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Generating Neuronal Diversity in the Mammalian Cerebral Cortex

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Abstract

The neocortex is the part of the brain responsible for execution of higher-order brain functions, including cognition, sensory perception, and sophisticated motor control. During evolution, the neocortex has developed an unparalleled neuronal diversity, which still remains partly unclassified and unmapped at the functional level. Here, we broadly review the structural blueprint of the neocortex and discuss the current classification of its neuronal diversity. We then cover the principles and mechanisms that build neuronal diversity during cortical development and consider the impact of neuronal class-specific identity in shaping cortical connectivity and function.

Contents

INTRODUCTION

The neocortex is the crowning achievement of brain evolution, containing unparalleled cellular diversity that has evolved to support complex behaviors. The diversity of neocortical cell types, the sophisticated local and long-distance cortical circuits, and the remarkable functional capacities of the neocortex have made the study of cortical development, evolution, and function a topic of very high interest over a span of decades marked by continuing discoveries. An additional motivation for this research is that dysfunctional cortical networks or abnormal cortical development often translates into prominent neurodevelopmental and neuropsychiatric diseases, which remain poorly understood and largely untreated.

The basic structural and functional features of neocortical organization have been identified for many years, but much remains to be clarified regarding the cellular composition of the neocortex and its relationship to cortical function. Several questions are still at center stage in the field regarding the classification of cortical neurons and the strategies that build neuronal diversity during developmental corticogenesis. For example, at what point during the progression from progenitors to neurons are lineage bifurcation decisions controlled, and what is the regulatory logic that allows the development of so many neuronal classes? How, mechanistically, do these large numbers of neurons maintain their class-specific features for the life of the organism? Finally, how do so many neurons integrate during development of the local cortical circuit to guarantee balanced cortical activity and function?

This article first covers key concepts in the structural and functional organization of the cerebral cortex, highlighting select discoveries that have led to our current understanding of cortical organization. It then reviews the cellular composition of the cortex, focusing on neuronal diversity and the strategies that build it during development. Finally, it considers how neuronal, class-specific identity informs connectivity choices, integration into the local circuit, and the behavior of glia.

EVOLVING UNDERSTANDING OF TISSUE ORGANIZATION AND NEURONAL COMPOSITION OF THE NEOCORTEX

Basic Principles of Cortical Organization: Areas, Layers, and Columns

At the beginning of the nineteenth century, neuroscientists were already aware that regions with specialized functions could be identified on the surface of the cerebral cortex. This led to the theory of localization of functions, which preceded the identification of the first functional cortical area by neuroanatomist Paul Broca (1865), who demonstrated that speech was localized in a specific region of the frontal lobe. It is now well established that the neocortex is tangentially parcellated into many functional areas that process specific sensory modalities (e.g., vision, hearing, and touch). Cortical areas have defined tangential boundaries, different cytoarchitectonic features, and specialized patterns of afferent-efferent connectivity. For example, areas devoted to processing visual information are located in the caudal neocortex (the occipital cortex) and largely receive input from the lateral geniculate nucleus of the thalamus, which in turn is the target of afferent input from the retina (**Figure 1*a***). By contrast, areas that process auditory stimuli are located in the temporal cortex, rostrolateral to the visual cortex, and receive input from a different thalamic nucleus, the medial geniculate nucleus (**Figure 1*a***). The mouse neocortex consists of four primary areas: the somatosensory cortex (which processes sensory modalities, such as input from the vibrissae), the auditory cortex (which processes sound), the visual cortex (which processes the sense of sight), and the motor cortex (which outputs information to control fine motor behavior) (**Figure 1*a***). For an excellent review covering the development and structure of cortical areas, we refer the readers to Rash & Grove (2006).

The cortex displays unique cytoarchitectural characteristics, which arise from the organization and composition of the constituent cell types and circuits and vary in an area-specific manner. One distinctive feature of the neocortex is the organization of neurons into six horizontal layers, historically defined as supragranular (layer I/II–III), granular (layer IV), and infragranular (layers V and VI) (**Figure 1*b***). Layers contain different classes of neurons and vary in thickness and tissue architecture depending on their areal identity (reviewed in Greig et al. 2013).

