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Cortical Specification and Neuronal Migration

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Abstract

The extraordinary complexity of the mammalian neocortex is the result of millions of years of evolution. Elucidating the principles underlying its development and function has been a major goal in the neurosciences. How a seemingly uniform group of neuroepithelial stem cells produces the vast array of electrically responsive cell types, and how these resulting cells establish such a rich variety of circuits in the mature neocortex remains, in particular, a key focus of the field. This chapter reviews seminal advances in understanding the production, specification, and migration of neocortical neurons prior to the establishment of mature circuits.

Introduction

Since the introduction of the basic principles of neocortical development at the Dahlem Workshop in 1987 (Rakic 1988a), such as the *protomap hypothesis* and the *radial unit hypothesis*, advances in molecular biology and imaging techniques have revolutionized our ability to interrogate developmental processes in the brain. These technical capabilities have uncovered many new molecular and cellular pathways that operate during neocortical growth, many of which confirm and extend the above-mentioned theories. Here we review the basic principles of neocortical development and highlight key controversies and emerging areas where additional studies are needed.

Although considerable differences exist across mammalian species in terms of timescale and architecture of cerebral cortical development, several major events occur in all mammals. The first stages of neocortical development are characterized by rapid proliferation that leads to exponential expansion of the neuroepithelial progenitor cells (NEPs) within the pseudostratified neuroepithelium (or the ventricular zone, VZ) lining the lateral ventricles of the prosencephalon. This earliest period of precursor proliferation has been termed the "founder cell expansion phase" and is one of the most important phylogenetic

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determinants of cortical size between species (Rakic 1988b, 1995) (Figure 2.1). Once the telencephalon emerges, rapid NEP proliferation continues until the molecular specification of apical radial glial cells (aRGCs), a cell type now considered to be neocortical stem cells, as they possess the ability to self-renew as well as to generate the mature cell types of the neocortex. The transition from NEP to aRGC occurs at different gestational times, depending on species (e.g., at E10 in mice and ~E40 in primates), prior to the generation of the first neocortical neurons—an event which occurs quickly thereafter. The duration of the founder cell expansion phase controls the resulting number of individual precursor cells. It is thought that these founder cells represent the primordial cortical units that establish columns of neurons in the neocortex.

Following the establishment of aRGCs in the VZ, the first excitatory neurons are born from asymmetrical divisions that result in a newborn neuron and a self-renewed aRGC (Malatesta et al. 2000; Hartfuss et al. 2001; Miyata et al. 2001; Noctor et al. 2001; Tamamaki et al. 2001; Noctor et al. 2002). These neurons exit the VZ and establish the first postmitotic layer of neocortical neurons. In rodents, this first layer is called the preplate and lies just superficial, or basal, to the VZ. In rodents, subsequently generated neurons migrate along the basal fibers of aRGCs (so-called gliophilic migration) to split the preplate

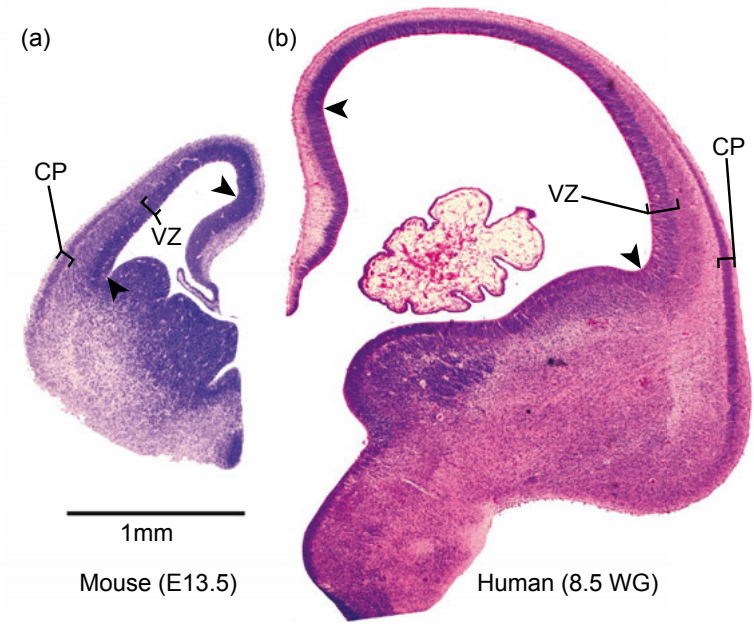

Figure 2.1 Species-specific effects of founder cell expansion on neocortical size. Coronal sections of E13.5 mouse (a) and 8.5 WG human (b) brains—comparable stages of fetal development—highlight the increase in area of the human ventricular zone (VZ), between arrowheads, due to founder cell expansion. Both brains display the onset of cortical neuron arrival into the cortical plate (CP). Adapted from Tyler and Haydar (2010).

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into the deep subplate and the superficial marginal zone (neocortical layer 1). In primates, the firstborn neurons are thought to directly form the cortical plate (Smart et al. 2002). In all mammals, migrating neurons then form the remaining cortical layers (2–6) in an inside-out manner based on their date of birth. This temporal specification results in the later-born neurons migrating past the earlier-generated neurons to form each successive superficial neocortical lamina (Rakic 1975). Once they have arrived at their proper destination, neocortical neurons differentiate molecularly (expressing specific laminar marker genes) as well as cellularly (extending axons to postsynaptic targets and elaborating complex dendritic trees). In addition to producing neurons directly, another property common to all mammals is the ability of aRGCs to generate intermediate precursor cells (IPCs) during the course of neurogenesis. As will be detailed below (see section on "Precursor Heterogeneity"), these IPCs contribute to the expansion of neocortical layers by producing additional neurons. While it is well-established that IPCs come in a variety of different forms, whether and how each cell type uniquely contributes to neocortical formation is only now becoming understood.

