Deconstructing cortical folding: genetic, cellular and mechanical determinants

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Abstract | Folding of the cerebral cortex is a fundamental milestone of mammalian brain evolution and is associated with dramatic increases in size and complexity. New animal models, genetic tools and bioengineering materials have moved the study of cortical folding from simple phenomenological observation to sophisticated experimental testing. Here, we provide an overview of how genetics, cell biology and biomechanics shape this complex and multifaceted process and affect each other. We discuss the evolution of cortical folding and the genomic changes in the primate lineage that seem to be responsible for the advent of larger brains and cortical folding. Emerging technologies now provide unprecedented tools to analyse and manipulate cortical folding, with the promise of elucidating the mechanisms underlying the stereotyped folding of the cerebral cortex in its full complexity.

Gyri
Also known as 'folds' or 'convolutions'. Rounded elevations of cortical tissue between two sulci that contain all six neuronal layers bending outwards, such that the deep layers on either side of a gyrus come close together.

Sulci
Also known as 'fissures'. Depressions or grooves of cortical tissue that contain all six neuronal layers bending inwards, such that the superficial cortical layers come close together on either side of a sulcus.

Gyrencephalic
The characteristic of a brain presenting cortical folds, giving a convoluted or wrinkled appearance.

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One of the most prominent and characteristic features of the human brain is the folding of the cerebral cortex into gyri and sulci. However, we still have a very limited understanding of how this emerges during development. Unfortunately, the tangibility of this phenomenon has in the past prompted many seemingly intuitive misconceptions regarding its origins. Intense research over the past decade has exponentially increased our understanding of cortical folding, identifying some of the key genetic, cellular and mechanical mechanisms involved. Until now, cortical folding has been viewed and approached as a phenomenon that is either purely mechanical (that is, resulting from physical forces) or purely biological (resulting from cellular or molecular factors) (see REFS[1–5] for recent reviews). However, it is becoming evident that, although important for cortical folding, neither of these two aspects is in itself sufficient. Relevant cellular and molecular mechanisms occur days to weeks before actual tissue folding[6], and the effect of mechanical factors depends on specific initial conditions that must be set in advance by early developmental processes[7]. Hence, the initial simplistic view is rapidly changing towards an integrative one in which cortical folding depends on genetics and cell biology to set the stage for tissue mechanics, as supported by recent experimental evidence[8].

In this rapidly evolving scenario, we present here an updated, comprehensive and integrative review of determinants of cortical folding. Following a description of the anatomical features of cortical folding, we review models and evidence concerning the mechanics of this process and the cell biological processes underlying stereotyped cortical shaping. We then focus on the genetics and transcriptomics that define and regulate these cellular and mechanical factors and review the current understanding of the evolution of cortical folding. Two main themes of our Review are the importance of mutual interactions between mechanical and cellular or genetic factors, and the importance of the regulation of gene expression in the development, evolution and disease of cortical folding.

Anatomical features

The folding of the cerebral cortex of gyrencephalic species is characterized by the alternation of folds (outward bending) and fissures (inward bending) of the cortical mantle. In the mature brain, fissures display remarkable periodicity across the cortex, with highly regular spacing between them, measured as folding wavelength. Cortical folding wavelength across species correlates with thickness of the grey matter: brains with thicker neuronal layers have wider folds than those with thinner grey matter[9–11]. This correlation emerges from the necessity of folds to contain the full complement of all six neuronal layers plus their descending axons, which varies considerably between species. For example, cetaceans have remarkably thinner grey matter and a much smaller folding wavelength than other clades, translating to a very high gyrification index (GI) (for example, 5.55 in the Pacific pilot whale compared with 2.56 in humans)[9].

In highly folded brains, including those of humans, cortical fissures are hierarchically organized into primary fissures, which are the deepest and first to form during development; then secondary fissures, which form by subdivision of the former; then tertiary fissures; and so on. The sulcal pits of primary fissures

occur at highly stereotyped locations between individuals[12]. In animals in which folding involves only the formation of primary fissures, their pattern is similarly well conserved[10]. Different mechanisms seem to underlie the formation of each type of fissure, as presented in the following sections.

The thickness of the neuronal layers varies considerably along folds, being thickest at the gyral crown and thinnest at the sulcal fundus, whereas the thickness along the lateral walls remains relatively constant[10,13] (FIG. 1a). The combination of these variations in shape and thickness of the neuronal layers along cortical folds corresponds with variations in the morphology and the arrangement of their constituent elements. In gyral crowns, the layers and columns of neurons are more sharply defined, cell bodies are less densely packed and myelinated fibres are denser and more vertically oriented than in fissures, where the latter mostly run horizontally[13,14]. Pyramidal neurons in crowns have longer and more elaborately branched apical and basal dendrites than do pyramidal neurons in sulcal fundi. Moreover, in the gyral crowns, these neurons are more vertically oriented and show an elongated cell soma and basal dendrites in vertical or oblique orientations[15]. By contrast, the morphology of pyramidal neurons at sulcal fundi largely follows the tangential axis, with a flattened cell soma, short apical dendrites with long collateral branches, and tangentially extended basal dendrites[15] (FIG. 1a). Primary descending axons follow similar overall patterns of orientation, with predominantly radial orientation in folds and tangential orientation in fissures. In lateral walls, most of these features are intermediate between those of the gyri and sulci. As an exception, the most superficial and cell-sparse layer 1 is thicker in fissures than in folds[13], and this difference is proposed to be the consequence of the high density of apical dendritic tufts of pyramidal neurons in fissures[10]. The meningeal membranes, which are associated with a protein-rich extracellular matrix (ECM) on the surface of the brain, faithfully follow the basal border of layer 1 at the cortical surface, fully overlaying all cortical folds and fissures.

In contrast to the remarkable folding of the six layers of cortical grey matter, the outer surface area of which dramatically expands during development, the cortex inner surface, which, in the adult brain, is composed of white matter and ependymal cells, is completely smooth[1]. The smoothness of this inner cortical surface derives from the equally smooth embryonic ventricular zone (VZ), the primary germinal layer.

These two distinct features of cortical folding under physiological conditions — outer folding and inner smoothness — are reproduced in some experimental models but not all (reviewed elsewhere[1]). Bona fide cortical folding occurs, by definition, in naturally gyrated model species such as ferrets, cats, sheep, macaques and obviously humans[13,16–18]. Several experimental models also faithfully recapitulate these features: in vivo in transgenic mice[19–21] and through transient manipulation of the developing cortex in ferrets and mice[22–29], and recently in vitro, through manipulation of early human brain slices[8]. By contrast, other models fail to reproduce these features of true folding, including transgenic mice in vivo[30–33] and in vitro cultures of mouse cortex or human cerebral organoids[34–36].

Several pathological conditions alter normal folding, and most of these largely affect the number and periodicity of folds[37]. Such malformations usually result from abnormal cortical development and include severe alterations of the above-mentioned architectonic features, such as the layering, density and orientation of cortical neurons, as well as the formation of cellular ectopias (BOX 1).

Mechanical factors

Cortical folding is a long process that takes place in the very last period of forebrain development, coincident with the cytoarchitectural differentiation of cortical areas and synapse formation[2,6,10,38,39]. Several hypotheses have been proposed over the past decades to explain the process of cerebral cortical folding. Early ideas proposed that cortical folding could be the passive result of external mechanical forces acting on the expanding and developing brain, including hydraulic pressure from the cerebrospinal fluid, the impression from major blood vessels or growth constraints from the limited volume of the cranium[10]. These ideas were soon abandoned for important reasons. For example, cranial sutures do not ossify until the brain has finished growing, and experimental evidence in cats demonstrates that alleviation of cranial pressure does not reduce cortical folding[10]; in fact, abnormally large brains can force the expansion of the surrounding cranium[30]. In 1997, Van Essen published one of the most attractive and influential hypotheses on the biomechanics of cortical folding, in which he proposed that internal hydraulic pressure and patterned axonal tension between specific cortical areas were the driving forces to initiate and shape cortical folding[40] (FIG. 1b). This notion was consistent with the fact that axons in the living brain exist and grow in a state of tension[41–43] and was consistent with axonal tracing evidence in the macaque[44]. However, subsequent direct experimental measurements very elegantly and definitively refuted the idea by showing that, contrary to the basis of the Van Essen hypothesis, the tensile forces from cortical axons do not act along the orientation that would be required to bring the walls of developing gyri together[42].

An alternative hypothesis proposed that cortical folding is the result of forces originating from the differential growth in the developing cerebral cortex, in which an outer shell of tissue undergoes faster tangential expansion than an inner core[45] (FIG. 1c). Subsequent theoretical modelling with more sophisticated mathematical methods, and recent experimental testing, have provided further support to this idea[7,42,46–49]. For example, circular slices of gel, in which an outermost layer expands by swelling, have been used as a physical model of differential growth, showing that this outer layer folds with wavelength depending on its thickness and elastic modulus[46]. In a series of remarkable experiments, these experimental studies of differential growth were extended to three dimensions by combining two polymer gel preparations: one forming an inner core and

Gyral crown
Also known as the 'gyral crest'. The top or outermost part of a gyrus.