From a functional perspective, neurons connect horizontally within and across cortical areas but also radially within functional columns that contain neurons from different layers connected in a highly stereotypical fashion (da Costa & Martin 2010). Functional columns were first defined in the cortex by Mountcastle (1957), who proposed the columnar hypothesis, which states that the cortex is composed of discrete, modular columns of neurons, characterized by a consistent connectivity profile. This discovery, originally built on data from electrophysiological recordings in the somatosensory cortex of monkeys, was a turning point in the understanding of neocortical organization. The columnar theory has been prominent in the field for more than 50 years, although investigation is still ongoing to determine whether columnar organization applies across the entirety of the neocortex (see the Blue Brain Project at **http://bluebrain.epfl.ch/page-52063.html** and Markram 2006) and whether, as the original formulation of the columnar hypothesis proposes, these columns are repetitive, modular, and canonical.

Despite some outstanding questions, several well-established examples of the columnar organization of the cortical circuit exist. The rat somatosensory cortex (or barrel cortex) illustrates this concept well (Petersen & Sakmann 2001). In rodents, sensory information travels from the sensory neurons in the whisker follicles through the brainstem to the thalamus, which then relays this information to the somatosensory cortex. Thalamic axons carrying information from individual whiskers form clusters of synapses, called barrels, in layer IV of the somatosensory cortex, whose spatial organization delineates a topographic map in the cortex corresponding to the arrangement