Whereas excitatory neurons are produced in the dorsal telencephalon, inhibitory interneurons are generated from precursor cells in the ganglionic eminences of the ventral telencephalon. Once generated, nascent interneurons migrate tangentially into the dorsal telencephalon along axonal fibers (so-called neurophilic migration) and may switch to gliophilic migration as they near their final destination (Polleux et al. 2002). Thus, production of the proper ratio of excitatory to inhibitory neurons is a critical aspect of proper neocortical development (and is altered in some developmental disabilities). Moreover, how the tangentially migrating interneurons coalesce with their radially migrating excitatory cousins in a proper laminar fashion, and with appropriate density, has not yet been fully discovered. It is now known that key, molecularly different germinal fields within the ganglionic eminences produce specific subtypes of interneurons, and the genetic code for this spatiotemporal specification is beginning to be understood (Flames et al. 2007). This causal relationship between differential gene expression in the germinal zones and the fate potential of the daughter cells generated in those areas also exists in the dorsal telencephalon, as we explore further below.

The developmental events outlined above occur in all known mammals, but there are important species-specific differences that are important to consider when arriving at a comprehensive understanding of the developmental mechanisms that govern growth and formation of the neocortex. These differences separate "smooth brain" lissencephalic species (e.g., rodents) from gyrencephalic species (e.g., carnivores and primates). In general, peculiarities in the structure of the gyrencephalic cerebral wall results from precursor cell compartmentalization during fetal development. This organization is thought to play a role in the development of the larger and more complex circuitry found in the gyrencephalic neocortex. One of these specialized architectonic

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features is the splitting of the subventricular zone (SVZ) into inner and outer compartments by the inner fiber layer (IFL) (Smart et al. 2002). While the inner SVZ resembles the SVZ found in rodent neocortex, the outer SVZ (oSVZ) is greatly expanded in ferret and primates. The predominant precursor cell type in the oSVZ is the basal RGC (bRGC) which has been shown to self-renew and generate neurons via asymmetrical divisions (Fietz et al. 2010; Hansen et al. 2010). The concurrent expansions in the numbers of bRGCs and the size of the oSVZ are thought to underlie the increased radial growth as well as the convoluted surface of the gyrencephalic neocortex. In addition, there are several neuronal groups that appear to be unique to gyrencephalic neocortex, including subpial granular neurons and an expanded population of subplate neurons (Kostovic and Rakic 1990; Meyer et al. 2000). All of these findings suggest that while key mechanisms of neocortical development can, and for many reasons must, still be elucidated in lissencephalic species, the field of neocortical development has arrived at a stage where novel findings of fundamental mechanisms must be confirmed, or at least queried, in gyrencephalic brains as well.

Control of Mode of Division

The cellular transitions that enable the switch from NEP → aRGC → neuron (direct neurogenesis) or from NEP → aRGC → IPC → neuron (indirect neurogenesis) have a large impact on the eventual size and neuronal complexity of the neocortex. Here we define "mode of division" as the mechanisms that operate within or upon a dividing cell to result in either symmetrical or asymmetrical divisions. Symmetrical divisions occur when the resulting daughter cells share the same fate, whereas asymmetrical divisions lead to daughter cells with different fates. The importance of control of mode of division in neocortical development was first promulgated in the radial unit hypothesis three decades ago (Rakic 1988a, b). Since then, many studies have shown that cell-cycle duration, cleavage plane orientation, diffusible factors (extracellular cues), nascent gene expression, and changes in precursor morphology together control the proper timing and extent of these transitions.

During the founder cell expansion phase prior to the onset of neurogenesis, NEP numbers grow exponentially as the cells symmetrically produce two new NEPs. Following this, NEPs must undergo "consuming" symmetrical divisions since they are rapidly replaced by RGCs, but the factors controlling the transition between these two types of apical precursor cells have not been conclusively identified. Once generated, aRGCs mainly divide asymmetrically, signaling the onset of the neurogenesis period, to produce either neurons or other IPCs which will themselves generate neurons. Many of the extrinsic and intrinsic factors influencing these aRGC divisions have been identified, including Shh, Wnt, BMPs, FGF, IGF, and *FOXG1* (Grove et al. 1998; Lako

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et al. 1998; Hanashima et al. 2002; Assimacopoulos et al. 2003; Abu-Khalil et al. 2004; Hanashima et al. 2004; Medina et al. 2004; Shimogori et al. 2004; Storm et al. 2006; Clowry et al. 2018). However, as we discuss below (see section on "Precursor Heterogeneity"), all of the IPC cell types known to exist in the mammalian cerebral wall appear to be derived from aRGCs by asymmetrical divisions (i.e., divisions yielding a self-renewed aRGC and an IPC). The factors controlling the genesis of each of these IPC classes have not been identified, and whether this process is stochastic or tightly programmed is as yet unknown. This is a critical knowledge gap, especially since many of these precursor types are simultaneously present during neurogenesis and contemporaneously produce daughter neurons at any given time. In addition, there is evidence that IPC diversity is altered in certain developmental disabilities, such as Fragile X and Down syndrome (Saffary and Xie 2011; Tyler and Haydar 2013). It is also well established that the aRGC cell cycle gradually lengthens during neurogenesis due to increases in S and G1 duration (Takahashi et al. 1995; Turrero Garcia et al. 2016). This increase in cell-cycle length is thought to play a primary role in neuronal production, and recent data also indicate that lengthening the M-phase can lead to precocious neurogenesis at the expense of the precursor pool (Pilaz et al. 2016). Following the asymmetrical division phase of neurogenesis, the aRGC population is largely exhausted by "consuming" symmetrical divisions resulting in two daughter cell neurons. During this stage, it has also been established that aRGCs are direct precursors both to cortical astrocytes and the neural stem cell population that persists in the adult brain, although the factors regulating these developmental pathways remain to be conclusively identified.