Sulcal fundus
Also known as the 'sulcal pit'. The bottom or deepest part of a sulcus.

Lateral walls
Portions of cortex between gyral crowns and sulcal fundi.

Cellular ectopias
Also known as 'cellular heterotopias'. Cells positioned in an abnormal location.

Hydraulic pressure
Force exerted by a fluid onto the surrounding tissue that contains it under pressure.

Cranial sutures
Fibrous joints between the cranial bones.

Modulus
Measure of a mechanical property of a material. The elastic modulus is the measure of the resistance of an object to being deformed elastically after stress is applied.

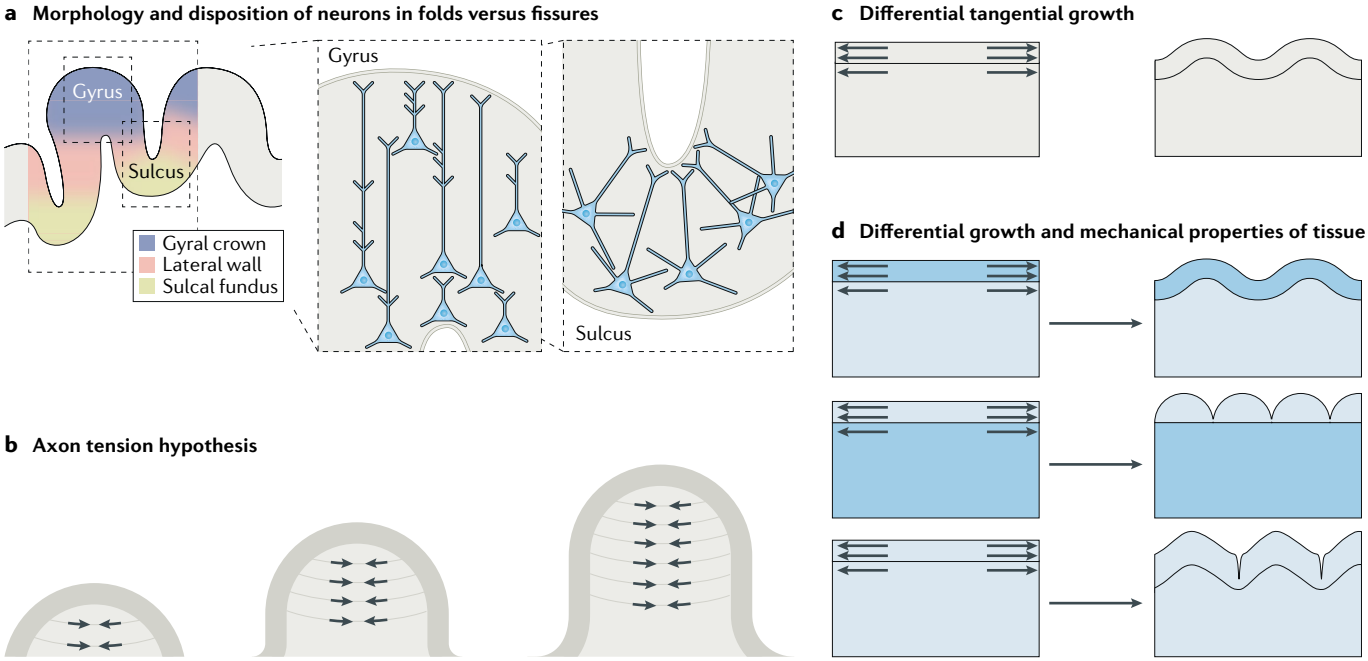


Fig. 1 | **Anatomy and mechanics of cortical folding: push, not pull. a** | Key cortical folding terms are illustrated, along with a schematic of the general morphology and orientation of cortical neurons within gyri and sulci. Cells have a different orientation and a more extensive horizontal branching of apical and basal dendrites in sulci compared with gyri. **b–d** | Biomechanical hypotheses of cortical folding. According to the axon tension hypothesis (part **b**), axons connecting cortical areas are under tension, and the more connected these regions are (grey lines), the greater the tension (arrows) and the closer the regions become, resulting in increased folding. According to the differential tangential growth hypothesis (part **c**), the cerebral cortex is divided into two regions, with the outer layer growing faster than the inner core. This differential growth is predicted to result in the folding of the outer layer. Another hypothesis of cortical folding is based on the notion that, on top of differential growth, differences in the mechanical properties of the tissue (such as stiffness and elasticity) define its capacity to fold more or less (part **d**). Darker blue indicates the stiffer material; lighter blue indicates the softer material. The cerebral cortex is intrinsically connected with subcortical structures that have different mechanical properties. Variations in the difference of stiffness between superficial and deep regions influence the degree and mode of folding. Wrinkling occurs in a smooth, sinusoidal way. Evagination of the grey matter leads to the formation of cusped folds. If the grey and white matter have similar properties, both will fold. Part **a** is adapted from REF.[10], Springer Nature Limited. Part **d** is adapted with permission from REF.[49], Proceedings of the National Academy of Sciences (PNAS).

another forming an outer layer, bound to the former. Upon exposure to a solvent, the outer shell polymer expanded faster than the inner core. When the stiffness of the outer material was similar to that of the inner material, it wrinkled similarly to a folded cortex[49] (FIG. 1d). When the physical size, shape and material properties (that is, stiffness) of this compound gel structure were all made similar to those of the smooth fetal human brain before folding, the fast-expanding outer shell formed folds with remarkable similarity to those in the human cortex, in both wavelength and pattern[7]. Indeed, it is generally held that the stiffnesses of grey and white matter in the human brain are not as different as initially postulated[45]. Importantly, gel models and in silico 3D simulations demonstrate that certain initial conditions are crucial for determining the eventual pattern and wavelength of cortical folds, whereby even subtle differences in these parameters lead to dramatically different outcomes. These parameters include the initial size and precise geometry of the expanding brain, the thickness of the outer zone and factors that affect the rate of tangential expansion of the outer zone relative to the inner zone (for example, differences in solvent absorption)[7,48–50].

What are the actual biological substrates involved in the differential expansion models of the mechanical forces driving cortical folding? Current hypotheses propose that the neuron-dense cortical plate (CP), or grey matter, may act as the rapidly expanding outer shell, whereas the underlying, transient embryonic layers, including the fibrous intermediate zone and the germinal zones, which eventually become the white matter, may act as the slowly expanding inner zone[2]. Differences in stiffness and expansion rate between these two general compartments seem compatible with these factors being driving forces for cortical folding, but developmental changes in these and other variables that may also have a critical impact on the process remain to be evaluated[2]. Folding has also been observed in the neuroepithelium of various cerebral organoid preparations[35,36], and biomechanical measurements in these systems support the differential expansion model[36]. Notably, as mentioned above, the degree of cortical folding varies considerably between mammalian orders, and this variability may result from variations in the thickness of grey matter, which will change the mechanical resistance of the cortex to folding[51]. Likewise, substantial variations in white-matter

Stiffness
Property of a material that defines its resistance to being deformed after force is applied to it.

Cortical plate
(CP). Transient layer of the developing cortex, located beneath the marginal zone and containing the neurons that most recently finished radial migration.

Viscoelastic instability
Property of nearly inertia-less, non-Newtonian, flowing, complex fluids, such as polymer melts and solutions.

Anisotropy
The characteristic of materials of having different physical or mechanical properties when measured along different axes.

volume or thickness (for example, as observed between elephants and manatees[52]) may also exert effects similar to variations in grey-matter thickness on the mechanics and degree of cortical folding.

The differential expansion hypothesis depends on the rapid tangential growth of the CP. This fast tangential growth of the CP was recently proposed to emerge from the differentiation of its constituent neurons, the growth of their cell bodies and surrounding neuropil, the elaboration of dendritic and axonal arborizations, and the formation of synapses[53]. This notion is well supported by the temporal correlation between the developmental trajectories of cortical maturation and folding in ferrets, macaques and humans[2,53–55]. Accordingly, variations in the initial packing of neurons in the CP may have a fundamental impact on the cortical folding phenotype.

Remarkable as they are, these differential expansion models fail to replicate two important features of cortical folding: the stereotyped location of primary folds and fissures across individuals, and the growth of the subcortical white matter. At a microscopic level, axons elongate in response to, and maintain a small level of, tension[43,56–58]; similarly, at the macroscopic level, white matter probably responds to stress by growing in the direction of axonal fibres — consistent with experimental observations[41,42] — and thus may affect macroscopic folding. Taking this growth into consideration, the (inner) subcortical tissue can be modelled as a viscoelastic material that displays a distinct viscoelastic instability (buckling on a viscoelastic foundation) in response to growth of the CP. That is, mechanical stress in the (inner) subcortical tissue induced by relatively rapid CP growth will relax with a characteristic time constant, depending on the ratio of viscous resistance to elastic stiffness. This instability, and the wavelength of the resulting surface, depend on the rate of cortical growth relative to the rate of mechanical relaxation of the subcortical tissue[4]. Computer models that incorporate tensile-stress-induced growth reproduce several qualitative features of cortical folding, including its dependency on cortical thickness, growth rate and stiffness of the outer layer[47]. More elaborate mathematical models that represent subcortical white matter as a 3D continuous viscoelastic foundation can predict the effects of different growth rates on the wavelength of cortical folds, such that the larger the ratio of cortical to subcortical growth rates, the shorter the folding wavelength[48,50,59]. This model of tensile-stress-dependent growth was also further extended to include subcortical anisotropy, such that axons elongated more in the prevailing direction of their orientation than in other directions[60]. This model predicted that tissue anisotropy is also an important parameter in cortical folding, as the changes in the orientation of subcortical axons could alter the location of gyri and sulci.