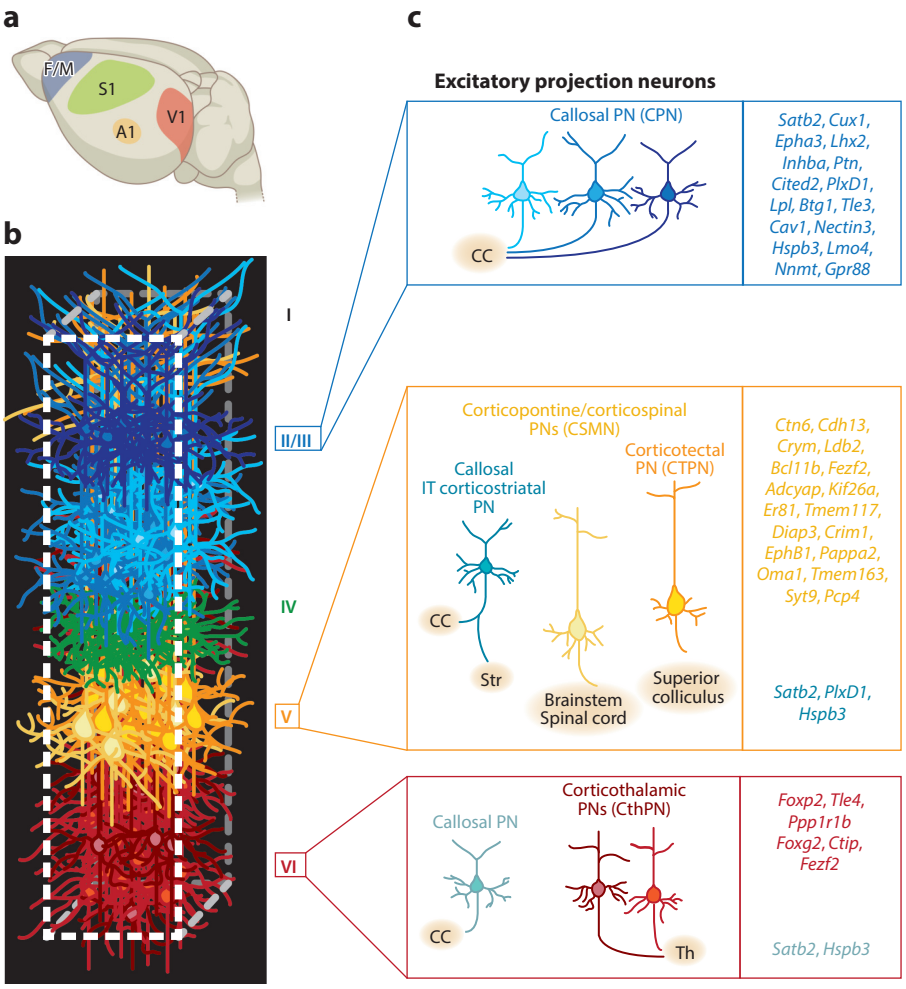

Figure 1

The neocortex is organized into areas, layers, and columns populated by a great diversity of excitatory and inhibitory neuronal subtypes. (*a*) Schematic representation of primary neocortical areas dedicated to processing distinct sensory modalities and governing fine motor control. (*b*) Cortical columns contain horizontally arranged layers with very diverse neuronal compositions. Only select examples are depicted here. (*c*) Layer II/III contains different classes of commissural neurons, primarily of distinct CPN identities. Layer V contains CPNs, often maintaining distinctive collaterals to the striatum (IT type of corticostriatal PNs), and different classes of subcerebral PNs that connect to the brainstem, spinal cord, and superior colliculus. Layer VI has different classes of CThPNs, connecting to separate thalamic nuclei and CPNs that connect through the CC. Cortical PN subtypes express unique gene signatures that in specific combinations identify each class (*listed on right*). Roman numerals refer to the six cortical layers. Abbreviations: A1, auditory cortex; CC, corpus callosum; CPN, callosal projection neuron; CTPN, corticotectal projection neuron; CThPN, corticothalamic; F/M, frontal/motor cortex; IN, interneurons; IT, intratelencephalic; PN, projection neuron; S1, somatosensory cortex; Th, thalamus; V1, visual cortex.

of whiskers on the animal's face (Frostig 2006). From layer IV, the whisker-specific signal spreads mostly vertically within a column to pyramidal neurons located in layer II/III, in which the signal is processed and integrated by horizontal transmission to neighboring neurons before reaching output neurons in layer V, which concludes the columnar processing of sensory information from the whiskers (Petersen & Sakmann 2001).

Neurons of the Neocortex

How many classes of neurons does the neocortex contain? Even ignoring that the answer is probably different in different species, it is fair to say that the current classification of cortical neuron diversity is at best incomplete. That the cortex contains many types of cells (including a variety of neurons) has been appreciated since early neuroanatomists, most prominently Ramón y Cajal, first began to investigate its cellular components. By employing a staining technique invented by Camillo Golgi (the *reazione nera*; today better known as the Golgi method), Ramón y Cajal (1909) generated extremely detailed morphologic depictions of individual cells in the cerebral cortex, uncovering great cellular diversity. One conclusion that has emerged from a now large body of work is that the neocortex has evolved an extreme heterogeneity of neuronal subtypes, subtypes that are still only partly classified and whose functions are not fully mapped.

Recent single-cell transcriptional profiles of adult cerebral cortex suggest the existence of molecularly distinct clusters of cells that appear to represent new types of neurons, as they cannot be assigned to classically defined populations (Johnson et al. 2015, Zeisel et al. 2015). Much more work is required to establish whether these are genuinely new, previously unappreciated neuronal types and whether these new populations have any distinct functional roles. However, it is likely that new neuronal groups await discovery and classification. As famously observed by Ramón y Cajal [1967 (1906), p. 240], "Unfortunately, nature seems unaware of our intellectual need for convenience and unity, and very often takes delight in complication and diversity." This statement still holds true today. Below we begin reviewing the current classification and developmental origin of both excitatory pyramidal (or projection) neurons (PNs) and inhibitory local cortical interneurons (INs).

Cortical Projection Neurons: The Principal Cells

PNs are excitatory, glutamatergic neurons that connect the cerebral cortex to the entirety of its distal intracortical, subcortical, and subcerebral targets. These cells make up the vast majority of neurons of the cortex (approximately 70–80%) and are stereotypically distributed within the layers. The nomenclature in use today to distinguish PN subtypes is still primarily based on hodology, i.e., long-distance connectivity, as originally proposed by Ramón y Cajal (1909). However, nowadays PN subtype-specific identity is defined by the intersection of several molecular, electrophysiological, morphological, and connectivity traits (**Figure 1*c***).