Cleavage Plane Orientation and Segregation of Apical Factors

The angle of the mitotic cleavage plane in relation to the apicobasal polarity of dividing precursors was first identified as critical for specifying mode of division in yeast and in *Drosophila* and *Caenorhabditis elegans* (Skop and White 1998; Theesfeld et al. 1999; O'Connell and Wang 2000; Doe and Bowerman 2001). The overall mechanism at play is that cleavage angle modifications can lead to even or uneven partition of fate-determining molecules to the resulting daughter cells. Numb, Prospero, Pon, and the Par complex (among others) have been identified as key players in this process, and their distribution is affected by vertical or horizontal divisions in many species (Chenn and McConnell 1995; Huttner and Brand 1997). While many groups have shown that mitotic cleavage plane is also important for mode of division in the developing neocortex (especially in the VZ), it is now clear that most VZ divisions occur with relatively little cleavage angle variation (most cleavages occur with vertical cleavage planes). However, even minor deviations from the vertical cleavage plane can result in unequal partitioning of the apical plasma membrane and its associated components, such as cadherin, prominin, and apical junctional

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complexes (Wang et al. 2009; Kim et al. 2010; Postiglione et al. 2011). Thus, a consensus view has emerged that cleavage plane modification is part of the coordinated process of controlling neurogenesis in the mammalian neocortex. Significant controversy remains, though, in terms of the precise fate of the resulting daughter cells following different orientations (i.e., which daughter differentiates in asymmetrical divisions). This is due primarily to technical challenges in using real-time imaging to follow a cell through mitosis and then track the resulting daughter cells until their fate can be determined. Moreover, because the three-dimensional environment (including cell–cell interactions and gradients of signaling factors) is critical for this process, *in vivo* live imaging is necessary to describe fully how mode of division is controlled during fetal neocortical development.

Association between Cell Cycle and Mode of Division

In addition to the shift in cleavage plane orientation during the fetal neurogenesis period, the neural precursor cell cycle lengthens during neurogenesis, primarily in G1 phase, and these two physiological changes cooperate to yield the proper numbers of neurons in the overlying cortical plate. Moreover, regional changes in cell-cycle kinetics across the developing neocortical wall fine-tune areal differences in neuronal number and laminar thickness. For example, the neighboring Brodmann areas 17 (primary visual) and 18 (visual association) of primate neocortex differ considerably in the number of supragranular neurons (more in BA17), presumably leading to a discrete functional competence in the visual cortex. Seminal work in 2005 demonstrated significant shortening in cell-cycle duration in the oSVZ of BA17, modulated by differential expression of $p27^{Kip1}$ and Cyclin E, enabling supragranular layers in BA17 to expand in comparison to the adjacent layers in BA18 (Lukaszewicz et al. 2005). While this study and others demonstrate some of the cellular mechanisms that lead to final control of neuron number within each area, it remains unclear how specific cell classes within layer 4, such as the spiny stellate neurons, are differentially produced. Regardless, the Lukaszewicz et al. (2005) study illustrates how the primary signals of areal demarcation can be operationalized as the supragranular layers in a given area are generated. In the relevant sections below, we discuss how intrinsic differences, diffusible factors, and various feedback mechanisms within the neocortical wall may provide the initial map and control the implementation of this developmental program.

Precursor Heterogeneity

It is now known that a variety of neural precursors contribute to the generation of excitatory neurons and the overall expansion of the neocortex. At the center of this process, aRGCs serve as stem cells: they give rise to neurons

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that form all six layers during the neurogenic interval and subsequently contribute to the production of glial lineages. After the founder cell expansion period ends, aRGCs give rise to an increasing number of IPCs, which in turn amplify neuronal output (Figure 2.2). To date, three distinct classes of IPCs have been characterized in the dorsal germinal compartment of all mammals based on differences in gene expression, morphology, and location of mitoses. These include basal IPCs (bIPCs), apical intermediate progenitors (aIPCs), and bRGCs. The first to be identified were the bIPCs: multipolar cells which form a second proliferative area (the SVZ) that overlays the VZ. These cells express the transcription factor T-box brain protein 2 (Tbr2, or Eomes) protein and undergo primarily symmetric divisions to produce neurons, with increased numbers contributing to the formation of the supragranular layers (Englund et al. 2005; Kowalczyk et al. 2009). Like bIPCs, aIPCs undergo exhaustive symmetric divisions to produce neurons. However, aIPCs reside in the VZ, express paired box 6 (Pax6) but not Tbr2 protein, and divide at the ventricular surface (Gal et al. 2006; Stancik et al. 2010; Tyler and Haydar 2013). Like aRGCs, aIPCs exhibit a radial morphology and maintain contact with the ventricle via an apical process but lack a basal process that extends to the pia mater (Mizutani et al. 2007; Elsen et al. 2013; Nelson et al. 2013; Pilz et al. 2013). While aIPCs constitute a considerable portion of the progenitor pool during mid-neurogenesis in the rodent (Gal et al. 2006; Tyler and Haydar 2013), many details about their numbers and contribution to neuronal production during early and late neurogenesis and across species remain to be elucidated. It should be noted that apical neural precursors without pial-contacting basal processes were recently described in the fetal human brain (Nowakowski et al. 2016). Whether these cells represent the primate version of aIPCs is currently unresolved. A third class of IPCs, bRGCs, undergoes mitoses in the intermediate zone (IZ) and oSVZ, and maintains a long basal fiber extending to the pia. bRGCs were first identified in the developing human brain as an expanded population of neurogenic progenitors in the oSVZ (Fietz et al. 2010; Hansen et al. 2010; Shitamukai et al. 2011; Betizeau et al. 2013). At the molecular level, bRGCs resemble aRGCs in that they express canonical stem cell markers including Pax6 and Sry-box2 (Sox2). Furthermore, bRGCs exhibit a greater propensity to undergo self-renewing or transient-amplifying divisions compared to bIPCs and aIPCs. While these descriptions denote the major classes of neuron-producing progenitors, further complexity has been suggested by evidence which shows that bRGCs and bIPCs are also heterogeneous with respect to their morphology and division parameters. For example, at least five distinct bRGC types with unique lineal attributes have been identified in the primate (Reillo et al. 2011; Pilz et al. 2013; Pfeiffer et al. 2016). Thus, emerging evidence suggests the existence of subclasses of precursors within each of these three IPC types.

