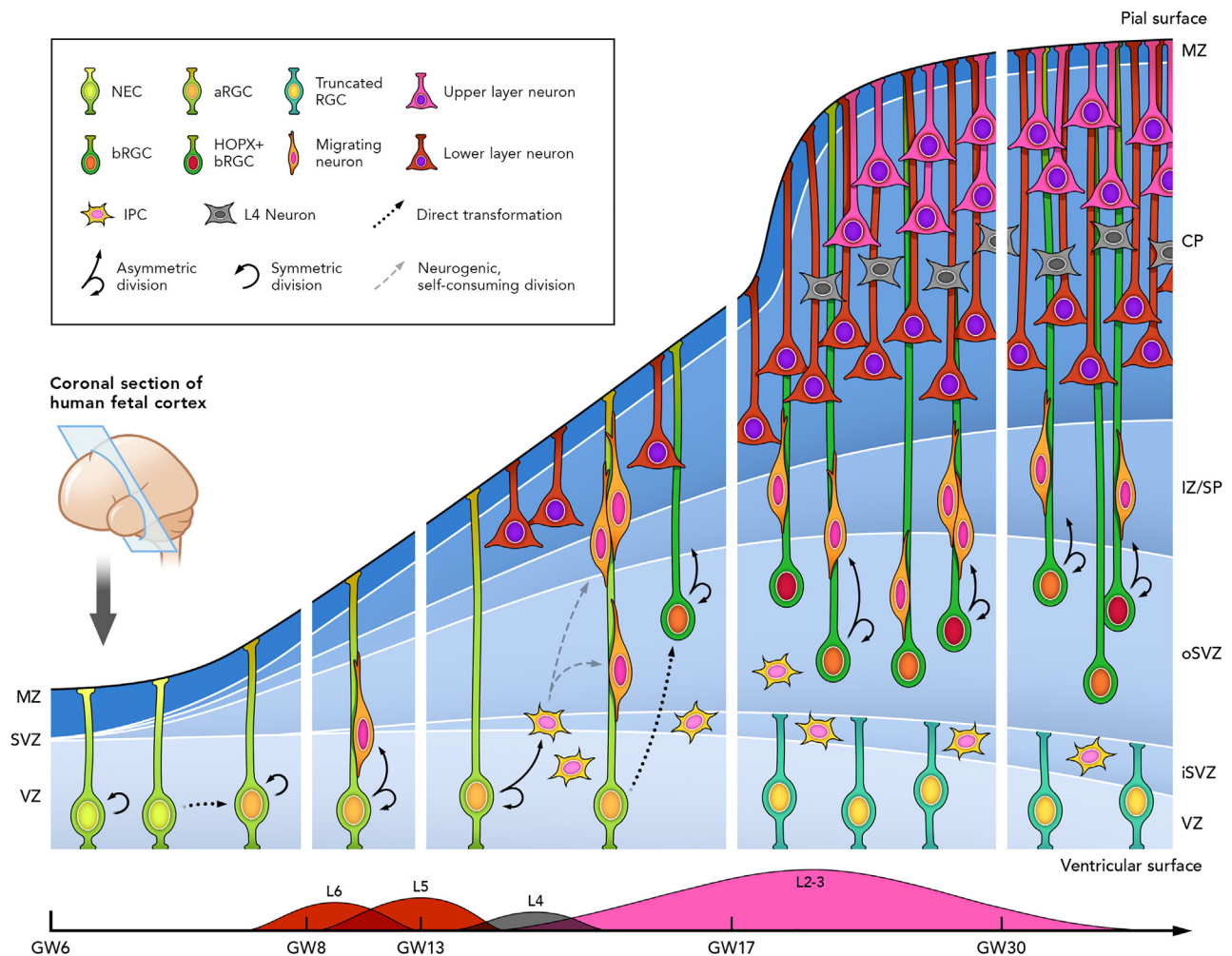


Figure 1. Temporal stages and progenitor types in the human developing neocortex

During early human brain development, a layer of neuroepithelial cells (NECs), spanning from the ventricular surface to the pial surface, populates the developing neural tube and undergoes self-renewing divisions to generate more NECs (symmetric divisions) in early developmental stages. They then elongate and differentiate into radial glial cells (RGCs), which also undergo symmetric divisions to expand the population of progenitors. During the neurogenic period, RGCs begin to divide asymmetrically to generate neurons while self-maintaining the progenitor pool, either generating neurons directly or producing neurons indirectly through intermediate progenitor cells (IPCs). Apical radial glial cells (aRGCs) are defined by residing in the ventricular zone (VZ) and by establishing contacts at both the apical and basal surfaces of the developing cortex. Later in development, aRGCs can also give rise to basal radial glial cells (bRGCs) by delamination of the apical belt of adherens junctions attached to the ventricular surface and translocation of their somas to the subventricular zone (SVZ). Based on marker expression, bRGCs have multiple subtypes, including HOPX+ bRGCs. Migrating neurons generated by aRGCs or bRGCs use the RG scaffold of both types of progenitors to migrate through the intermediate zone (IZ) into the developing cortical plate (CP), which contributes to the growth of the CP. By the second trimester of pregnancy (gestational week 17), aRGCs transform into truncated RGCs with their basal process terminating in the border between the inner and outer layers of the SVZ (iSVZ/oSVZ); thus, the RG scaffold becomes truncated at the iSVZ/oSVZ border. Cortical neurons are born in an inside-out fashion, with neurons destined to deeper layers (L6) born first and neurons destined to superficial layers (L2) born last. The density plots shown on the bottom represent the different neurogenic stages that preferentially generate neurons committed to each cortical layer. The extended neurogenic period for superficial layer neurons (L2–L3) coincides with the expansion of bRGC proliferation and is considered a hallmark of human brain evolution.