Cellular mechanisms

A key corollary from the section above is that cortical folding varies considerably depending on the conditions immediately before the onset of folding, particularly the shape of the cortex and regional variations in neuron density. What previous developmental events, then, influence these onset conditions? Cortical shape and the density of neurons in the CP depend on two fundamental processes: neurogenesis and neuron migration.

Neurogenesis

Studies in ferret, macaque and human brain show that, in species with a folded cortex, the rate of neurogenesis is heterogeneous along the developing cortical mantle. Regions that exhibit high neurogenesis go on to undergo great surface area expansion and folding, and alternate

Box 1 | Human malformations of cortical folding

Cortical development involves a plethora of mechanisms that are under fine genetic control. Mutations in genes controlling these processes cause malformations in cortical development that may affect cortical folding in several ways, either increasing or decreasing the number or size of folds.

Polymicrogyria is a heterogeneous malformation characterized by the generation of many small folds, usually confined to a particular area. Depending on the extent of the malformation, it may be unilateral or bilateral, symmetric or asymmetric, and may occur in combination with other pathologies or as a part of a complex syndrome. Lissencephaly ('smooth brain') includes various disorders of varying severity, in which the folding pattern is either simplified (leading to pachygyria) or absent (leading to agyria). Both mild and severe forms may appear together in the same affected individual, with gradients in cortical fold simplification usually appearing along a rostro-caudal or caudo-rostral axis. Cases of polymicrogyria are usually associated with thinning of the cortex, whereas lissencephaly generally coincides with increased cortical thickness.

Genes mutated in patients with these pathologies are listed here and are grouped by the molecular mechanism in which they are implicated. Variants of the same gene — for example, *LAMC3* or one of the tubulin-encoding genes (*TUBB2B*, *TUBB3*, *TUBA1A* and *TUBA8*) — can have differential effects on cortical folding, depending on the specific mutations or context.

Molecular mechanism	Mutated genes or genomic mutation	Refs
Polymicrogyria		
Synaptic function	*GRIN1* and *SRPX2*	94,179
Regulation of cortical patterning	*GPR56* and *RTTN*	169,180–184
Specification and proliferation of IPCs and their progeny	*TBR2*	107
DNA repair	*NHEJ1*	185
Neuronal migration and cortical lamination	*TUBB2B*, *TUBB3*, *TUBA1A*, *TUBA8* and *LAMC3*	186–189
Vesicle transport	*KBP* and *KATNB1*	188,190
Tight junction maintenance	*OCLN*	191
Unknown	*WDR62*, microdeletions in 22q11, monosomy 1p36 and duplication of Xq26.1–26.2	192–197
mTOR pathway	*CCND2*, *PIK3CA* and *PIK3R2*	91,198
Pachygyria–agyria		
Neuronal migration and cortical lamination	*RELN*, *VLDLR*, *ACTB*, *LIS1*, *ACT1*, *DCX*, *TUBA1A*, *TUBB2B*, *TUBB*, *TUBA8*, *TUBB3* and *LAMC3*	91,120,122, 199–207
Vesicle and organelle motility and mitosis spindle assembly	*DYNC1H1*, *KIF2A*, *KATNB*, *KIF5C* and *CDK5*	187,190,208–210
Maintenance of specific neuronal types	*ARX*	211,212
Neuronal apoptosis	*CRADD*	206
Centrosome duplication and the formation and function of mitotic spindle	*YWHAE*, *NDE1*, *LIS1* and *TUBG1*	187,206,209,213

IPC, intermediate progenitor cell; mTOR, mechanistic target of rapamycin.

with regions of low neurogenesis that go on to show limited expansion and folding[17,61–63]. Regional differences in neurogenesis rate are particularly striking in the basal germinal zones (away from the ventricular surface), the so-called inner and outer subventricular zones (ISVZ and OSVZ, respectively). Regional differences in embryonic neurogenesis translate, in part, into differences in the density of immature neurons in the CP before cortical folding[1,17]. However, neuronal density and layer thicknesses are quite homogeneous across the mature cortex (except area 17 in macaque, which shows twofold neuron density compared with other areas[62,64]), seemingly as a result of greater surface area expansion and folding of regions with higher neurogenesis[17,63]. This notion is consistent with the current biomechanical model in which the cortex folds owing to a greater tangential expansion of the CP as a consequence of neuronal differentiation and neuropil growth[2].

Tangential dispersion

In addition to differences in neuron density in the CP, patterned expansion and folding of the cortical surface area also result from the tangential dispersion of neurons during their radial migration. The importance of radial neuron migration for cortical folding was first made evident by genetic studies of cortical malformations in humans[65] (BOX 1) and was further highlighted recently by studies showing that alterations to the dynamics of radial migration can induce folding of the mouse cortex[19] or the loss of folds in the ferret cortex[27].

Newborn cortical neurons migrate radially from the germinal layers to the CP along a scaffold of radial glial fibres (RGFs), the basal processes of radial glial cells (RGCs). In species with a smooth, non-folded cortex, such as the mouse, RGFs follow strictly parallel trajectories, such that neurogenic progenitors and their daughter neurons remain radially aligned[66,67]. In species with a folded cortex, the trajectory of RGFs varies across regions, being dramatically divergent in regions where the CP will later undergo the greatest expansion and folding[17]. As a result, radially migrating neurons exhibit considerable tangential dispersion in these regions, thus greatly contributing to expansion of the local cortical surface area[17,68].

The divergence of the RGF scaffold emerges from the generation of basal RGCs (bRGCs)[17], also known as outer RGCs[69]. These are neural stem cells that are very similar to the classical apical RGCs (aRGCs) in the VZ — in that they show similar marker expression and both have a radial process extending to the pial surface of the cortex — but instead have the cell soma located in the ISVZ or OSVZ, where they undergo mitosis[17,69–71]. bRGCs are generated by delamination from aRGCs in the VZ[17,72–74] or by self-amplification in the ISVZ and OSVZ; in both cases, newly generated bRGCs extend a new radial fibre that intercalates among the pre-existing RGF scaffold[17,75,76]. The generation of large numbers of bRGCs, each with their new radial fibre, leads to the divergence of this scaffold in the layers that are superficial to the OSVZ[17,77]. As a consequence, cortical areas with greater proliferation in the OSVZ and ISVZ before cortical folding exhibit the greatest divergence of radial fibres, the greatest tangential dispersion of radially migrating neurons and the highest degree of surface area expansion and folding[78] (FIG. 2), again with the exception of A17 in macaque[62]. Moreover, the degree of cortical folding in a given species increases exponentially with the relative abundance of proliferation in the OSVZ[17,79].

The above model has been tested experimentally in mice and ferrets. In ferrets, which have a folded cortex and abundant bRGCs, genetically enhancing OSVZ progenitor proliferation in a particular cortical region leads to a considerable increase in the surface area and folding of that region[24], whereas reducing the proliferation of bRGCs leads to smaller folds[17]. In mice, which naturally have a smooth cerebral cortex and very rare bRGCs[73], genetically increasing bRGC abundance consistently leads to the formation of cortical folds[20,22,23].

Neurogenesis is not enough

In gyrencephalic species such as ferrets and macaques, much neurogenesis takes place in the OSVZ[62,80,81], particularly from bRGCs[75,76]. An alternative possibility to the model of RGF divergence[77] is that cortical folding depends solely on the number of cortical neurons, which would suggest that the main role of the OSVZ and bRGCs is to increase neuron production rather than to modify and build the RGF scaffold. This might be the case in the human cortex at late stages of development, where it has been recently reported that the RGF scaffold splits between the VZ and ISVZ[82]. This recent finding remains controversial, as it is in conflict with evidence in ferrets, macaques and humans from multiple laboratories[17,38,62,83,84]. Neurons born from aRGCs need RGFs to migrate from the VZ to the CP. If, in human embryos, the RGF scaffold is truncated at late stages and aRGCs cease to contribute to it, late aRGC-born neurons cannot migrate all the way to the CP and thus cannot become part of the cortical grey matter. In that case, only late neurons born in the OSVZ have an RGF scaffold uninterrupted to the CP, and therefore bRGCs in the OSVZ may be the only, and thus key, contributors to late neurogenesis in our species[52,82]. Contrary to the notion that cortical folding depends solely on the number of cortical neurons, however, human lissencephaly (that is, a pathological absence of cortical folds) can occur without a substantial reduction in brain volume and cortical size, as in lissencephalic mutant ferrets[85]. Similarly, the marmoset is a New World monkey with thick cortical layers, rich in neurons, but a relatively low proportion of bRGCs located in the OSVZ and a near absence of folding[79,86,87]. Conversely, the considerable reduction in neuron number and brain size observed in human microcephaly (pathological small brain size) may occur with seemingly normal cortical folding (BOX 1). Likewise, in ferrets, microcephaly can be induced through early genetic depletion of VZ progenitor cells, without leading to a loss of folding or a loss of bRGCs in the OSVZ[85,88]. Thus, cortical folding is driven by differences in neurogenesis and the tangential dispersion of radially migrating neurons along the developing cortex and by the accumulation of neurons at different densities at the CP.