The biological significance of neural precursor variation is only now beginning to be understood. Precursor diversity has been postulated to contribute

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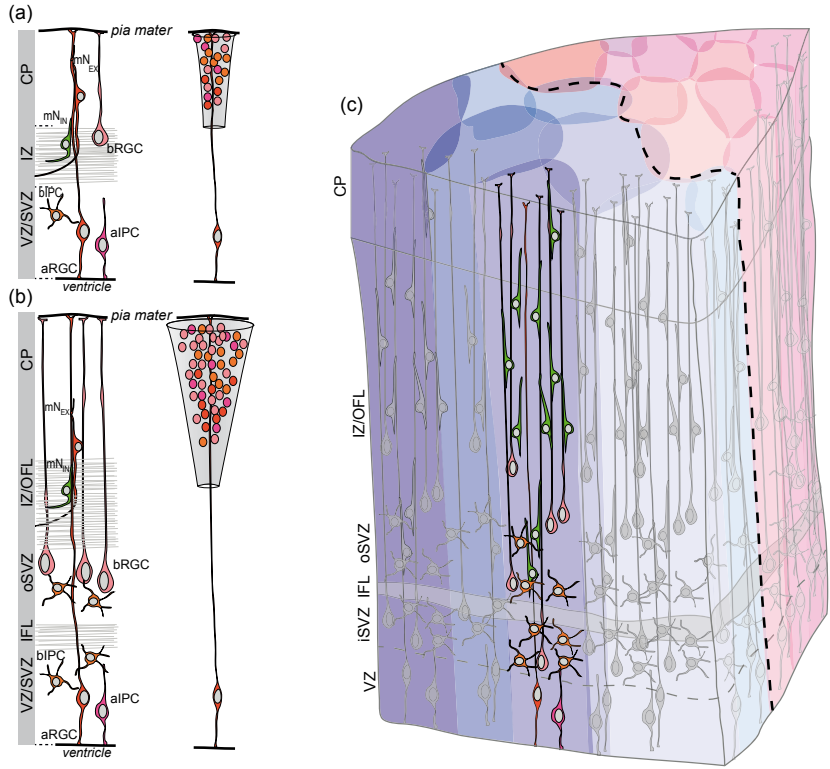

Figure 2.2 Precursor diversity and clonal output during dorsal cortical neurogenesis. Mouse (a) and primate (b) neocortical progenitors include apical radial glial cells (aRGCs), apical intermediate precursor cells (aIPCs), basal intermediate precursor cells (bIPCs), and basal radial glial cells (bRGCs). Migrating excitatory and inhibitory neurons (mN_{EX} and mN_{IN}) climbing to the cortical plate (CP) through the intermediate zone/outer fiber layer (IZ) are also depicted. While both lissencephalic and gyrencephalic neocortices contain similar precursor types, there are comparatively more bRGCs and bIPCs in the gyrencephalic brain neocortical wall, leading to a larger neuron output from each aRGC. Furthermore, these precursors are split into the inner and outer subventricular zones (iSVZ and oSVZ) by the inner fiber layer (IFL). Increased output from the aRGC-derived precursor cells during the late stages of neurogenesis in gyrencephalic species leads to a conical shape of the radial unit due to increased production of supragranular neurons. (c) Cartoon depicting a section of gyrencephalic neocortical wall taken near the boundary of two cortical areas (pink and blue areas separated by dashed line). Each area is populated by the clonal distribution of neurons from aRGCs. Each aRGC generates a cone of neurons through direct and indirect neurogenesis (shaded radial stripes) and the boundaries of these cones of neuronal allocation are thought to slightly overlap.

to two main processes that (a) increase the size of the neocortex, potentially leading to gyrification, and (b) generate neuron diversity. A primary focus of the field for the last decade has been the role of precursor heterogeneity and

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how it relates to the neocortical growth of higher mammalian species. Several studies have noted that brain size is correlated with an enlargement of both subdivisions of the SVZ and with increased numbers of IPCs. In particular, the gyrification index of a wide array of species has been linked to the size of the bRGC population (Fietz et al. 2010; Hansen et al. 2010). While species with a lissencephalic cortex contain bRGCs, and some (e.g., marmoset) even develop a distinct oSVZ compartment (Garcia-Moreno et al. 2012; Kelava et al. 2012), the numbers of bRGCs within these brains are reduced relative to gyrencephalic species. Nonetheless, how bRGCs contribute on a macro scale to cortical formation is still at a theoretical stage. For instance, the human brain has expanded more in the lateral dimension than in the radial dimension (thickness) and it is unclear how and whether bRGCs lead to a preferential increase surface area rather than thickness. Because of these unanswered questions, a primary mechanism driving species-specific differences in brain growth remains the expansion of the founder cell population prior to the onset of neurogenesis (Figure 2.1). Taken together, differences in brain size across the phylogenetic tree are likely due to the synergistic effects of a larger founder cell population and species-specific differences in the composition of the IPC pool as well as changes in the total neuronal output per precursor type.

A second role for the precursor heterogeneity in brain development may be that it contributes directly to neuronal diversity. The classes of excitatory neurons which comprise the six layers of the neocortex have been characterized by their birth date, molecular expression, electrophysiology, and target-specific projections. Clonal analysis by genetic fate-mapping has shown that individual aRGCs can produce neurons that span all of the neocortical layers, suggesting that aRGCs may be progressively tuned over developmental time to generate different types of excitatory neurons. Transplantation experiments indicate that isolated aRGCs generate neuron types appropriate for their birth date, even when placed in a heterochronic environment (McConnell and Kaznowski 1991), further supporting the notion that aRGCs undergo temporal fate restriction. Another assumption contained within this progressive fate restriction model is that aRGCs of any given developmental age are all identical, but *in vivo* evidence for this assumption is thus far unconvincing. It is also important to note that the molecular mechanisms underlying this temporal fate restriction have not yet been identified. Nevertheless, while this model may explain a primary mechanism for producing the different neuronal cell types across neocortical layers, it does not describe a method for generating different types of neurons within each layer.