Thus, regulation of the cell cycle and division mode of different types of RGCs is a determining factor for brain size and cortical thickness.^{56,57}

Although the fundamental components of neurogenesis described above are conserved in all developing mammalian cortices examined,^{58,59} the relative abundance of progenitor cell types and their behaviors vary with brain size and folding pattern across species, which provides correlative implications about the cellular bases of cortical thickness, size, and gyrification.^{50,57} Gyrencephalic species like ferrets and humans have thicker and more complex SVZs relative to lissencephalic

mice. Beyond increased frequency in the developing cortex, basal progenitors have greater diversity in subtype and neurogenic potential in primates than in mice.^{38,50,56,57,60} A basal progenitor type reminiscent of human bRGCs, with marked epithelial features, appears to be present only in small numbers in the SVZ of mice, which primarily rely on aRGCs and IPCs during their short neurogenic period.^{48,61} By contrast, bRGCs, rather than aRGCs, predominantly produce cortical neurons in primates, including humans, and are considered essential in the generation of superficial layer neurons, which have extraordinarily expanded in numbers and diversity in humans.^{3,50,56,57,62}

MECHANISMS OF CORTICAL FOLDING

The process of cortical folding undergoes several developmental stages—spanning from embryonic stages through postnatal development in humans—and requires a complex interplay of mechanisms.^{18,50} On the one hand, physical studies of cortical folding suggest that mechanical forces from differential tangential expansion of the CP relative to the germinal layers (VZ and SVZ) are sufficient as an important factor to induce cortical gyration.⁶³ On the other hand, twin studies indicate that gyration patterns are heritable, with patterns of sulci and gyri being significantly more similar between mono-zygotic twins than between control pairs. Higher-order gyri and sulci show greater variability across individuals relative to lower-order ones.^{14,15,17} Together, these data suggest that physical models may require additional influences to account for patterns of gyrification, and that genetic factors might regulate the emergence of cortical gyrification, to a certain degree, in a deterministic fashion, especially of the primary, deeper sulci.^{13,50,64} In the next subsections, we will review the mechanical forces and cellular processes underlying cortical folding and discuss how the interactions between these mechanisms form the bases for the current model of cortical folding.^{22,50,56,57,65,66} This model proposes a central role for RGC dynamics in producing cortical gyration, based on a wealth of studies identifying genetic programs expressed in these progenitors during development that guide the expanding cortex to fold in a controlled manner. These developmental programs produce the necessary conditions for mechanical forces to fold the cortex—such as regions of relative cell production and expansion neighboring regions of slower growth—while maintaining minimal stochasticity for the development of primary gyri. The increased variability in higher-order gyri appears to reflect a greater degree of stochasticity in their formation. Studying the interplay between both factors (mechanical forces and genetic and cellular programs) is essential to gain a better understanding of the mechanisms that orchestrate folding in the developing cortex.

Mechanical forces underlying cortical folding

Prior to 2010, numerous models of the physics of cortical folding were debated, including one in which external force from the skull caused folding of the developing cortex,¹⁴ or one in which internal tension forces from axonal growth pulled cortical regions into a wrinkled shape.^{67,68} However, these two hypotheses are not strongly supported by more recent data: the developing skull does not place mechanical constraint because cranial sutures do not ossify until the completion of brain growth, and reducing cranial pressure does not influence gyrification in animal models.¹⁴ However, influence in the reverse direction is possible, and pressure from an expanding brain or fluid compartment can increase head size, as seen in cases of macrocephaly, both in mice and humans.^{14,69,70} The axon tension model is also inconsistent with experimental investigations demonstrating that localized severing of axons in gyrencephalic brains during development does not influence cortical folding.⁷¹

The prevailing physical model of cortical gyration is based on differential tangential growth of the basal portion of the developing cortex (CP and MZ) relative to the underlying germinal zones (VZ and SVZ).⁶³ The foundations for exploring this model were laid by observations across animals that cortical folding

is best correlated with cortical gray matter thickness and surface area rather than with brain size,⁷² and computational models showing the sufficiency of differential tangential expansion to produce convolutions resembling gyri in structures like the developing cortex.^{73,74} Building on these analyses, studies with physical models using polymer gels constructed of an inner region that expands less than an outer basal region produced patterns of gyration that are grossly similar in scale and pattern to those seen in the human cortex.^{75,76} By using simple polymer gel expansion models with initial parameters derived from fetal MRI imaging at 20 weeks of gestation, these physical models yielded striking similarity to human cortical gyration patterns.⁷⁷ Variations in the stiffness of the inner and outer regions of these compound gel structures impacted the pattern and wavelength of folding, suggesting that the material properties of the tissue play key roles in folding.^{76,77} These findings are consistent with the notion that mechanical force from differential tangential expansion of the outer layer (CP and MZ) relative to the inner layer (VZ and SVZ) of the developing cortex is sufficient to cause folding in a pattern similar to that of the human cortex.

Even though studies of the physical causes of cortical gyration are compelling, they do not capture all the features of cortical folding seen *in vivo*. First, although mechanical models of cortical folding can correctly predict how folds arise from specific initial conditions, presumably these initial conditions explain the recurrence of specific folding patterns across individuals and, hence, still require mechanistic understanding. Second, mechanical models do not yet encompass the physical properties of the distinct developmental layers and only recently have started to incorporate contributions that influence folding at microscopic rather than macroscopic levels, although it is striking how accurate they are already. Finally, these models require tuning of initial parameters such as specific thickness of the layers modeled and rate of expansion pulled from biological estimates, and changes in these parameters have dramatic impacts on the outcomes of predicted gyrification.^{77,78}

Roles of RGCs in cortical folding

If differential tangential expansion during cortical development exerts mechanical forces that can induce folding, what is the cellular substrate responsible for the emergence of such forces in the developing cortex? Regional variation in neuronal density is viewed as a determinant factor in the differential growth of the cortical mantle.^{50,57} Not only this differential accumulation of neurons can lead to region-specific rates of tangential expansion of the cortical surface area, but also the subsequent maturation of neurons (e.g., dendritic arbor development) has been suggested to further contribute to differential growth in developing gyri and sulci.⁷⁹ Multiple lines of evidence over the past decade support a major role for the behavior of cortical progenitors (proliferation and neurogenesis) in the differential accumulation of neurons, ultimately influencing cortical folding. Initial anatomical analyses of primate developing cortices showed regional differences in the size of germinal layers, in particular the oSVZ.⁴⁶ Subsequent studies demonstrated that the distribution of dividing cells is heterogeneous across the cortical mantle in gyrencephalic species, observations consistently reported in ferrets and monkeys,^{40,80,81} and several studies documented the differential distribution of these basal progenitors in regions that will