PNs can be broadly classified into intracortical and corticofugal neurons. Intracortical PNs are present in all six layers, but they are predominantly represented in the upper layer II/III. They can be further divided into associative and commissural PNs (CoPNs). PNs that project their axons either to targets in the same hemisphere or to different layers of the same area or column are called associative PNs (Molyneaux et al. 2007). By contrast, CoPNs project their axons to targets located in the opposite hemisphere, usually in a topographic manner, through one of two major fiber commissures: the corpus callosum (CC) or the anterior commissure (AC). The AC represents the most evolutionarily conserved commissure of the brain, and in nonplacental mammals, such as marsupials, which lack the CC, it is the only route by which bilateral information can be exchanged between homologous areas of the cortex. In rodents and in primates, including

humans, most CoPNs connect through the CC and are known as callosal PNs (CPNs); only a small number of neurons, primarily located in the lateral cortex, project through the AC (Aboitiz et al. 2003) (**Figure 1*c***).

The other main class of PNs, corticofugal PNs (CFuPNs), are primarily located in the deep layers and send their axons to distal targets outside of the cortex; they can be further classified into corticothalamic PNs (CThPNs) and subcerebral PNs (ScPNs). CThPNs are a heterogeneous population of PNs located in layer VI that project to different nuclei of the thalamus to modulate incoming sensory information. ScPNs reside in layer Vb across multiple areas and project their axons to distinct targets below the brain, predominantly to the pons and other nuclei of the brainstem, in which case they are called corticopontine PNs; to the superior colliculus, in which case they are called corticotectal PNs (CTPNs); and to the spinal cord, in which case they are called corticospinal motor neurons (CSMNs) (Molyneaux et al. 2007) (**Figure 1**).

Some classes of PNs send axons to multiple targets and do not easily fit into any of the classes described above. These include (*a*) the subset of ScPNs that have backward projections and extend axons to both subcerebral targets and the ipsilateral caudal cortex (Cederquist et al. 2013) and (*b*) the intratelencephalic type of the corticostriatal PNs, which are present in layers II–VI and project to the ipsilateral and contralateral striatum and also innervate the contralateral cortex (Shepherd 2013) (**Figure 1*c***).

This canonical system for PN nomenclature has been integrated with other classification criteria that consider, for example, electrophysiological and molecular properties. PNs with distinct morphologies and patterns of long-distance connectivity have distinctive electrophysiological properties, including modes of firing action potentials and intrinsic membrane properties. Although a fine-grained characterization of the intrinsic electrophysiological properties of specific classes across a range of PN subtypes is not available, this suggests that distinct subtypes of PNs process incoming information in ways that match their function and, possibly, that the molecular composition of each neuron affects the ultimate choice of electrophysiological behavior.

Over the past 10 years, several studies have tackled the difficult problem of isolating and molecularly profiling classically defined PN populations. Labeling approaches have included retrograde tracing of distinct neuron types (Arlotta et al. 2005, Catapano et al. 2008, LaVail et al. 1973, Molyneaux et al. 2009), immunopanning (Dugas et al. 2008), and, more recently, genetic labeling (Huang & Zeng 2013) and intranuclear immunostaining with antibodies (Molyneaux et al. 2015) to permit fluorescence-activated cell sorting of differentially labeled neuronal subtypes and subsequent molecular profiling. These studies have collectively provided the field with the first sets of PN class-specific signature genes, which can be used both to molecularly identify distinct classes and to investigate mechanisms of PN subtype-specific development and function (Greig et al. 2013, Molyneaux et al. 2015; S. Lodato & P. Arlotta, unpublished results) (**Figure 1*c***).

CPNs and CSMNs are among the best-characterized PN subtypes at the molecular level; distinct combinations of genes have been identified that can uniquely separate them. Examples of these molecular identifiers are *Fezf2*, *Cntn6*, *Cad13*, *Bcl11b*, *Cry-mu*, and *Ldb2* for CSMNs (Arlotta et al. 2005, Lodato et al. 2014a) and *Cux2*, *Inhba*, *Btg1*, *Lpl*, *Cited2*, and *PlexinD1* for CPNs (Molyneaux et al. 2009, 2015) (**Figure 1*c***). Molecular differences also allow one to distinguish between more closely related classes of CFuPNs, such as CSMNs and CThPNs, and between distinct classes of ScPNs, such as CSMNs and CTPNs (Arlotta et al. 2005). For example, *Diap3* labels CSMNs but not CTPNs, *Tle4* marks CThPNs but not CSMNs, and CSMNs uniquely express *Er81*, for which CThPNs are negative. For a searchable database of transcripts differentially expressed in developing CPNs, CThPNs, and ScPNs, we refer the readers to DeCoN (**http://decon.fas.harvard.edu**).