Morphological and electrophysiological studies have defined distinct intralaminar populations of excitatory cells and shown that intralaminar diversity differs across areas. At any given time during the neurogenic interval, neurons are contemporaneously produced directly from aRGCs as well as indirectly from multiple classes of IPCs. As shown from birth-dating studies, these neurons are destined for the same neocortical layer, and mouse studies have

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suggested that neurons born via these parallel routes mature into distinct types of neurons. In particular, we have recently shown in mouse frontal cortex that layer 2/3 neurons generated from Tbr2-expressing progenitors differ from contemporaneously produced non-Tbr2-derived neurons with respect to their electrophysiological properties and the complexity of their apical dendritic arbors (Tyler et al. 2015). These results indicate that neurons are seeded with information from their parental lineage; this information is retained as they migrate to the appropriate layer and manifests a lineage-specific morphological and electrophysiological profile. To test whether this precursor lineage model for intralaminar neuronal diversity is a general rule that applies also to deeper layers and across different areas of the neocortex, we repeated this effort in mouse somatosensory neocortex by fate-mapping the same lineages during production of layer 4 (Guillamon-Vivancos et al. 2018). Results indicate that neurons derived from Tbr2 and non-Tbr2 lineages establish unique settling patterns within the somatosensory barrels and are different in terms of dendrite complexity and firing patterns (as in layer 2/3 of frontal cortex) as well as in their synaptic coupling with thalamocortical afferents.

Collectively, these studies suggest that precursor programs may directly influence how neocortical neurons participate in neocortical microcircuits. However, the lack of understanding of the true scale of precursor heterogeneity is a current roadblock. For example, several different types of aRGCs may exist and there may also be many subtypes of bIPCs and bRGCs. Indeed, there has been a rapid increase in identified neocortical precursor cell types over the past decade, including subapical progenitors (SAPs) (Pilz et al. 2013), quiescent or laminar-fated aRGCs (Franco et al. 2012), and, most recently, truncated RGCs in second trimester human neocortex which resemble aIPCs (Nowakowski et al. 2016). These cells are primarily identified based on morphological criteria and division site, as well as by time-lapse imaging in cortical slices. Despite this type of evidence, newly identified precursor types will remain controversial until they can be molecularly identified. Indeed, one of the rate-limiting steps in this line of research is the development of molecular markers for *in vivo* use of all of the different types of morphologically identified precursor cells. Only once the true scale of precursor heterogeneity is elucidated can comprehensive studies that investigate the role of individual cell types in cortical development and/or evolution be established.

In recent years, single-cell transcriptomics has provided new insight into the molecular signatures of neural precursors in humans, primates, and rodents. Several studies have successfully identified the core gene expression patterns of aRGCs, bIPCs, and to a lesser extent bRGCs, as well as specific differences which may underlie the properties of neuronal progenitors across species. However, the regional differences in aRGC expression predicted by the protomap hypothesis are as yet absent in single-cell RNA sequence analysis. While some studies have presented molecular signatures for potential subpopulations of progenitors, for the most part the number of unique cell types

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identified has not reflected the full scope of heterogeneity predicted by histological and time-lapse studies. For instance, the five types of bRGCs identified in the primate brain (Betizeau et al. 2013) have not yet revealed themselves as distinct cell classes at the RNA expression level, nor has the genetic fingerprint of aIPCs, SAPs, and truncated RGCs been elucidated. It has already been established that genes like *Trnp1* and *AP2γ* may be expressed in aRGCs producing bIPCs (Pinto et al. 2008, 2009; Stahl et al. 2013), but whether these genes mark divisions yielding other IPCs, such as aIPCs and bRGCs, is not yet clear. Are there molecular differences between these aRGCs (i.e., are there individual subtypes of aRGCs), or is each one of these asymmetrical divisions a stochastic choice? One of the basic assumptions in the field is that any given particular division outcome is reflected by a specific gene expression, yet these molecular signatures remain elusive. The lack of conclusive data in this area raises important questions regarding what defines a cellular "type" versus a distinct cellular "state." One possibility is that the full scope of cellular phenotypes may not be revealed at the RNA level. Instead, translational control may exert an as yet unappreciated influence on progenitor biology. In support of this hypothesis, several studies in progenitors have suggested that certain transcripts may be expressed but not translated into protein, providing a priming mechanism to accelerate the differentiation/commitment of their daughter cells upon cell division (Pinto et al. 2009; Albert et al. 2017).

Regional Specification of the Neocortical Map

The idea first ramified in the protomap hypothesis—that regional identities first emerge within the neural precursors and that this sets the stage for the mature functional cortical areas—is now widely accepted. Intrinsic characteristics of neocortical precursor cells, such as gene expression, cell-cycle duration, and fate potential, vary across the dorsoventral and rostrocaudal axes of the developing neocortex prior to the influence of subcortical input. In general, the concept that diffusible morphogens circulate across the developing precursor cells and instigate proliferative responses has been proven in multiple CNS areas and in many different species. In rodents and primates, the signaling centers for these various factors, including Shh, Bmps, Wnts, COUP-TFs, and FGFs, develop in multiple and discrete regions of the nascent telencephalon and release their contents during the founder cell expansion phase (Sur and Rubenstein 2005; Clowry et al. 2018). The crossing gradient fields established by these disparate signaling centers lead to regional expression of transcription factor genes in the precursor zones, including *PAX6*, *EMX2*, *COUP-TF1*, *SP8*, *PEA3*, *BHLHB5*, and *OLIG2*. These transcription factors set in motion a cascade of gene expression events that consolidate both the genetic and positional identities of the constituent precursor cells. Recent evidence also suggests that neurogenic factors released into the cerebral spinal fluid (CSF) generate

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proliferative responses in the neocortical germinal zones. Released into the CSF from the choroid plexus during fetal development, IGF1 and IGF2 can also modulate cortical growth (Lehtinen et al. 2011). There are potentially dozens of molecules in the CSF that can exert similar effects, but whether these factors initiate or merely act upon regional differences in the neocortex remains unclear. The absence of discrete sites of release of these factors and the circulation of fluid within the ventricles makes them an unlikely primary factor for regional diversity, for example, between directly adjacent areas such as BA17 and BA18.