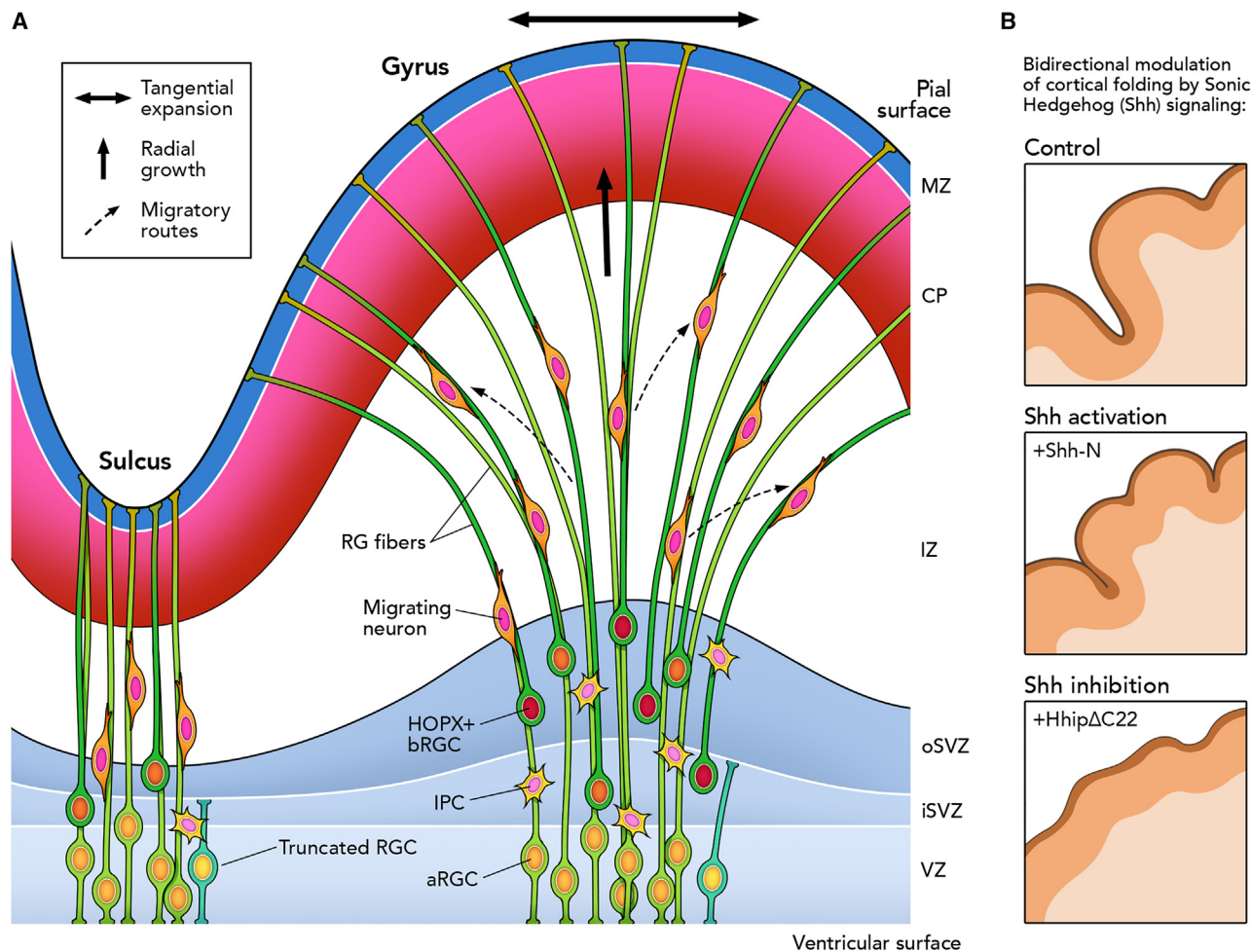


Figure 2. Cellular and molecular mechanisms of radial glial cells promote cortical folding

(A) The current model of cortical folding proposes a key role for basal radial glial cells (bRGCs), which are characterized by a highly proliferative capacity and are especially abundant in the outer subventricular zone (oSVZ). Regional differences in neurogenesis are found across the developing cortical mantle in gyrencephalic species (especially noticeable in the oSVZ): regions destined to prospective gyrus (proto-gyri) have greater densities of dividing progenitor cells than regions destined to prospective sulci (proto-sulci).^{40,81} Consistently, a subclass of bRGCs labeled by the marker HOPX in the developing ferret cortex is found in greater densities in proto-gyri.³¹ The differential density of basal progenitors results in differential production and accumulation of neurons in the developing cortical plate (CP), which causes a greater degree of tangential expansion (horizontal double arrow) in proto-gyri relative to proto-sulci. Moreover, there is a progressive divergence in the trajectory of radial glial (RG) fibers during gyrus formation due to the highly proliferative population of bRGCs that intercalate their fibers with pre-existing ones.⁴⁰ This fanned array of RG fibers in prospective gyral regions contributes to the tangential spread of migrating neurons guided by this scaffolding. By contrast, radial glial fibers typically show parallel trajectories in proto-sulci, which likely limits the tangential spread of migrating neurons.