Delamination
Detachment from the apical adherens junction belt, followed by basal movement, away from the ventricular zone.

Lissencephaly
The characteristic of a brain without cortical folds, smooth or unfissured.

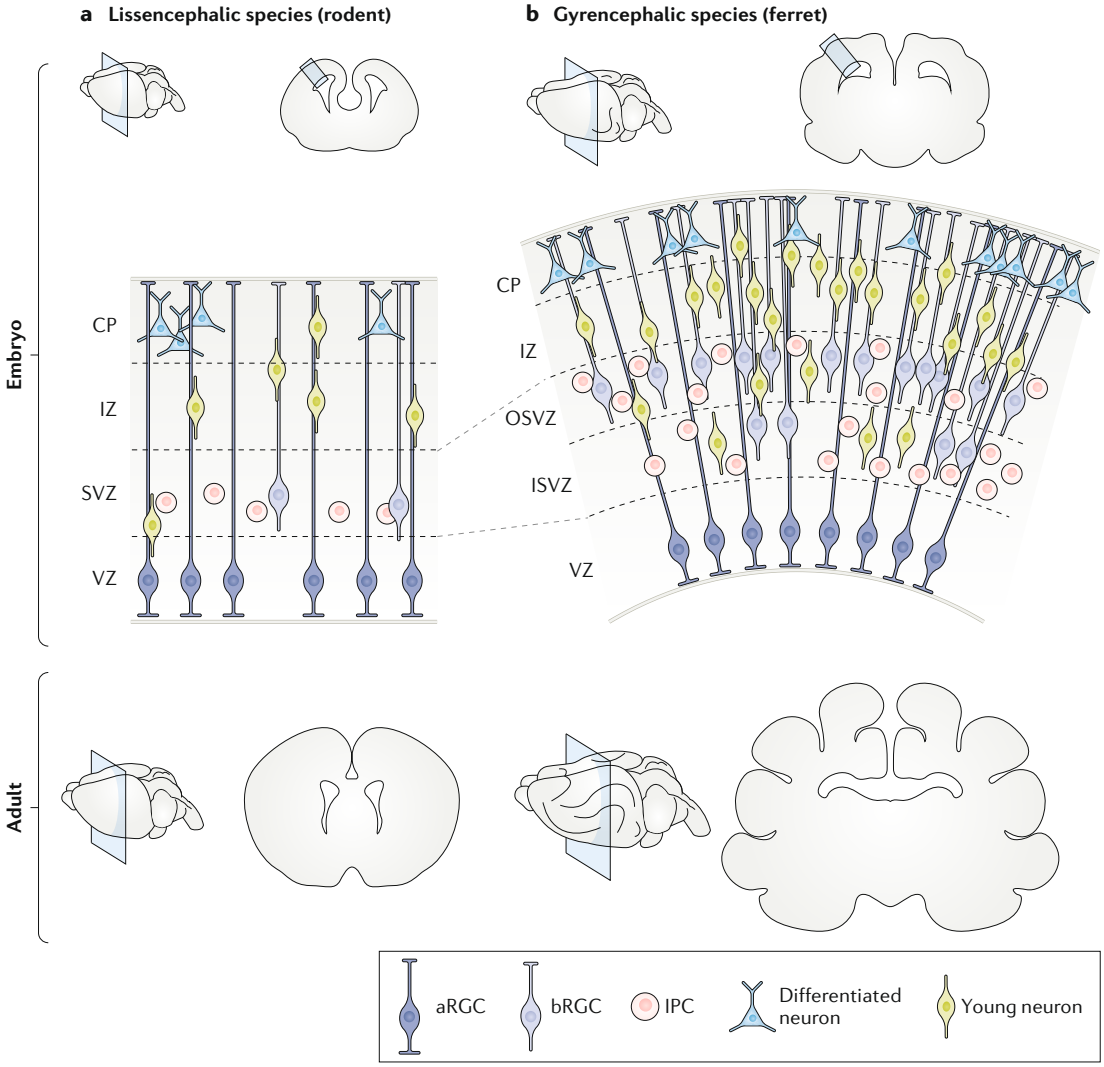


Fig. 2 | **Cellular mechanisms of cortical growth and folding: progenitor cells.** Different scenarios in lissencephalic (part **a**) and gyrencephalic species (part **b**) at embryonic and adult stages. Row 1 shows a representation of a rodent (part **a**) and ferret (part **b**) brain at embryonic stages in external view and coronal section. Row 2 shows schematics of the cellular composition and organization of the developing cerebral cortex. In lissencephalic species, most cortical progenitors are apical radial glial cells (aRGCs) and intermediate progenitor cells (IPCs), whereas basal radial glial cells (bRGCs) are scarce. After neurons are generated, they migrate intimately associated with radial glial fibres, which follow strictly parallel trajectories. In gyrencephalic species, the subventricular zone (SVZ) is greatly expanded and specialized in two germinal layers: the inner subventricular zone (ISVZ) and the outer subventricular zone (OSVZ). The ISVZ and OSVZ are both rich in bRGCs and IPCs. Species with a folded cortex present a much greater abundance of bRGCs than do lissencephalic species, particularly in the OSVZ. Each bRGC extends its own basal fibre, which creates a dramatic divergence of the radial fibre scaffold. This divergence leads to the tangential dispersion of radially migrating neurons (light green) and hence to the tangential expansion and folding of the cortical surface. Row 3 shows a representation of the adult brains of both types of species in external view and in coronal section: a smooth cortex in lissencephalic species in contrast to an expanded and folded cortex in gyrencephalic species. CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone.

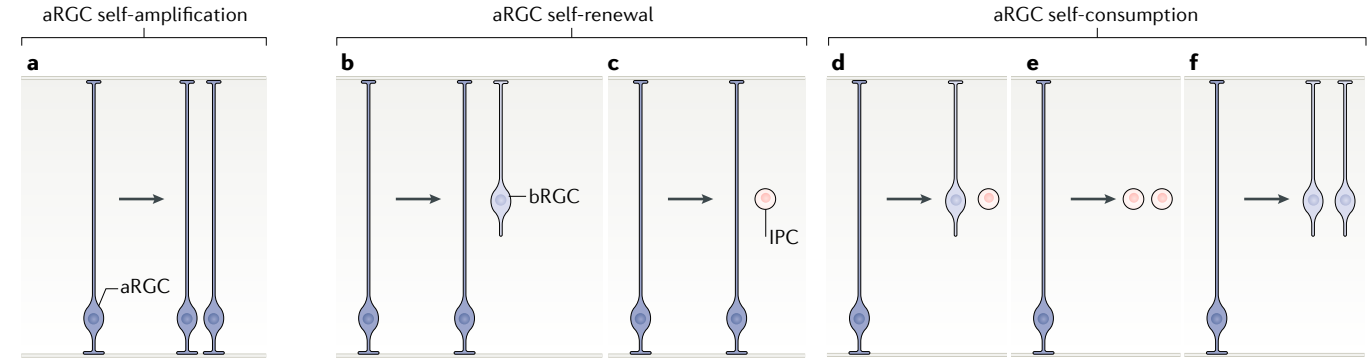
Genetic factors

Over the past two decades, human genetic studies have identified an ever-increasing list of mutations that severely perturb the development of the human cerebral cortex[37]. These mutations result in macroscopic malformations of cortical development and are usually associated with severe defects of cortical folding that underlie intellectual disability and epilepsy (BOX 1). Thus, the cellular mechanisms involved in cortical folding during brain development are under tight genetic regulation[89]. Moreover, the expression of genes involved in cortical folding is itself precisely regulated in both time and space[3].