Once the protomap has been established in the precursor cells, part of the intrinsic program includes the expression of cell surface molecules, including cadherins, protocadherins, neurexins, and ephrin receptors that distinguish each area. These molecules are expressed on the cell membranes within each area as well as within the local extracellular matrix. The expression of this "areal marking" mechanism on the axonal membrane of cortical efferents is also thought to be critical for attracting the proper classes of reciprocal thalamic projections as well as ingrowing interneurons that migrate from the basal telencephalon (handshake hypothesis). Once the interneurons have entered the neocortex, cell-adhesion molecules within their growth cones linked to their cytoskeleton mediate the final decision of whether or not to integrate into a particular area.

While the radial migration of cortical pyramidal neurons along the basal fibers of aRGC occurs within each radial unit and does not necessarily rely on areal information, the long-range migration of cortical interneurons and the extension of axonal fibers to disparate targets require mechanisms that confer a navigation system to the developing cortical map. Surprisingly, several pieces of evidence suggest that the spherical telencephalon is organized by a rectilinear map onto which migration routes and axonal pathways are superimposed, much in the way that lines of longitude and latitude organize the surface map of the earth.

Evidence for a Rectilinear Map

Two major pieces of data indicate that the telencephalon may be organized by an orthogonal grid. First, when tangentially migrating interneurons are imaged near the neocortical pial surface, either in time-lapse imaging studies or by electron microscopy, they are oriented predominantly along perpendicular axes. Intriguingly, the Cajal-Retzius (CR) cells overlying these migrating cells are also oriented along the same axes, indicating that the same cues organizing CR morphology may also direct interneuron migration (Ang et al. 2003). Second, recent diffusion magnetic resonance imaging has shown that cortical fiber pathways in primate brain also conform to a three-dimensional grid, with pathways crossing each other along three main axes (Wedeen et al. 2012). Furthermore, axons labeled with tract tracers turn with

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near 90-degree precision along these grids and axons from individual neurons labeled with the Golgi impregnation technique (Mortazavi et al. 2017), or with intracellular fluorescent tracers, have long been known to emanate collaterals from 90-degree branch points along the main axonal shaft. More work is needed to identify the molecules that may form such a grid and to determine if the white matter and interneuron migration grids are identical. Such a grid, if present, would constitute a coordinate system laid over the intrinsic determinants of the protomap, enabling a framework for long-range migration and tract formation as the neocortical surface grows and convolutes during development.

Local Implementation of the Neocortical Map

Once cortical areas have been established in the progenitor zones, several mechanisms ensure that the local program for each area is maintained during radial expansion of the overlying neocortical wall. In all mammals, the fact that regions across the neocortical map develop along different timelines elucidates a general maturational gradient. Within the context of this gradient, changes in mode of division, cell-cycle duration, and precursor subtype control the number and types of neurons across the neocortical laminae within the local area. However, there are many areas of the neocortex that deviate from this gradient-based development, where major differences in gray matter thickness are quite evident between adjacent areas. Moreover, the numbers and types of cells within each lamina are variable across areas and this again is most strikingly observed in the transition between BA17 and BA18 of the primate neocortex. In particular, stellate neurons in the granular layer 4 of BA17 are numerous while they are absent or at least sparsely present in layer 4 in the neighboring BA18. This indicates that areal identity, transmitted initially very early during formation of the telencephalon, is maintained throughout neurogenesis so that specific cellular landscapes develop in each area across the neocortex. These landscapes include markers that recruit ventral interneuron cell types, and this unique cellular and molecular milieu, combined with the ensuing afferent synapses, yield the final architectonic character and function of each cortical area. The fact that some of these area differences are very stark (i.e., not simply a gradation when compared to neighboring areas) indicates that local mechanisms can independently control radial size of the neocortex as well as the intralaminar neuronal diversity within each area. It is important to emphasize that cortical afferents are known to have the ability to modulate area-specific programs of development and to participate in the morphological development of key structural areas in the neocortex including barrels and functional columns. However, it is the combination of the unique marks that identify an area and the precise level of diversity in neuronal number and type that provides the playing field for these afferents.

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The transmission of this local developmental program necessitates at least two biological events: the first requirement is a unique molecular "agenda" that is maintained in the precursors during neuronal production so that the proper numbers and types of neurons are produced. This manifests as local control of cell-cycle duration, mode of division, and precursor heterogeneity. Several key studies indicate that gene expression can specify the type of neuron to be produced so that the full panoply of cortical excitatory neurons can develop in the expanding neocortical wall. As an example, we can consider the molecular mechanisms specifying subcortical projection neurons. The gene for the zinc finger protein Fezf2 (or Zfp312) is necessary for subcortical projection neuron morphology and formation of the corticospinal tract. Fezf2 is expressed highly in aRGCs during early phases of neurogenesis when the projection neurons of layers 5 and 6 are generated and is then downregulated during later phases of neurogenesis. As the first-generated neurons migrate to their proper layers, additional transcription factor genes, such as *Sox5* and *Tbr1,* repress *Fezf2* activity in layer 6 neurons, resulting in a layer 5-specific contribution to the corticospinal tract (Chen et al. 2005a, b; Kwan et al. 2008; Han et al. 2011; Guo et al. 2013). While these studies have begun to unpack how gene expression can control neuronal character, the molecular code underlying the specification of the many other types of cortical neurons have not yet been discovered.