(B) Schematic illustrating the effects of Sonic Hedgehog (Shh) signaling in cortical folding. HOPX+ bRGCs were shown to respond to activation and inhibition of Shh signaling activity in inverse ways: Shh stimulation (with the amino-terminal fragment of Shh, Shh-N) results in increased densities of HOPX+ bRGCs, whereas Shh suppression (with the competitive inhibitor HhipΔC22) results in a reduced population of HOPX+ bRGCs.³¹ These effects lead to bidirectional changes in cortical folding: Shh stimulation results in larger gyri, whereas Shh inhibition leads to smaller gyri.³¹

generate prospective gyri (proto-gyri) and regions destined to become prospective sulci (proto-sulci)^{39,40,82} (Figure 2A). The cellular density in the oSVZ relative to other germinal layers during development predicts the overall degree of cortical folding across mammalian species, independently of cortical size, such that the size of the oSVZ in development is not just correlated with brain size but also specifically with the gyrification index.^{40,41} More recently, experimental manipulations showed that increasing or reducing proliferation of bRGCs in ferrets leads to bidirectional modulation of cortical folding,^{40,82–84} and amplifying the limited pool of bRGCs in mice results in some degree of cortical folding^{85–87} (further discussed in the following sections).

These findings formed the bases for a proposed mechanism underlying the emergence of primary gyri and primary sulci: elevated rates of neurogenesis in proto-gyri during corticogenesis will result in a higher accumulation of neurons and rapid tangential expansion of the CP, whereas lower rates of neurogenesis in proto-sulci (neighboring regions to proto-gyri) will lead to a lesser degree of accumulation of neurons and tangential growth, thus the developing cortex will begin to form folds and fissures (Figure 2A). A potential source for differential bRGC distribution across the developing cortex, and perhaps even local bRGC behavior, may be the differential gene expression observed between proto-gyri and proto-sulci in the developing cortex, and

such expression patterns (also called protomaps of gene expression) are proposed to reflect the presence of early region-specific genetic programs guiding cortical folding and arealization in a stereotypical manner.^{13,64,88,89} It remains to be investigated whether the development of tertiary or higher-order gyri—characteristic of highly folded cortices such as the human cortex and evidently variable across individuals—follows similar principles than the formation of primary gyri, which are present in ferrets, the animal model used in most experimental investigations into gyrification mechanisms so far.

Neurogenesis from bRGCs, which explains part of the broad mechanism of differential tangential expansion, does not appear to account for the degree of gyrification seen *in vivo* entirely.⁶³ Aside from proliferation, bRGCs have also been found to contribute to cortical folding through the RG fiber scaffold they provide to nascent neurons, which guides and defines their radial migration and final positioning in the CP. Once bRGCs are initially born from aRGCs by delamination from the ventricular surface (roughly at embryonic day 34 in ferrets and gestational week 12 in humans), their basal RG fibers begin to intercalate with those from pre-existing aRGCs.^{39–41} The progressive accumulation of basal processes adds complexity to the fiber arrangement, causing fanning outward of the RG scaffold (Figure 2A). This is more prominent in regions with increased bRGC abundance, which correlates with prospective gyri.^{39,40} These data suggest that bRGCs contribute to differential tangential expansion of the CP not only by increasing the neurogenic potential but also by promoting tangential dispersion of neurons in the developing cortex by increasing the complexity of the RG fiber network. Additionally, aRGCs appear to transform their basal processes at gestational week 16.5 in humans (and during postnatal development in ferrets) to a shorter fiber that becomes truncated at the border with the oSVZ, referred to as “truncated” RGCs, with these truncated progenitors potentially producing ependymal and astroglial cells.^{35,42} These truncated RGCs define a RG scaffold in humans that becomes discontinuous or interrupted between the iSVZ and oSVZ (with the short process of truncated aRGCs spanning the depth of the VZ and iSVZ and the long process of bRGCs extending from the oSVZ up to the CP) in the second trimester of gestation, posing intriguing questions about the development and cell fate of these progenitors and how this phenomenon may shape cortical architecture.

In addition to these RGC-driven mechanisms, there is evidence that mechanisms intrinsic to migrating neurons can also influence cortical folding. Migrating neurons associate with numerous fibers of the RG scaffold promiscuously to take more tortuous and divergent paths (by branching their leading process) in developing gyrencephalic cortices than they do in developing lissencephalic cortices, where clonal analyses of migrating neurons show a more limited conical distribution.^{40,52,53,90,91} Accordingly, the expression of FLRT1 and FLRT3—genes encoding cell adhesion molecules—by migrating neurons in mice appears to restrict their tangential dispersion, and their deletion leads to the emergence of cortical sulci; by contrast, the downregulated expression of FLRT1/3 found in ferrets and humans was suggested to be a regulatory mechanism potentially underlying the distinct migration patterns observed in the gyrencephalic neocortex.^{92,93} Thus, the architecture of the RG fiber scaffold, together with cell-autonomous mechanisms of neuronal migration, critically influences

cortical folding by impacting the tangential dispersion of migrating neurons.