Genetic regulation of cellular mechanisms

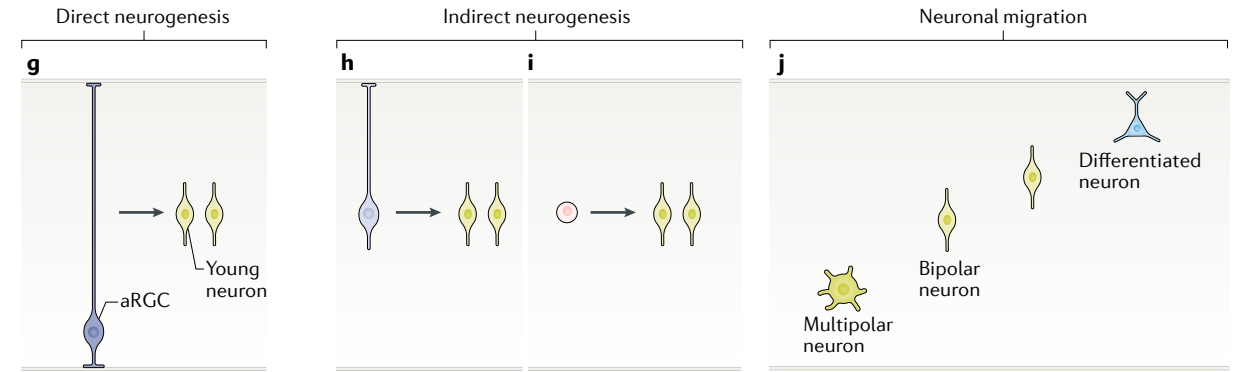
Progenitor cell proliferation versus neurogenesis. Regulation of the balance between cortical progenitor cell proliferation and neurogenesis (in all germinal layers) is under the control of multiple signalling pathways, but few thus far have been tested for an effect on cortical folding (FIG. 3).

Progenitor production



Apical domain maintenance AJ, cyclin D2 and PAR3		**Proliferation or differentiation and delamination** PAX6, TBR2*, TRNP1, ARHGAP11B, CTIP*, TMEM14B, PDGFRβ, ROBO1, ROBO2, HES1, cyclin D1, cyclin E1, ASCL1, NGN2, miRNA-15b and BAF170
Proliferation Integrins, ARHGEF2, IGF1, FGF2, CDK, cyclin D1 and SLIT–ROBO signalling		**Cellular division mechanisms** LIS1*, CDC42*, CENPJ* and ASPM*
Other NOTCH, WNT, BMP and FGF8B*		**Other** NOTCH, WDR62* and PRDM16

Neurogenesis and neuronal migration



Apical domain maintenance AJ and PAR3	**Proliferation or differentiation** Cyclin D1, CDK4, PAX6, TBR2, BAF170 and AP2γ*	**Cytoskeleton** FLNA*, CDK5* LIS1* and DCX*
Proliferation ROBO1, ROBO2, DLL1 and CD133		**Adhesion molecules** CDH1, E-cadherin, CX, FLRT1 and FLRT3
Other RA, NOTCH and CELSR3	**Other** HTT	**Other** RHO GTPases*, PIP3, RELN* and DAB1

* Variants associated with cortical malformations in humans.

Fig. 3 | **Genetic regulation of cortical folding: progenitor cells and neuronal migration.** The division of cortical progenitor cells may result in different lineage outcomes. Regarding progenitor production, the main type of cortical progenitor cell, apical radial glial cells (aRGCs), may self-amplify (part **a**); self-renew (parts **b**,**c**), generating one aRGC plus either a basal radial glial cell (bRGC) or an intermediate progenitor cell (IPC); or undergo self-consumption by producing two basal progenitor cells (in each combination; parts **d**–**f**). In addition, aRGCs may produce neurons via direct neurogenesis (part **g**). Basal progenitors may also generate neurons (so-called indirect neurogenesis) (parts **h**,**i**). Newborn neurons change their morphology from multipolar to bipolar before starting their migration through the cortical thickness to reach their final position in the cortical plate, where they differentiate (part **j**). Listed are some of the gene products that are critical for these lineage decisions and mechanisms. Cortical malformations in humans are associated with mutations in the genes encoding the products that are marked with an asterisk. AJ, adherent junction complex; AP2γ, transcription factor AP2γ; ARHGAP11B, RHO GTPase-activating protein 11B; ARHGEF2, RHO guanine nucleotide exchange factor 2; ASCL1, achaete-scute homologue 1; ASPM, abnormal spindle-like microcephaly-associated protein; BAF170, BRG1-associated factor 170 (also known as SMARCC2); BMP, bone morphogenetic protein; CD133, prominin 1; CDH1, cadherin 1; CDK, cyclin-dependent kinase; CELSR3, cadherin EGF LAG seven-pass G-type receptor 3; CENPJ, centromere protein J; CTIP, CTBP-interacting protein (also known as RBBP3); CX, connexin; DAB1, disabled homologue 1; DCX, neuronal migration protein doublecortin; DLL1, Delta-like protein 1; FGF2, fibroblast growth factor 2; FLNA, filamin A; FLRT, leucine-rich repeat transmembrane protein; HES1, transcription factor HES1; IGF1, insulin-like growth factor 1; HTT, huntingin; LIS1, lissencephaly 1 protein; NGN2, neurogenin 2; PAR3, partitioning defective 3 homologue (also known as PARD3); PAX6, paired box protein 6; PDGFRβ, platelet-derived growth factor receptor-β; PIP3, phosphatidylinositol-3,4,5-trisphosphate; PRDM16, PR domain zinc finger protein 16; RA, retinoic acid; RELN, reelin; ROBO, Roundabout homologue; TBR2, T-box brain protein 2; TMEM14B, transmembrane protein 14B; TRNP1, TMF-regulated nuclear protein 1; WDR62, WD repeat-containing protein 62.

Overactivation of fibroblast growth factor (FGF) signalling by infusion of FGF2 or FGF8 in the telencephalic ventricle increases progenitor proliferation and induces cortical folding in mice, or polymicrogyria (extra folding) in ferrets, respectively[25,28]. In agreement with a role for FGF signalling in regulating cell cycle proteins, direct overexpression of *Cdk4* and *Ccnd1* also causes extra folding in ferrets. However, this manipulation was not sufficient to induce folding in mice[24], highlighting the molecular and cellular complexity of this process[90]. Retinoic acid signalling also regulates the proliferation of cortical progenitors and induces folding in the otherwise smooth mouse cortex[31].

Other genes involved in signalling pathways that regulate progenitor proliferation are mutated in human malformations of cortical folding; for example, mutations in *SRPX2*, or *PIK3CA* and *PIK3R2* (which encode components of the mechanistic target of rapamycin (mTOR) pathway) induce polymicrogyria in children[91–94] (BOX 1). Although mice with mutations in these genes do not develop cortical folds, the emergence of transgenesis in more suitable animal models, such as ferrets and marmoset monkeys, should aid understanding of the contributions of these and other genes in progenitor proliferation and cortical folding[85,88,95–97].

Formation and amplification of bRGCs. New bRGCs may form de novo from aRGCs, or from basal progenitors, usually by self-amplification[72,75,76] (FIG. 3b). The formation of bRGCs from aRGCs involves two main processes: asymmetric division of aRGCs and cell delamination from the VZ. Changes in the orientation of the mitotic cleavage plane from vertical to horizontal usually lead to asymmetric cell division, although this kind of cell division may also happen from vertical cleavage planes, as does symmetric cell division. The orientation of the cleavage plane is regulated by factors such as the mosaic protein LGN, inscuteable protein homologue (INSC), nuclear distribution protein-like 1 (NDEL), lissencephaly 1 protein (LIS1) and the WNT–planar cell polarity (PCP) pathway, and strongly influences the acquisition of asymmetric cell fates by cortical progenitors[74,98–101]. However, these factors influence cell fate differently. Changes in mitotic cleavage plane orientation by LGN and cadherin 1 (CDH1) regulate the formation of bRGCs from aRGCs[74,76], whereas INSC favours the formation of intermediate progenitor cells (IPCs)[100]. The asymmetric division of aRGCs is also regulated by FGF and ROBO signalling, but this signalling promotes the formation of IPCs or neurons rather than bRGCs[102–104], a difference in cell fate decision that is not well understood.

Once bRGCs are born from aRGCs, they must delaminate from the VZ and translocate to the ISVZ and OSVZ. Most genes that have been manipulated or knocked in to induce cortical folding in mice, or that are necessary for cortical folding in ferrets, are involved in the delamination of basal progenitors, including bRGCs. These genes include those encoding the cell-adhesion molecules CDH1 and contactin 2 (CNTN2; also known as TAG1)[76,105], FGF receptors[26], insulinoma-associated protein 1 (INSM1)[106], sonic hedgehog (SHH) signalling factors[20] and TMF-regulated nuclear protein 1 (TRNP1)[22,76]. The transcription factors scratch, paired box protein PAX6 and T-box brain protein 2 (TBR2; also known as EOMES) also regulate cell delamination and the formation of bRGCs, and misregulation of TBR2 expression in humans produces severe folding abnormalities[107]. By contrast, manipulating these factors in mice has mild or no effects on cortical folding[108–110]. Importantly, some of the few newly emerged genes in the recent primate and hominid lineages, such as *ARHGAP11B*, *TBC1D3* and *TMEM14B*[21,23,111,112], induce the abundant formation of bRGCs and folding of the mouse cortex, whereas others, such as *NOTCH2NL*, amplify IPCs but not bRGCs[113–115].