Second, this areal identity must be transmitted to neurons as they are produced so that they can support the function-specific aspects of synaptic development that occur only after they achieve their proper positions and differentiation status. While the identity of the molecules imparting the area-specific maps (e.g., cadherins, Protocadherins, Eph) have been partially discovered, new techniques, such as microdissection/bulk RNASeq and single-cell RNASeq, may provide a more comprehensive list of these areal marks in the near future.

Two mechanisms leading to local area development have also been suggested more recently. The first, feedback from the emerging neocortical layers to the precursor zones, presumably operates in each area. The second, local control of precursor heterogeneity, must entail molecular programming that is intrinsic to each area.

Radial Feedback in Progression of Cortical Growth

In all neocortical areas, neurons of the deepest cortical layers are born first, followed in temporal succession by neurons destined for the more superficial layers. As mentioned above, this temporal specification of neocortical layers could be accomplished by an intrinsic "progressive tuning program" in aRGCs whereby they transition molecularly as they age during neurogenesis. Alternatively, electrical, biochemical, and genetic feedback from the growing

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laminae could modulate aRGC production parameters to fine-tune the overall extent of neuronal numbers during the neurogenic period. While these two ideas are not mutually exclusive and the system likely operates with both mechanisms, several lines of evidence indicate that coupling of cells within a radial clone could operate a feedback pathway. For example, single-cell fluorescent dye injections are known to label a discrete population of precursor cells surrounding an injected aRGC, and it has been found that the dye-coupled clusters are electrically coupled by gap junction channels (Bittman et al. 1997; Bittman and LoTurco 1999). Indeed, calcium waves initiated near the pial surface spread apically into the VZ and are transmitted for significant distances within the germinal zones (Owens and Kriegstein 1998; Owens et al. 2000). In addition, clonal labeling experiments have shown that neurons born sequentially from a single aRGC and destined for different layers are also gap junction coupled to one another and to their mother aRGC; later, these sister neurons preferentially form chemical synapses (Yu et al. 2009b; Gao et al. 2013; He et al. 2015). Thus, fast intracellular signaling methods have evolved to couple neurons within the developing neocortical layers to their precursors lying below. Moreover, gene expression feedback loops (Toma et al. 2014) as well as gene expression/growth factor loops are another identified mechanism, the latter highlighted in the connection between Sip1 expression by post-mitotic neurons and the release of signaling factors to underlying precursor cells (Seuntjens et al. 2009; Parthasarathy et al. 2014). Several other released factors have been implicated in this type of feedback as well, including nitric oxide and ATP.

While regulation of intracellular Notch signaling is thought to be a basic mechanism regulating cellular diversity, including in the neocortical VZ (Rasin et al. 2007; Kopan and Ilagan 2009; Ables et al. 2011; Pierfelice et al. 2011), recent work suggests that there is a Notch-based feedback mechanism between bIPCs and aRGCs during neurogenesis. In particular, Delta 1 and Delta 3 expressing bIPCs contact Notch-expressing aRGCs to modulate their mode of division and repress their differentiation (Mizutani et al. 2007; Kawaguchi et al. 2008; Yoon et al. 2008; Nelson et al. 2013).

Importantly, these feedback circuits enable the germinal zones not only to fine-tune cortical production but also to compensate for developmental or environmental insults by altering division parameters in the VZ and SVZ. For example, potentially in response to reduced neurogenesis and radial growth during early corticogenesis, the bIPC population in the Ts65Dn mouse model of Down syndrome is amplified during the later stages of neurogenesis, largely correcting (in bulk numbers) a severe paucity of early-born neurons with an overproduction of later-born neurons (Chakrabarti et al. 2007). While large insults to the developing system cannot be compensated for and may lead to lasting changes in cortical thickness or surface area (such as microcephaly), there is evidence supporting a level of plasticity in the germinal zones that responds to feedback from cells in the overlying neocortical layers.

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Mechanisms of Gyrification

One of the most remarkable features of the neocortex is the patterned folding of its surface during the evolution of certain mammals. The stereotypical gyri and sulci that develop are thought to be a general mechanism for fitting a neocortex with greater surface area into the volume required to pass through the narrow birth canal and to fit within the confines of the calvarium. Within all gyrencephalic species, the pattern of folding is highly concordant with the neocortical map, such that functional areas routinely lie across the same gyri or sulci in the brains of different individuals. Due to the importance of this event in specifying the size and function of the brain in gyrencephalic species, and the recognition that developmental disorders arising by gene mutations can significantly alter the degree of gyrencephalization, how these folds occur in such a repeated fashion across individuals is a hotly debated topic in the field of cortical development. The primary data supporting each of the theories outlined below come from comparative analysis of lissencephalic and gyrencephalic brains, gene perturbation studies, and complex computer simulations based on imaging studies.

Three overarching theories have been proposed to explain how the neocortex is folded and how this occurs in such a stereotypical pattern within a species. While each theory has a list of studies both supporting and opposing it (an exhaustive list of these corresponding studies is not described here), the most parsimonious explanation is that each of the three concepts partially identify some of the biological events and that they combine to yield cortical folding.

The *axonal tension hypothesis* considers axon connections to be the primary driving force of a folded neocortical sheet (Van Essen 1997; Holland et al. 2015). It proposes that corticocortical fibers primarily connecting two ipsilateral regions can create localized regions of tension, thereby generating a prolonged mechanical force that results in folding of the neocortical surface to produce a gyrus. Correspondingly, neighboring regions that are less well-connected will form the reciprocal event—the formation of a sulcus. The *radial expansion hypothesis* (Richman et al. 1975) suggests that differential production of neurons across the 6 neocortical layers, for example, increased generation of supragranular neurons compared to infragranular neurons, can result in localized tangential spread, or wedging of the neocortical sheet, and that this convexity may later blossom into a gyrus. Conversely, overproduction of deeper layer neurons compared to the superficial layers can result in incipient concavity and formation of a sulcus. Lastly, the *differential tangential expansion hypothesis* (Ronan et al. 2014) suggests that isolated regions of the developing neocortical wall undergo different rates of tangential expansion. This could occur during the founder cell expansion phase or during the period when specific classes of IPCs emerge and begin to divide. The tangential forces generated between neighboring areas then lead to buckling of the neocortical sheet and to formation of gyri and sulci.