MOLECULAR MEDIATORS REGULATING RADIAL GLIAL CELL BEHAVIOR

Experimental studies in both mice and ferrets have provided direct evidence of the role of RGC behavior, and especially of bRGCs, in cortical folding and identified molecular signaling pathways involved in the genetic regulation of RGC function. Several ectopically expressed factors in mouse cortex can induce pseudogyration (folding of the entire developing cortical thickness, including ventricular surface, usually due to increased size), but not convincing true ectopic gyration (cortical folding without disruption of the apical membrane shape, usually due to increased tangential dispersion). Thus, parallel experiments in ferrets investigating those same factors have now examined them in an animal model—the ferret—with the cellular substrate to modulate true cortical gyration. For example, local overexpression of fibroblast growth factor 2 (FGF2) or FGF8 via *in utero* electroporation in mice is sufficient to cause local growth reminiscent of gyration, attributed to increased self-renewal of aRGCs, the inverse of the phenotype described in an *Fgf2* knockout (KO) mouse model.^{94,95} A similar experiment of FGF8 misexpression in ferrets caused frank polymicrogyria (a cortical malformation characterized by many small gyri creating excessive folding), and histological examination revealed an increased density of bRGCs not seen in the mouse experiments that is potentially driving the change in gyration.⁹⁶ In a similar pattern, modulating cell cycle of RGCs through overexpression of the cell cycle proteins *Cdk4/CyclinD1* via *in utero* electroporation in mice promoted increased *Tbr2*+ IPCs in the SVZ, which increases cortical surface area in mice but did not distinctly lead to local gyri-reminiscent growth.⁹⁷ Remarkably, the same manipulation in ferrets generates new cortical folds with normal laminar organization.⁸² These differences in phenotypes between mouse and ferret models may be explained by the distinct cytoarchitecture of the gyrencephalic developing cortex, characterized by expanded basal progenitors in the SVZ, that might be necessary for such phenotype. Alternatively, the underlying molecular programs involved in this phenotype in ferrets may not be shared by the mouse developing cortex.^{82,97} A unique case of a single gene perturbation in mice causing a phenotype of true cortical gyration is the loss of *Trnp1* in the embryonic mouse cortex, loss of which led to an expansion of the small population of bRGC-like progenitors found in mice, thus causing an expanded oSVZ, increased fanning of the local RG scaffolding, and formation of cortical folds.⁸⁵ Importantly, the cortical folding induced in these mice emerged from radial growth of the germinal zone followed by the tangential expansion of neurons in the CP, recapitulating the endogenous process of gyrification described in the gyrencephalic cortex.⁸⁵

Beyond these examples of single gene perturbations in both murine and gyrencephalic models, numerous other genetic factors have been shown to modulate RGC behavior through studies using *in utero* electroporation, KO models, or drug treatments (excellently reviewed by Del-Valle-Anton and Borrell⁶⁶). These factors, including *IGF1*,⁹⁸ *INSM1*,⁹⁹ *NT3*,⁹⁵ lysophosphatidic acid,¹⁰⁰ or modulation of beta-catenin signaling,⁶⁹ modulate

RGC proliferation, and can cause cortical lamination or even cortical shape changes, although none of these induce true gyration in their murine models. Thus far, it seems that modulating genetic factors in lissencephalic animal models lacking the key cellular components of the developing cortex required for folding (that is, an abundant population of bRGCs) may not be sufficient to induce true cortical gyration. Likely, given the evolutionary history of mice and the emergence of pseudogyration in most experiments described above, it seems most plausible that mice have shed or suppressed much of the cellular substrate needed to form folds during corticogenesis and, as a result, developed a lissencephalic cortex. Thus, future experiments in gyrencephalic models such as ferret may be able to examine gyrification mechanisms specifically in a developing cortex with a more endogenous substrate required for cortical folding.

Molecular cues from the extracellular environment have also been found to play a crucial role in cortical progenitor behavior, hence influencing cortical shape. In this context, the constituents of the extracellular matrix (ECM) are essential for cellular and tissue organization and dynamics in the human developing cortex, and in particular the proliferation of cortical progenitors, which has been thoroughly reviewed elsewhere.^{101,102} These functions of ECM proteins have been shown to be mediated both by modulating the activity of major signaling pathways (e.g., FGF signaling and Hedgehog signaling) and by direct signaling via ECM receptors (e.g., laminin binding to integrins). A seminal study identified three ECM components (HAPLN1, laminin, and collagen I) involved in regulating the morphogenesis of the human developing cortex: treatment of 11–16 gestation week fetal neocortical slices with these ECM proteins as soluble factors induced folding within 24 h of culture.¹⁰³ Importantly, signaling mediated by integrins is required to promote the proliferation of both types of basal progenitors (bRGCs and bIPCs) in ferrets and mice.^{45,104–106} Beyond ECM-related molecular signaling, other cell-extrinsic factors, such as retinoic acid, have been shown to regulate proliferation of RGCs.¹⁰⁷ Taken together, extracellular signals are essential in modulating the behavior of cortical progenitors and, therefore, act as determinant factors in the emergence of shape in the developing cerebral cortex.

SHH SIGNALING AND CORTICAL GYRATION

Recent findings indicate that Shh signaling is a key regulator of cortical gyration through its function in bRGCs, further extending the broad spectrum of Shh roles in nervous system development (such as its well-described role in the specification of the dorsal-ventral axis of the neural tube and spinal cord as well as cortical patterning). Shh is the best studied of the three Hedgehog signaling ligands, and its expression is expanded in its breadth and concentration in the developing cortices of gyrencephalic animals relative to lissencephalic ones.^{31,108} Shh acts as a mitogen in the developing cortex and promotes the proliferation of RGCs, stimulating their amplification over neurogenic divisions.^{109–111} Although Shh might act as a mitogen in aRGCs in some capacity early in neurogenesis, its effects are more apparent on the proliferative capacity and stemness of bRGCs and IPCs in the SVZ and on the generation of bRGCs from aRGCs.^{43,48,112} Shh function in the SVZ promotes the capacity of bRGCs and IPCs to self-renew through symmetric divisions

rather than terminally differentiate, thereby expanding bRGC and IPC populations.³¹ The role of Shh as a mitogen can be illustrated further by case examples that examined the effects of titrating Shh signaling activity during cortical development: (1) increased Shh signaling by constitutively activating the pathway in RGCs caused macrocephaly and even some cortical folding (pseudogyration) in mice⁸⁷; (2) decreased Shh due to defective Shh secretion into the cerebrospinal fluid led to a smaller neocortex and cerebellar hypoplasia due to decreased RGC proliferation in *CHMP1A*-null mice¹¹³; and (3) prenatal treatment of ferrets with Shh signaling modulators in the critical window for bRGC production fine-tuned the density and dynamics of bRGCs: an Shh inhibitor led to decreased bRGC abundance, and an Shh agonist resulted in augmented bRGC pool size.¹¹² This experiment did not induce noticeable effects on the aRGC population, suggesting a specific role for Shh signaling activity in basal progenitors.¹¹²