The amplification of bRGCs seems to be regulated by various molecular pathways, some of which have been identified, including Notch signalling and integrin signalling[69,71]. Many other signalling mechanisms have been identified in recent targeted transcriptomic analyses — including those comparing developing cortical layers in mice and humans[116], different progenitor cell populations in mice and humans[23], and single-progenitor cells in humans and ferrets[113,117–119]. Although the signalling mechanisms identified in these analyses have not yet been shown to affect cortical folding, they may also be relevant in the bRGC amplification that leads to it.

Neuronal migration. Several genes essential for neuronal migration have a substantial impact on cortical folding[65] (FIG. 3j). For example, in humans, mutations affecting the reelin signalling pathway or in the gene encoding the neuronal migration protein doublecortin (DCX) — two key regulators of radial migration and cortical lamination — severely impair cortical folding[120–124], and *Dcx*-knockout ferrets completely lack cortical folds[85]. Overexpression of a dominant negative mutant allele of *Cdk5*, which is important in mouse neuron migration, impairs the radial migration of upper-layer neurons and the formation of cortical folds in ferrets[27]. Leucine-rich repeat transmembrane protein 1 (FLRT1) and FLRT3 are cell-adhesion molecules important in radial migration of cortical neurons[125], and mice lacking both FLRT1 and FLRT3 develop cortical folds[19].

Genetic regulation of mechanical tissue properties

The stiffness and other viscoelastic properties of biological tissues are determined by their molecular composition[126]. This molecular composition is related to protein density and composition of the ECM, as well as to cell adhesion, either between cells or with the ECM. Thus, the mechanical properties of the developing brain, which strongly contribute to cortical folding, depend on the expression levels and half-lives of various ECM, cell-adhesion and cytoskeletal proteins. Whereas much remains to be investigated on this front, transcriptomic analyses have identified several integrins, collagens and laminins that are differentially expressed between layers of the developing human cortex[116] and between prospective gyral and sulcal regions in the developing ferret brain[127]. Importantly, FLRT1 and FLRT3 are differentially expressed between gyri and sulci in ferrets, whereas in mice they are

Intermediate progenitor cells
(IPCs). Germinal cells born from apical radial glial cells that populate the subventricular zone (basal from the ventricular zone) and produce neurons.

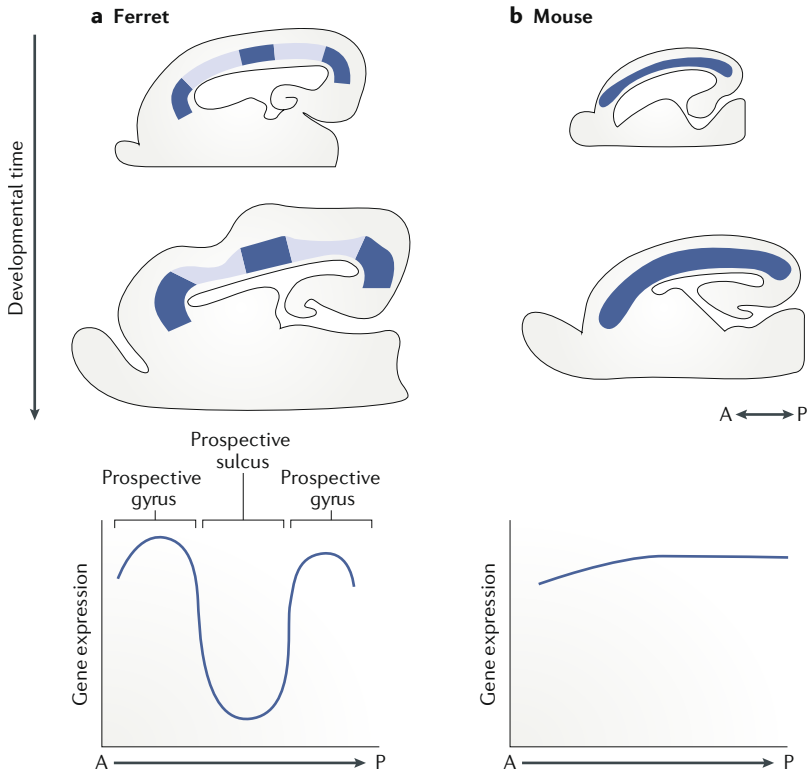
expressed by only a subset of cells distributed in a 'salt-and-pepper' pattern. In mice lacking FLRT1 and FLRT3, the neurons that were deficient for these molecules (which were genetically labelled) instead clustered together to form columns, and cortical folds developed[19], strongly suggesting that the modular arrangement of the neurons expressing these genes is important to drive cortical folding.

Patterned gene expression

Cortical folds form in stereotyped patterns among individuals of a given species, with a predetermined location, size and shape. This stereotypy is very clear in species with few folds, such as ferrets and cats[10]. In species with a highly folded cortex, such as humans, these patterns are more complex and variable, but primary fissures, which develop the earliest and end located at the deepest positions (sulcal pits), are still highly conserved[12,128]. Folding patterns also follow remarkably conserved trends among phylogenetically related species (for example, among carnivores)[10,77]. Altogether, these similarities indicate that the patterns of cortical folding are under strong genetic regulation, during development and across evolution[129].

In ferrets and cats, the locations along the developing cortex where primary folds will form display increased neurogenesis, radial fibre divergence and amplification of bRGCs — particularly in the OSVZ — compared with the locations of future primary fissures, suggesting a possible causative link[17]. In support of this idea, local genetic manipulations of OSVZ proliferation in ferrets substantially alter the size and shape of the overlying folds[17,24]. Insights into the genetic regulation of cortical folding versus fissuring first came from transcriptomic analyses in developing ferrets. A comparison of the transcriptional signatures of germinal layers in prospective folds and fissures in ferrets identified thousands of differentially expressed genes (DEGs), mostly in the OSVZ and VZ[127]. DEGs include genes that are important for cortical progenitor proliferation, neurogenesis and folding, such as *Trnp1, Ccnd1, Tbr2, Flrt1, Flrt3, Fgfr2* and *Fgfr3*, as well as genes encoding components of the Notch, SHH, MAPK and WNT signalling pathways. DEGs from this analysis also included 80% of the genes that are homologous to those mutated in humans with cortical malformations, such as *RELN, DAB1, CDK5, PAX6, PAFAH1B1, TUBA8, TUBA1A, TUBB2B* and *GPR56* (REF.[127]) (BOX 1). Strikingly, in the germinal layers of ferret and human cortex, but not in lissencephalic mouse cortex, many of these DEGs are expressed in modular patterns, with alternating blocks of high and low expression[127,130,131] (FIG. 4). These patterns faithfully map the prospective location of folds and fissures, strongly supporting a role for the OSVZ and DEGs in defining the stereotyped patterning of cortical folds[17,52,127,129,132].

The OSVZ is important in cortical expansion and folding, as explained above. The abundance of progenitor cells in the OSVZ correlates positively both with cortical size and the degree of folding, both within and between species[17,79]. Studies in ferrets demonstrate that the OSVZ forms through the seeding of bRGCs generated from aRGCs in the VZ[76]. Unlike seeding of the ISVZ, this seeding of the OSVZ from the VZ is transient, occurring during a very brief time window of embryonic development, after which the founder bRGCs self-amplify until the OSVZ reaches its full size[76]. This critical time period for OSVZ formation is defined genetically, with the expression of genes that are important for the formation and delamination of bRGCs from the VZ — namely, *Cdh1* and *Trnp1* — precisely regulated[76]. Thus, in ferrets and primates[75,76], the developmental stage at which the OSVZ is seeded, the duration of the seeding period and the duration of the subsequent period when the OSVZ self-amplifies are all proposed to dramatically affect the size of the OSVZ and hence the degree and complexity of cortical expansion and folding.



c DEGs

High in SG				High in LS
Human and ferret		Ferret		Ferret
• *TBR2*	• *FGFR3*	• *Lhx2*	• *St3gal1*	• *Cdh8*
• *GPR56*	• *FLNA*	• *Lmo7*	• *Slitrk6*	• *Nrxn3*
• *FGFR2*	• *TRNP1*	• *Cdk6*		

Fig. 4 | **Genetic patterning of cortical folds.** In gyrencephalic species such as the ferret, certain genes are expressed in modular patterns, with alternating high and low expression levels (relative grey tone) along germinal layers of the developing cortex (part **a**). By contrast, in lissencephalic species such as the mouse, gene expression levels are quite homogeneous (part **b**). Modules of expression levels concur with the prospective location of folds and fissures. Variations in expression are associated with differences in proliferation and neuron migration and, eventually, cortical expansion and folding. Differentially expressed genes (DEGs) reported in ferrets by in situ hybridization are listed (part **c**), distinguishing between those expressed at high levels in the splenial gyrus (SG) with respect to the lateral sulcus (LS), or vice versa. Some of these genes also display modular expression in the fetal human cortex. A, anterior; P, posterior. Parts **a** and **b** are adapted with permission from REF.[3], Elsevier.