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In general, these theories can be simplified as mechanisms operating at the level of the precursor cells within each neocortical area and to those influenced by migrating and differentiating neurons and to afferent cortical projections. For example, both of the expansion hypotheses rest on differential cell production, either in the radial domain (i.e., laminar differences) or the tangential domain (i.e., cortical column number). Both, therefore, suggest that isolated programs of neurogenesis along the protomap-specified areas yield the resulting pattern of cortical folding most appreciated after birth. Incidentally, while the axonal tension hypothesis primarily focuses on forces generated by differentiated neurons, it also invokes the area-specific projection and targeting patterns that must be conferred to the neocortical layers upon their generation from the underlying precursor cells. Thus, all theories for gyrification require a significant role for precursor cells in establishing the regions that will eventually undergo convolution.

Recent studies have provided a role for precursor gene expression and its consequences on neurogenesis as a predictor of gyrification. In particular, a key study identified the cell-adhesion molecules FLRT1 and FLRT3 in regulating area-specific formation of gyri and sulci (Del Toro et al. 2017). These two genes are upregulated in the lissencephalic mouse neocortical wall and downregulated in the gyrencephalic ferret and human neocortical wall in regions of incipient gyrus formation. This study shows that perturbations to lower the levels of FLRT1/3 expression lead to faster neuronal migration rates and to clusters of neurons expressing a similar level of these adhesion molecules. The increased radial and tangential pressure caused by these clustered neurons is postulated to lead to localized formation of a gyrus, even in the normally smooth mouse neocortex. Another gene recently identified in the germinal zones that plays a role in neocortical folding is *Trnp1*, which encodes a nuclear protein potentially involved in chromatin state (Stahl et al. 2013). Knockdown of *Trnp1* alters the pattern of cell division in the VZ, causing overproduction of bIPCs and bRGCs. The hypothesis of this study is that increases in the numbers of resulting neurons, and their tangential spread afforded by the increased number of bRGC fibers, result in gyrus formation in the overlying neocortical sheet. A study by de Juan Romero et al. (2015) offers perhaps the most convincing argument for a link between differential gene regulation in the germinal zones, expansion of bRGCs, and formation of overlying cortical convolutions. In this study, regions of the ferret neocortical wall, which later develop either a gyrus or a sulcus, were isolated at a stage in early development prior to the formation of these folds. Upon microarray profiling, many hundreds of genes were differentially expressed between these two areas, and clear expression of these genes in the oSVZ in the future gyrus site was contrasted to the lack of their expression in the oSVZ of the neighboring future sulcus site. In addition, a human RGC-specific gene, *ARGAP11B*, was shown to increase basal precursor proliferation and cortical folding when introduced into the developing mouse germinal zones (Florio et al. 2015).

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As described in the precursor heterogeneity section above, individual classes of precursor cells are thought to generate neurons with different dendrite morphologies, electrophysiology, and numbers of synapses with cortical afferents. Thus regions of neocortex with specific numbers and types of precursor cells could differentially produce specific neuron types and yield locally distinct rates of neuropil growth, based on differentiated neuron morphology and synapse capability. This could play a large role in the consolidation or growth of nascent convolutions during later stages of neocortical development. Altogether, several lines of evidence demonstrate that differential neurogenesis may lead to cortical convolutions, by promoting basal precursor production, by modulating neuronal migration rates and adhesive properties, or by providing unique areas for neuropil expansion.

A number of compelling studies also indicate a role in cortical afferent projections in gyrification. First, in enucleation studies, when input from an eye is removed during early cortical development prior to the formation of convolutions, the size and number of resulting gyri and sulci are significantly alterred (Rakic 1988b; Dehay et al. 1996). These studies show that afferent input into the area that will eventually form a gyrus is required for proper development of the convolution pattern. Second, MRI imaging studies from prenatal human brain indicate that gyral patterning is dependent on regional growth heterogeneity as well as axonogenesis and afferentation (Knutsen et al. 2013; Razavi et al. 2017; Wang et al. 2017). Taken together, all of these studies clearly indicate that the pattern and extent of gyrification in certain species is a combined result of programmed events in the germinal zones and influences of cortical afferent systems on synaptogenesis, as well as due to expansion of local regions of the neuropil.

Summary

Similar to the development of any body organ, formation of the cerebral cortex requires a complex choreography of stem and progenitor cell allocation, cell division, production of the requisite numbers and types of cells, and the eventual differentiation of these cells into mature functional components of the maturing organ. In this chapter, we have discussed several developmental events that pertain especially to the cerebral cortex, including the migration of neurons from their site of birth to their proper location, which can be over several millimeters in the primate brain. In addition, the numbers of excitatory neurons and inhibitory neurons must be tuned within each area and layer, an incredibly intricate process due to the distant proliferative zones from which these cell types are derived. How these cells initiate and consolidate the synapses and circuits necessary for complex function is being elucidated at a rapid pace, as are the genetic and molecular mechanisms which underlie all of these crucial developmental events. Focused effort must be paid in the near future

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to fully elucidate the molecular controls of cell commitment and allocation, in particular the precise relationships between neocortical precursor cells and their resulting neuron offspring. The exceptional advances over the past several decades described herein will soon culminate in a clear understanding of how cell number and type relates to circuit formation and eventually to behavior and cognitive function. When this is accomplished, a clear roadmap will exist not only for understanding the most complicated biological machine currently known, but also for the design of therapeutic approaches for developmental disorders that affect cognitive and intellectual function.

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