Recent work has described greater radial glial diversity in gyrencephalic mammals (ferret, macaque, and human) relative to lissencephalic mice.^{40,43,60} The specific sensitivity to Shh stimulation by a subclass of bRGCs expressing *HOPX* (encoding a homeodomain-containing protein) has been recently reported. The *HOPX*+ bRGC subclass has greatly expanded in gyrencephalic mammals and accumulates in proto-gyri in higher numbers compared with the *HOPX*– bRGC subclass³¹ (Figure 2A). Importantly, Shh activity specifically suppressed differentiation of *HOPX*+ bRGCs, thus increasing their neurogenic potential through progenitor self-renewal.³¹ Local suppression of Shh signaling in ferrets using a competitive inhibitor of Shh (*HhipΔC22*) led to decreased *HOPX*+ bRGCs and locally decreased cortical folding, demonstrating a necessary and sufficient relationship between Shh signaling, local gyrification, and the sustained local proliferation of bRGCs³¹ (Figure 2B). Overall, in agreement with cortical folding theory described above, these studies show the potential of *HOPX*+ bRGCs to promote local differential tangential expansion through an Shh-mediated mechanism inducing elevated proliferation in proto-gyri. These results extend pioneer studies showing that ectopic constitutive Shh stimulation in the VZ in mice was sufficient to form cortical folds in the otherwise lissencephalic brain.⁸⁷ Altogether, the function of Shh signaling pathway in *HOPX*+ bRGCs emerges as a central molecular mediator of cortical folding.

Evidence for the key role of Shh signaling in cortical folding also comes from described cortical malformation syndromes in which cortical gyration is abnormal. Although mutations in *SHH* have been traditionally associated with forebrain patterning malformations such as holoprosencephaly (a disorder in which the embryonic forebrain fails to form two distinct hemispheres), they have also been associated with microcephaly, polymicrogyria (characterized by an aberrant number of small folds), and schizencephaly (characterized by abnormal slits or clefts), as part of the phenotypic spectrum associated with dysfunctional Shh signaling.^{114–116} Mutations in genes associated with the Shh signaling pathway, such as receptors *PTCH1* and *CDON*, have also been associated with cortical malformations.^{116–118}

In addition, mutations in genes involved in modulating Shh signaling have also been linked to non-holoprosencephaly cortical malformations, providing further support to the notion that disrupted Shh signaling can lead to defects in cortical

folding. These include the microcephaly/cerebellar hypoplasia associated with recessive mutations in *CHMP1A*,^{113,119} and the recently described polymicrogyria syndrome caused by bi-allelic mutations in *TMEM161B*.^{120,121} Although disruption of *TMEM161B* through bi-allelic missense mutations in humans causes cortical gyration abnormalities, loss of *Tmem161b* function in mice causes several Shh-related malformations such as holoprosencephaly, eye defects, and craniofacial abnormalities, as well as morphologically abnormal primary cilia in the VZ of the developing cortex.¹²⁰ During cortical development, *TMEM161B* is expressed preferentially in HOPX+ bRGCs relative to other bRGC populations. Disrupting *Tmem161b* function in cortical progenitors in ferrets led to decreased gyral size and sulci depth, confirming its role in promoting normal cortical gyration.¹²⁰ Furthermore, *Tmem161b* also plays a role in the integrity of the RG fiber scaffold, which, as described above, is different locally between proto-gyri and proto-sulci.¹²¹ Overall, these findings, especially in context of the ferret studies examining Shh stimulation of HOPX+ bRGCs described above, suggest a decreased sensitivity to Shh signaling in a developing gyrencephalic cortex deficient in *Tmem161b*, which provides important insights into the pathogenic mechanisms underlying the polymicrogyria syndrome described in patients.

More generally than the example of polymicrogyria associated with *TMEM161B* mutation, cortical malformations associated with dysfunction in the primary cilium (the key cellular organelle necessary for downstream transcriptional activation induced by Shh ligand) frequently include microcephaly and polymicrogyria as part of their phenotypic spectra.^{122,123} Although primary cilia also are involved in numerous other signaling processes, disruption of normal Shh signaling due to abnormal primary cilia may be in part explanatory for the cortical folding malformations seen in primary ciliopathies such as Joubert or Meckel-Gruber syndrome.^{122,123}

EVOLUTIONARY EXPANSION OF RADIAL GLIAL CELLS

The evolution of neocortical circuits is thought to have enabled remarkable computational abilities required for human higher cognitive function.^{124,125} These evolutionary changes include the expansion of the neocortical cytoarchitecture (i.e., the cellular composition and distribution), and such enlargement is especially prominent in superficial layers 2 and 3 (also known as supragranular layers given their location above the granular layer 4)¹²⁶ (Figure 3A). Indeed, comparative transcriptomic analyses recently showed an extraordinary diversification of projection neuronal types within superficial layers of the human neocortex³ (Figure 3A). The enlargement and diversification of superficial layer neurons may, therefore, provide a biological substrate for increased intracortical, associative connectivity that is essential in higher cognitive abilities, given that superficial layer neurons form cortico-cortical synaptic connections. As discussed above, numerous studies have documented that bRGCs are more numerous and diverse in gyrencephalic species as compared with lissencephalic species, and in particular, the oSVZ is substantially expanded in the primate developing cortex.^{39,40,43–46} Therefore, it is thought that the expansion of basal progenitors might underlie changes in neocortical cytoarchitecture that occurred over the course of evolution. The

last decade has seen a quest for human-specific genomic changes that shaped the development of the cerebral cortex by controlling the behavior of cortical progenitors, thus elucidating molecular mechanisms that played an important role in human cortical evolution.