A mechanical and molecular tango

Several lines of evidence suggest that mechanical forces affect various important events during neural development, including progenitor cell proliferation, neuronal migration, cell differentiation and the formation of synapses. In chick embryos, neuroepithelial cells proliferate in response to mechanical impact[133,134]. The differentiation of stem cells into neurons in vitro is strongly affected by physical tension and the mechanical properties of surrounding growth substrates[135,136]. Neurite outgrowth in chicks, and synapse formation in *Drosophila melanogaster*, are strongly promoted by increased axonal tension[58,137]. In the developing rat cerebral cortex, mechanical pressure onto the basal process of aRGCs triggers calcium signalling and promotes their proliferation[138].

Cellular mechanotransduction is mediated by a number of elements, including through focal adhesions and/or by the ECM, primary cilia and cytoskeletal proteins[135,139,140]. During embryonic development, variations in the cytoskeletal system that result from mechanotransduction ultimately alter the intracellular localization and function of proteins, including transcription factors. For example, in developing *D. melanogaster*, mechanical pressure induces ectopic expression of the transcription factor Twist throughout the entire embryo[141]. Similarly, tension forces drive the activity of tension-induced proteins (TIPs) that, owing to their histone acetyltransferase activity, modify chromatin structure and thus substantially alter gene expression[142]. These changes in gene expression alter the genetic profile of neural cells during development and, consequently, their identity and biology.

The ECM is a key structural component of tissues that, depending on its specific molecular composition, helps to define their specific mechanical properties and functions[126]. For example, the assembly of complex fibronectin-based fibrillary structures in the ECM increases its stiffness, which is essential to support the movement and change in shape of cells during gastrulation[143]. Transcriptomic analyses of the developing cerebral cortex have identified differences in gene expression patterns between the smooth mouse cortex and the folded human cerebral cortex[23,116,118,119,144,145]. These analyses have shown that the expression of genes encoding ECM proteins is upregulated in human cortical germinal zones and progenitor cells, especially in the OSVZ[23,116]. In vivo and in vitro studies have demonstrated that ECM receptors can regulate the proliferation of cortical progenitor cells in mice and ferrets[71,146]. Most importantly, one recent in vitro study showed that application of a combination of three ECM components that are highly expressed in the human embryonic cerebral cortex (namely, hyaluronan and proteoglycan link protein 1 (HAPLN1), lumican and collagen I) onto human fetal brain slice cultures could induce cortical folding[8]. This treatment increases hyaluronic acid (HA) in the CP, and loss of HA in vitro reduces the nascent physiological folding of human fetal cortex. Moreover, treatment with these ECM components induced changes in stiffness of the cortical tissue, coherent with the localization of folds and fissures[8].

In summary, regulation of gene expression during cortical development may help to establish the necessary conditions to initiate folding, including patterned neurogenesis, neuron migration and differentiation as well as tissue stiffness and mechanical stress (tension or pressure). In turn, cellular strain and stress resulting from cortical folding may cause considerable changes in gene expression, as observed in several systems. Thus, the intimate and mutually influential relationship between genetics, cell biology and mechanical forces is likely to define their respective dynamic changes during development and to finally affect the cortical phenotype, both in health and disease[1,2].

The evolution of cortical folding

The advent of high-throughput genome sequencing and improved bioinformatics methodologies has enabled the phylogenetic relationships between extant mammals to be properly established[147]. By combining genomic information with measurements of cortical folding in different species, the evolution of cortical folding seems not to be a simple or linear story.

Starting from *Homo sapiens* and our closest relatives, analysis across the New and Old World primates shows that our common ancestor had a GI of 1.50–1.75 (REF.[87]). From this ancestor, Old World monkeys and primates evolved towards an exponential increase in cortical folding up to a GI of 2.56, as in humans, whereas New World monkeys followed the opposite trajectory and exhibit simplified cortical folding with a GI as low as 1.17, as in marmoset monkeys[87]. This divergence was confirmed independently by a phenomic character matrix (set of physical traits) of living placental orders and fossil species, which concluded that the ancestor of placental mammals had a folded cortex[148]. A specific analysis of cortical folding in 102 mammalian species including marsupials further confirmed that gyrencephaly is an ancestral mammalian trait (with the common mammalian ancestor estimated to have a GI of 1.36 ± 0.16) and that trajectories towards increased folding or decreased cortical folding, as in primates, have occurred multiple times across mammalian clades over the past ~100 million years of brain evolution[149]. Secondary loss of gyrencephaly may have occurred as a result of reductions in the number of basal neurogenic progenitors and bRGCs[77,87,150]. The ability of the mammalian brain to undergo dramatic phenotypic reversals and differential changes in cortical folding during evolution may reflect the remarkable adaptability of mammals along this process[151].

The genomic changes that occurred during evolution and that were directly responsible for the observed variations in cortical folding are unclear. The human genome shows sites of uniquely high sequence divergence from the genomes of close evolutionary relatives[152]. In fact, genetic evolution is still ongoing in humans; there are several hot spots in genes related to cortical development, including *ASPM* and *MCPH1*, and mutations in these hot spots are associated with microcephaly[153–155]. Recent transcriptomic analyses have identified 15 genes that exist only in primate genomes and that show enriched expression in cortical progenitor cells[113]. A subset of these genes emerged uniquely in the human

Gastrulation
Phase of early embryonic development during which the single-layered blastula is reorganized into a multilayered gastrula.

Hot spots
Regions in the genome that exhibit elevated rates of a specific event. In evolutionary hot spots, the local sequence of DNA has changed rapidly during evolution.

lineage during evolution through certain mechanisms — mostly through whole-gene duplication, as in the case of *NOTCH2NL*[114,115,156,157], or through partial gene duplication, as with *ARHGAP11B*[23,111,158,159]. In most other cases, new genes emerged through small insertions into or deletions from the ancestral genome that may have eliminated pre-existing stop codons, caused frameshifts or introduced new sites of RNA splicing[113]. Even within genes that are conserved between species, small differences in coding DNA sequences — including synonymous and non-synonymous base pair changes — have also occurred over evolution and may or may not have led to modified amino-acid sequences.

In addition to the appearance of new genes, changes in the regulation of gene expression have also contributed considerably to cortical evolution. A comparison of the human genome and the genome of the chimpanzee (which has a GI of 2.45) identified hundreds of human accelerated regions (HARs) — small DNA segments that exhibit highly divergent sequences in humans and rapid