A few lessons can be learned from these molecular studies. First, the laminar coordinates of a neuron do not fully define its class-specific identity. For example, layer V contains many different

PN subtypes, of both commissural and subcerebral identity. In addition, different classes of PNs populate layer V in separate areas; CSMNs (a subpopulation of ScPNs) in layer V of the motor cortex subserve control of motor behavior, whereas CTPNs (a distinct subpopulation of ScPNs) in layer V of the visual cortex are responsible to process vision-related movements.

Second, molecular profiling suggests the presence of a higher degree of heterogeneity within PN subtypes than is apparent from their long-distance connectivity. For example, although CPNs across multiple layers express some common callosal genes, such as *Satb2* and *Hspb3*, that distinguish them from the corticofugal classes, genes that are only expressed in subpopulations of CPNs also exist, exposing an additional level of parcellation that until recently went unrecognized (e.g., *Cux2* labels CPNs of layers II–IV, *Ptn* labels CPNs located in the deepest part of layer II/III, and *EphA3* and *Nnmt* are only expressed in CPNs of the most superficial part of layer II/III) (Molyneaux et al. 2009, 2015) (**Figure 1*c***). Indeed, first-generation, unbiased molecular profiling of single cells isolated from the adult cerebral cortex supports the existence of molecularly distinct neuron populations that cannot be easily assigned to current categories (Zeisel et al. 2015). Third, class-specific profiles of gene expression are temporally dynamic, changing dramatically as neurons undergo lineage bifurcation and mature. For example, *EphB1* is specifically expressed in CFuPNs during development but its expression dramatically declines by postnatal day (P)7 (Lodato et al. 2014a).

Finally, molecularly defining PN classes requires consideration of multiple genes and their expression levels; classes cannot be defined by single markers. For instance, both CThPNs and CSMNs express *Bcl11b*; however, CSMNs express high levels of *Bcl11b* in combination with *Ldb2*, whereas CThPNs express low levels of *Bcl11b* and are *Ldb2* negative.

Cortical Interneurons: The Short-Axon Neurons

Cortical INs represent approximately 20–30% of the total number of cortical neurons and make local connections within the cortex that may span multiple layers (Lodato et al. 2014b). INs of the cortex are thought to be extremely diverse, and their classification has been a work in progress for many years. Traditionally, INs have been subdivided into two broad classes: spiny pyramidal and stellate cells (Yamawaki et al. 2014) and aspiny (or sparsely spiny) nonpyramidal cells (Kepecs & Fishell 2014). Spiny INs are excitatory glutamatergic neurons located in layer IV that receive sensory inputs from the thalamus; aspiny cortical INs are inhibitory GABAergic neurons located in all layers of the cortex. Aspiny INs are the main inhibitory component of neocortical circuits, finely modulating PN activity by regulating both synaptic function and the timing of action potential generation (Kepecs & Fishell 2014). Cortical GABAergic INs are very diverse; they contain subtypes that differ in morphology, molecular identity, firing properties, and patterns of local connectivity, and although tremendous progress has been made in their classification, to date no accepted integrated taxonomy exists (Markram et al. 2004).

Three comprehensive and nonoverlapping groups of INs can be distinguished in the neocortex based on the expression of distinct molecular markers: parvalbumin (PV), somatostatin (SST), and the ionotropic serotonin receptor 5HT3a (5HT3aR) (Kepecs & Fishell 2014, Marín et al. 2012). PV- and SST-positive INs are primarily found in the deep layers of the cortex, and 5HT3aR-positive INs preferentially populate the upper layers (Lee et al. 2010) (**Figure 2**). However, compared with PNs, the laminar distribution of the molecularly distinct IN groups is much less precise.