Gene duplication is a major evolutionary driving force. Many recent studies have documented the essential function that the emergence of novel genes during evolution played in the specialized behavior of human basal progenitors (thoroughly reviewed in Pinson and Huttner⁵⁷ and Mora-Bermúdez and Huttner¹²⁷) (Figure 3B). *ARGHAP11B*, a gene that arose on the human lineage after the divergence from the chimpanzee lineage by partial duplication of the Rho guanosine triphosphatase-activating protein *ARRGAP11A* and subsequently underwent a splice-site mutation, increases basal progenitor proliferation, as revealed by overexpression experiments in mice, ferrets, and marmosets.^{83,86,128} *CROCCP2*, a gene duplicated in the hominini lineage (chimpanzees and humans) that is highly expressed during human corticogenesis, enhances the generation of basal progenitors.¹²⁹ Three human-specific paralogs derived from duplication of *NOTCH2* (*NOTCH2NLA*, *NOTCH2NLB*, and *NOTCH2NLC*) play critical roles in expanding the pool of cortical progenitors.^{130,131} The overexpression of *TBC1D3* (a great ape-specific gene) and of *TMEM14B* (a primate-specific gene) in mice promote the expansion of basal progenitors.^{132–134} As a result of the effects in basal progenitors, an increasing body of research in ferret models has revealed roles for many of these human-evolved genes in promoting both cortical neuron production and cortical folding (Figure 3).

Changes in gene regulation constitute another major force driving cortical evolution. A broad range of regulatory mechanisms have been found to be impacted not only by species-specific genomic changes during evolution but also by genetic variants associated with neurodevelopmental disorders. These range from mutations in enhancers and promoters to changes in alternative splicing,^{135–139} from the emergence of novel microRNAs and to that of novel long non-coding RNAs.^{140–142} Gene expression changes in the developing cortex can occur at different spatiotemporal scales, including cell types, cortical areas, and developmental windows.^{60,89,143–146} Such fine-tuned expression patterns likely reflect a positive selection necessary for precise functional organization of the cerebral cortex. Importantly, experimental manipulation in mice has shown a direct link between gene regulation and cortical expansion: the human accelerated region HARE5 upregulates the expression of FZD8, a receptor of the Wnt signaling pathway, and promotes accelerated cell cycle of cortical progenitors, resulting in increased cortical size.¹³⁸ Despite the increasing number of evolutionarily dynamic non-coding regions linked to cortical development, the functional evaluation of these loci in animal models remains limited, and therefore, their impact in regulating cortical shape and folding is still poorly understood.

Notably, several recent studies have revealed specialized molecular and cellular features of human cortical progenitors (Figure 3C). These include elevated expression of ECM proteins,^{45,103,147} responsiveness to extracellular signaling cues through innovations in receptor expression,^{148–150} molecular pathways controlling fatty acid synthesis,⁸⁴ specialized mitochondrial dynamics and metabolism,^{151,152} and ciliary dynamics