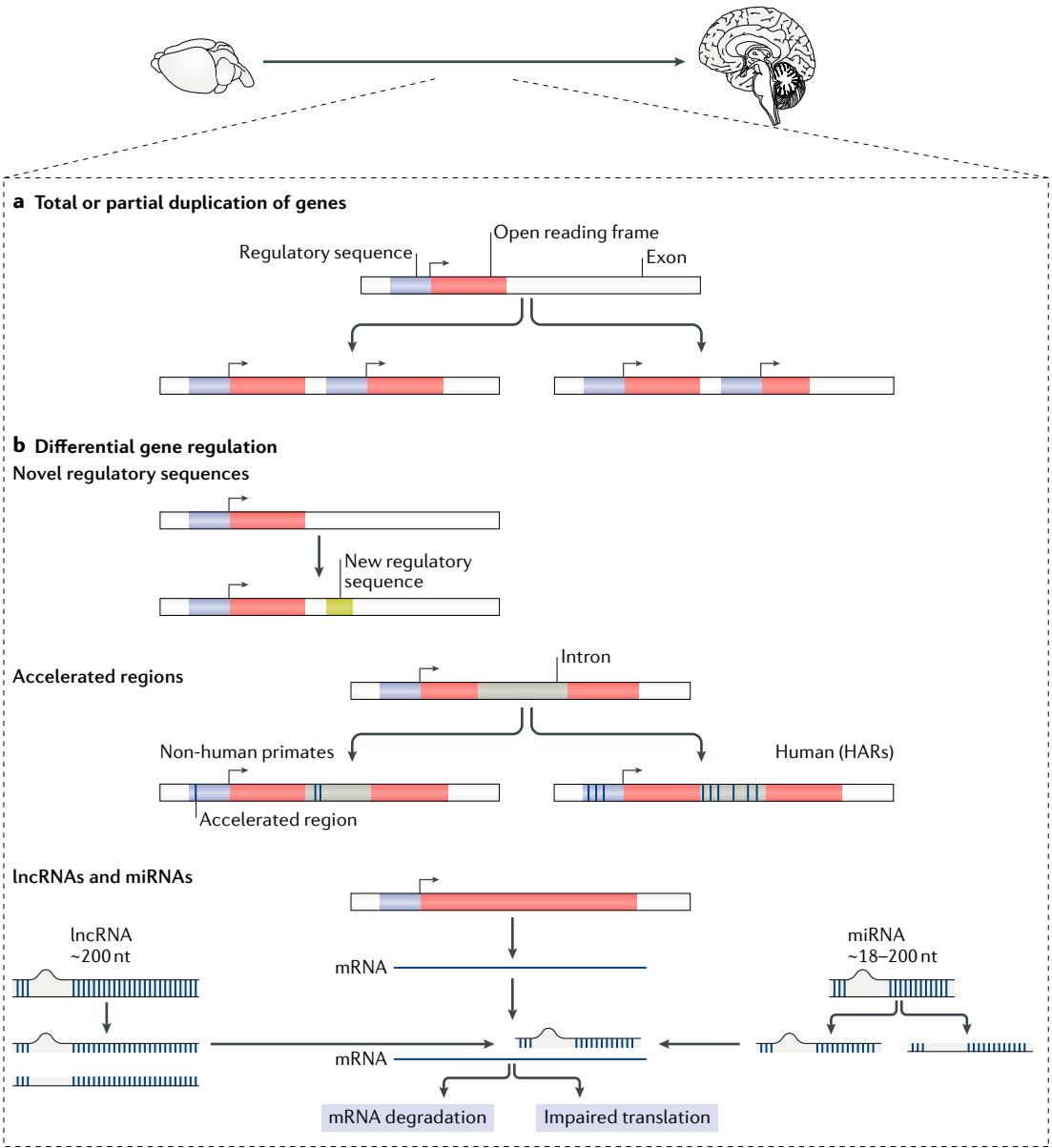


Fig. 5 | **Genetic evolution and cortical folding.** Different genetic variations that have occurred during evolution and affect cortical folding are summarized here in two main categories: duplication and gene regulation. **a** | Duplication (total or partial) of genes, both protein-coding and non-coding, allows the modification of the existing sequence without altering the original gene, preserving its functionality. One example of such duplication is *ARHGAP11*, which occurred specifically in the human lineage and resulted in the emergence of the new, human-specific gene, *ARHGAP11B*. **b** | The expression of different genes may be differentially regulated through the generation of novel regulatory sequences, including promoters or enhancers, accelerated regions and those encoding long non-coding RNAs (lncRNAs) and microRNAs (miRNAs). Interestingly, some of these regulatory elements are primate or human-specific, and many of these are related to cell cycle and neurogenesis. Blue, regulatory sequence; red, open reading frame; light grey, non-coding sequence; dark blue, accelerated regions. HAR, human accelerated region; nt, nucleotide.

nucleotide substitution in the human lineage — that are proposed to regulate gene expression and thus contribute to human-specific brain expansion and complexity[160–162]. At least 30% of HARs encode developmental enhancers that show activity in humans and chimpanzees[163] and therefore are good candidates as regulatory regions that may define human-specific spatiotemporal patterns of gene expression[160,164–168]. Although not a HAR, one study assessed the activity of the enhancer sequence at the *GPR56* locus, which is crucial for normal cortical folding, in different species[169]. In transgenic mice, reporter gene expression driven by the mouse *Gpr56* enhancer sequence displayed a pattern different from that driven by the enhancer sequences from species with folded cortices, such as dolphins, cats or humans. Gene expression is also regulated by non-coding RNAs, which have central roles in cortical development, and long non-coding RNAs have been proposed to be important in the evolution of gene regulation[170–172] (FIG. 5). Accordingly, in apical and basal progenitor cells of the developing macaque cortex, but not in the developing mouse cortex, hundreds of microRNAs are expressed that regulate the cell cycle and neurogenesis, particularly in the OSVZ, and thus may contribute to primate cortical complexification and possibly folding[62,81,173,174].

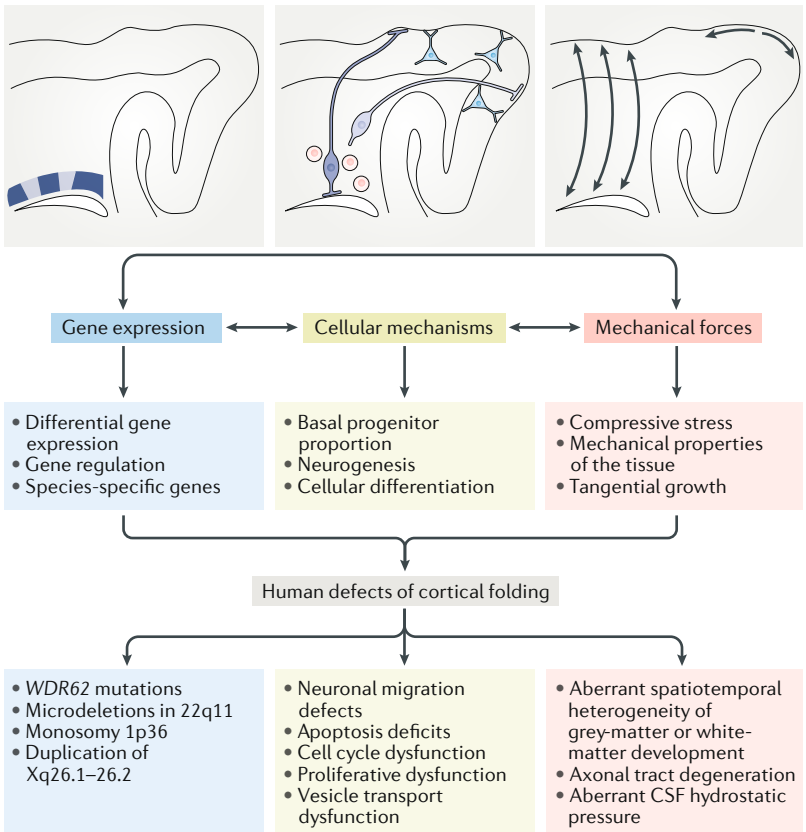


Fig. 6 | **Three key players in cortical folding: gene expression, cellular mechanisms and mechanical forces.** Each component and their specific role in cortical folding is represented schematically in the upper part of the figure. Arrows between gene expression, cellular mechanisms and mechanical forces represent their mutual influence. Some of the main mechanisms from each component are listed. The bottom panel lists examples in which alterations at any one of these three levels lead to defective folding of the human cortex (bottom). CSF, cerebrospinal fluid.

These changes in the regulation of gene expression translate into transcriptomic differences between species, as demonstrated in comparative analyses of single-progenitor cell transcriptomes that show greater similarity between humans and ferrets than between humans and mice[117]. On this basis, variations in enhancers and other regulatory elements may be at the core of the evolution of precise spatiotemporal patterns of gene expression that define cortical patterning and folding during development[3,76,127].

Future directions

The folding of the mammalian cerebral cortex remains one of the most fascinating phenomena of developmental neuroscience and evolution, but only with the combined efforts of several scientific disciplines are we beginning to clarify its most basic aspects. Not surprisingly, these efforts reveal that cortical folding results from the combined actions of factors at different levels and their interactions with one other — a notion that seems directly relevant for human disease (FIG. 6). Now that some of the key cellular and genetic elements involved in cortical folding have been identified, ongoing research is focusing on suitable experimental animal models, such as ferrets, marmosets and macaque monkeys, and in vitro systems such as cerebral organoids[35,36,85,96,175]. Taking full advantage of genome-editing techniques, gene-knockout and transgenic ferrets and marmosets are already being generated[85,88,176], and these will undoubtedly be one of the driving forces of this field in the future. In the quest for genetic mechanisms that regulate cortical development, and particularly progenitor cell diversity and lineage, considerable efforts are being made using high-throughput single-cell analyses (for example, Drop-Seq)[177,178]. Performing these analyses in ferrets, humans and non-human primates holds great promise for the identification of cell populations that are enriched in prospective folds or fissures and that may hence underlie differential cortical expansion rates and folding. Such cell populations may have distinctive features such as the potential to self-amplify, to produce bRGCs or neurons, or to undergo tangential dispersion. Single-cell analyses in various gyrencephalic species may reveal differences in expression of proteins that endow cells, and the tissue immediately surrounding them, with particular mechanical properties relevant to drive tissue buckling, including adhesion molecules, cytoskeletal proteins or ECM components[8].

We are beginning to capture the outstanding complexities of the developing cerebral cortex as a biological and mechanical system, with dynamic developmental trajectories and feedback mechanisms between cell biology, mechanics and genetics. Unfortunately, our knowledge in these fields is still too fragmentary to be combined in a unifying model. We have some detailed understanding of genetic and cellular events that occur early in development and that precede and underlie the eventual folding of the cortex. However, much less is known about the events that occur during folding, such as any changes in cell size and density, neuropil or ECM. By contrast, we have some notions about the mechanical features and constraints of tissue deformation that

are involved in actual cortical folding late in development, but not about how these emerge during early developmental stages and change later. Understanding cortical folding will require a full and quantitative characterization of all these relevant features throughout cortical development.

Once a unifying model is built that explains cortical folding, a great challenge will be to validate it by experimentally analysing the interactions between genetics, cell biology and mechanics and their highly dynamic changes during development. Which cells generate mechanical forces before and during folding, and how is this achieved? In turn, how do these cells respond to mechanical force during folding, and what are the effects of physical forces on the gene expression or function of progenitor cells and migrating neurons at different stages of cortical development and folding? Only by understanding these parameters and interactions in the developing cerebral cortex in vivo, at a quantitative level, will we finally elucidate the mechanisms that underlie the emergence of stereotyped folding of the mammalian cerebral cortex.

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