Within these three classes, many other subtypes can be identified based on soma morphology, the shape of axonal and dendritic arbors, and electrophysiological properties. PV-positive cells include fast-spiking (FS) INs belonging to two main morphological classes: large basket cells (which make synapses on the proximal dendrites and the somas of target PNs) and chandelier

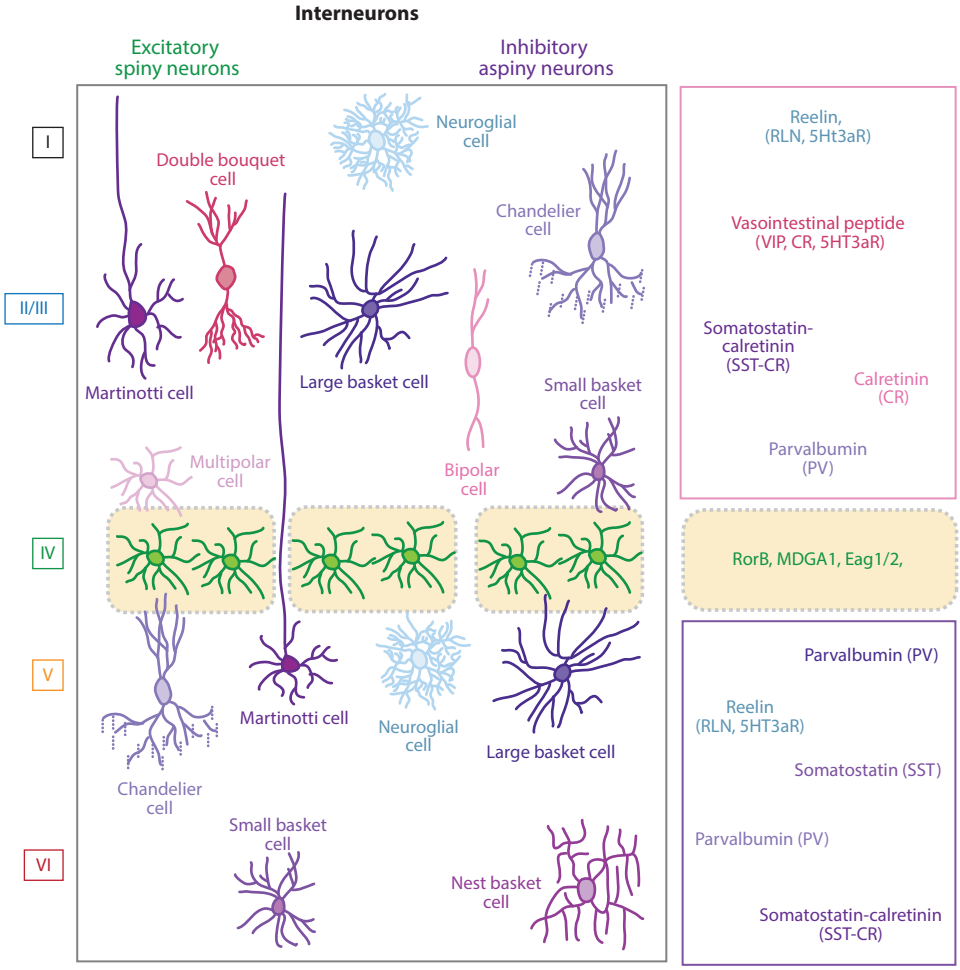

Figure 2

Neocortical interneurons are characterized by their short-range projections and can be broadly classified into excitatory and inhibitory interneurons. Here, we depict a schematic representation of the distinct excitatory and inhibitory interneuron classes within the six cortical layers. Excitatory spiny interneurons display mainly stellate and pyramidal morphology and are primarily located in the intragranular layer IV of the somatosensory cortex (barrel cortex, *shown in yellow boxes*). In contrast, each cortical layer contains different types of inhibitory interneurons, which display a wide array of morphologies and molecular identities. Both classes can be further classified into subtypes that express distinctive combinations of molecular markers (*listed on right*).

cells (which target the initial axonal segment of PNs). Nest basket cells are also PV-positive but exhibit a variety of firing properties (accommodating, nonaccommodating, and FS). SST-positive INs include small basket cells, which are not FS but target the soma and the proximal dendrites of pyramidal cells, and Martinotti cells, which are burst spiking, coexpress calretinin (CR), and target the distal dendrites of PNs in layer I (Ascoli et al. 2008, Markram et al. 2004, Vitalis & Rossier 2011) (**Figure 2**). 5HT3aR-positive cells include vasoactive intestinal polypeptide (VIP)- and CR-positive INs with bipolar morphology, targeting the proximal dendrites and firing in burst or adapting mode, and the double bouquet cells that mostly synapse onto other INs.