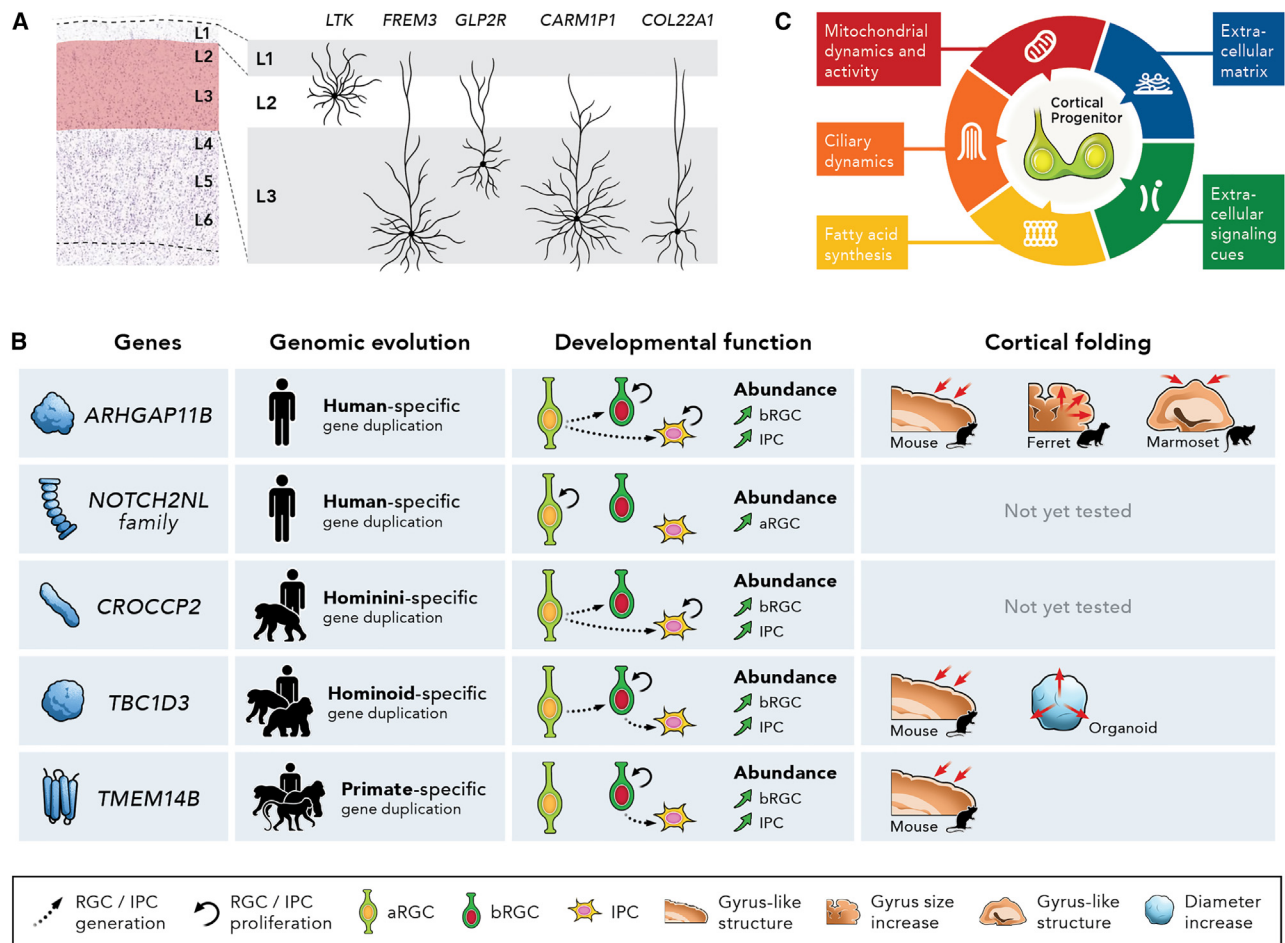


Figure 3. Evolutionary expansion of the human neocortex and cortical progenitors

(A) A hallmark of human neocortical evolution is the expansion of superficial layer neurons. Left: image of a coronal section of the human neocortex illustrating the relative thickness of cortical layers (L1–L6). Red shade highlights the expansion of superficial layers, especially L2–L3, in the human neocortex. Image adapted from the Allen Institute for Brain Science. Image credit: Allen Institute for Brain Science: <https://human.brain-map.org/ish/specimen/show/79946224> (specifically, Nissl-stained brain section from the right-hand side panel: image 7 of 8). Right: drawing illustrating the expanded neuronal diversity found in superficial layers of the human neocortex (as described in Berg et al.³), and labels indicate the marker genes of specific types of excitatory pyramidal cells.

(B) Gene duplications in evolution that regulate the behavior of cortical progenitors. *ARHGAP11B* arose 5 million years ago by partial duplication of *ARHGAP11A*. Three human-specific paralogs (*NOTCH2NLA*, *NOTCH2NLB*, and *NOTCH2NLC*) derived from duplication of *NOTCH2*. *CROCCP2* arose through partial duplication of exons 13–21 of *CROCC* in the Hominini lineage (chimpanzees and humans). *TBC1D3* arose in the Hominoid lineage (great apes) from a segmental duplication, with multiple copies found in the human genome and only a single copy found in the chimpanzee genome. *TMEM14B* arose in the primate lineage, including Old World and New World monkeys and apes. In parallel, experimental manipulations in mice, ferrets, and marmosets, as well as cerebral cortical organoids, have revealed a role for these genes in inducing cortical folding induction and/or cortical size expansion. Symbols represent the animal models in which cortical folding appeared as a result of overexpression experiments.

(C) Distinctive features of cortical progenitors during human brain development. The schema compiles 5 cellular features reviewed in this work: extracellular matrix components, responsiveness to extracellular signaling cues, regulation of fatty acid synthesis, mitochondrial dynamics and metabolism, and ciliary dynamics and trafficking.

and trafficking.¹²⁹ These mechanisms have been shown to modulate a variety of developmental processes, including not only the behavior of cortical progenitors but also the cell fate during neurogenesis as well as the protracted timing of neuronal maturation. Therefore, it is foreseeable that research over the next years will continue to decode evolutionary genetic mechanisms underlying these developmental processes, thus shedding new light into the organization of the human cerebral cortex. The recent development of novel comparative genomic strategies will provide new and exciting opportunities to approach these questions.^{153,154}

CONCLUSIONS AND PERSPECTIVES

In the past decade, a wave of studies have revealed genetic mechanisms that—as discussed in this review—regulate the highly proliferative capacity of basal RGCs and their effect on differential tangential expansion of the developing cortex, thereby establishing a direct link between the behavior of cortical progenitors to the emergence of cerebral cortical folding. Notwithstanding their significance, the study of how the human cerebral cortex acquires its shape during development appears to be at its beginning rather than its end. Advances in comparative genomics

have identified a rapidly increasing number of candidate human-specific regulators of cortical development, both in coding and non-coding regions of the genome.^{131,139,155} Neuroimaging approaches coupled with genome-wide analyses have uncovered many genomic loci significantly associated with various neuroimaging phenotypes related to morphological variables of sulci and gyri.¹⁵⁶ Single-cell transcriptomic analyses have found striking differences in gene expression across cortical regions and developmental stages, revealing molecular and cellular patterns for cortical arealization.^{89,143–145} Furthermore, it is striking to note the many variations in cortical gyrification patterns across mammalian species,^{157–159} the evolutionary specializations of defined cortical areas (especially within the frontal lobe),^{160–163} as well as the effects of environmental perturbations in cortical cytoarchitecture.^{40,164} Altogether, these data suggest that only a minor fraction of genetic programs involved in shaping the structural and functional organization of the neocortex have been functionally dissected so far, as recently discussed in the context of the evolution of cortical connectivity,¹²⁵ and many questions remain to be addressed. How do region-specific programs give rise to distinct functional cortical areas? What temporal windows define the specifications of these areas? Which evolutionary mechanisms determine species-specific variation in cortical shape and organization? We think that the next years will see intense research on the mechanisms of the emergence of structural and functional organization in the neocortex and that these developmental insights will have deep implications for our understanding of human cognitive functions.

Recent work is beginning to shed new light on additional cellular processes involved in shaping neocortical architecture. In later stages of development, cortical progenitors acquire new roles and shift their neurogenic fate into a gliogenic one, becoming committed to generate astrocytes as well as oligodendrocytes.³⁴ A recent study found an important role for localized astrogenesis in gyri formation, and this process is regulated by FGF signaling.¹⁶⁵ This is consistent with earlier observations in the macaque developing cortex that suggested a contribution of gliogenesis in folding of the cortical surface.¹⁶⁶ Since glial cells represent extraordinarily voluminous populations in the neocortex,^{7,8} further understanding of the role of gliogenesis in cortical folding will complement the current knowledge on neurogenesis-driven mechanisms and lead to more comprehensive models on the emergence of shape during cortical development.

Finally, technological and bioinformatic advances, together with increased sample sizes, have spurred the identification of genetic variants associated with cortical malformations, implicating rapidly increasing numbers of genetic factors in recent years.¹⁶⁷ This emphasizes the need to functionally interrogate novel genetic variants to continue dissecting the pathogenesis of these neurodevelopmental disorders. These studies will also be critical in expanding our understanding of molecular and cellular mechanisms involved in the development of cortical shape.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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