Also included in the 5HT3aR-positive category are neuropeptide Y (NPY)- and reelin-positive INs, which are located in the upper layers, are VIP negative, show multipolar or neurogliaform morphology, and contact the dendritic shaft and the blood vessels, respectively (Vitalis & Rossier 2011) (**Figure 2**). It is clear that INs are an extremely heterogenous group of neurons, and their classification has proved very complex and remains incomplete.

DEVELOPMENTAL GENERATION OF CORTICAL NEURONAL SUBTYPES

All neurons of the mammalian cerebral cortex are generated during a limited period of embryonic development whose length is species specific. In humans, cortical neurogenesis starts at gestational week (GW)5 and ends at approximately GW20 (Bystron et al. 2008). In rodents, neurogenic intervals are shorter, as shown by classic [^{3}H] thymidine labeling studies; cortical neurogenesis spans from embryonic day (E)11 to E19 in mice (Angevine & Sidman 1961, Caviness 1982, Takahashi et al. 1995) and from E13 to E21 in rats (Bayer & Altman 1991, Berry & Rogers 1965). These birthdate population studies, confirmed by more recent genetic studies (reviewed in Kohwi & Doe 2013), have shown that generation of cortical neurons proceeds in a precise temporal sequence, such that neurons of the deep layers are generated first, followed by those of the upper layers (Angevine & Sidman 1961). Although the initial experiments did not distinguish between PNs and INs, it is now firmly established that all classes of PNs and most early-born INs populate the cortex following an inside-out pattern of migration (Greig et al. 2013, Kohwi & Doe 2013). Interestingly though, a few classes of late-born INs do not obey this rule and populate the upper layers independent of birthdate (Miyoshi et al. 2010).

The molecular regulatory logic that builds this neuronal diversity during corticogenesis is likely to involve a plethora of distinct regulatory events. Here, we focus on the most recent findings on mechanisms employed at both the progenitor and postmitotic neuron stages to generate this unparalleled neuronal complexity.

Birth of Excitatory Projection Neuron Diversity: Decoding Progenitor Strategies

All excitatory neurons of the neocortex are generated from neural progenitors located in the dorsal telencephalon, within the anterior part of the neural tube. Before the onset of neurogenesis (~E9–E10 in the mouse), neural progenitors are neuroepithelial (NE) cells, which divide symmetrically to expand the early progenitor pool. As neuronal production begins, NE cells give rise to more committed progenitors, termed radial glial cells (RGCs). RGCs expand in the ventricular zone (VZ) lining the ventricle through multiple rounds of cell division before undergoing a terminal asymmetric, neurogenic division responsible for the generation of cortical PNs (Malatesta et al. 2000, Miyata et al. 2001, Noctor et al. 2001) (**Figure 3**). This process generates directly only 10–20% of the total number of excitatory PNs (Kowalczyk et al. 2009). Most excitatory neurons are instead derived from an additional type of cortical progenitor, intermediate precursor cells (IPCs), which are generated from RGCs via symmetrical, proliferative divisions (Haubensak et al. 2004, Miyata et al. 2001, Noctor et al. 2004). IPCs undergo mitosis away from the ventricle and over time generate a new germinal zone called the subventricular zone (SVZ), in which IPCs divide symmetrically to generate neurons (Fietz & Huttner 2011, Hansen et al. 2010, Haubensak et al. 2004, Taverna et al. 2014) (**Figure 2**). IPCs generate a substantial fraction of the cortical neuronal population, up to ~80% of all excitatory PNs across all cortical layers (Kowalczyk et al. 2009, Vasistha et al. 2015).

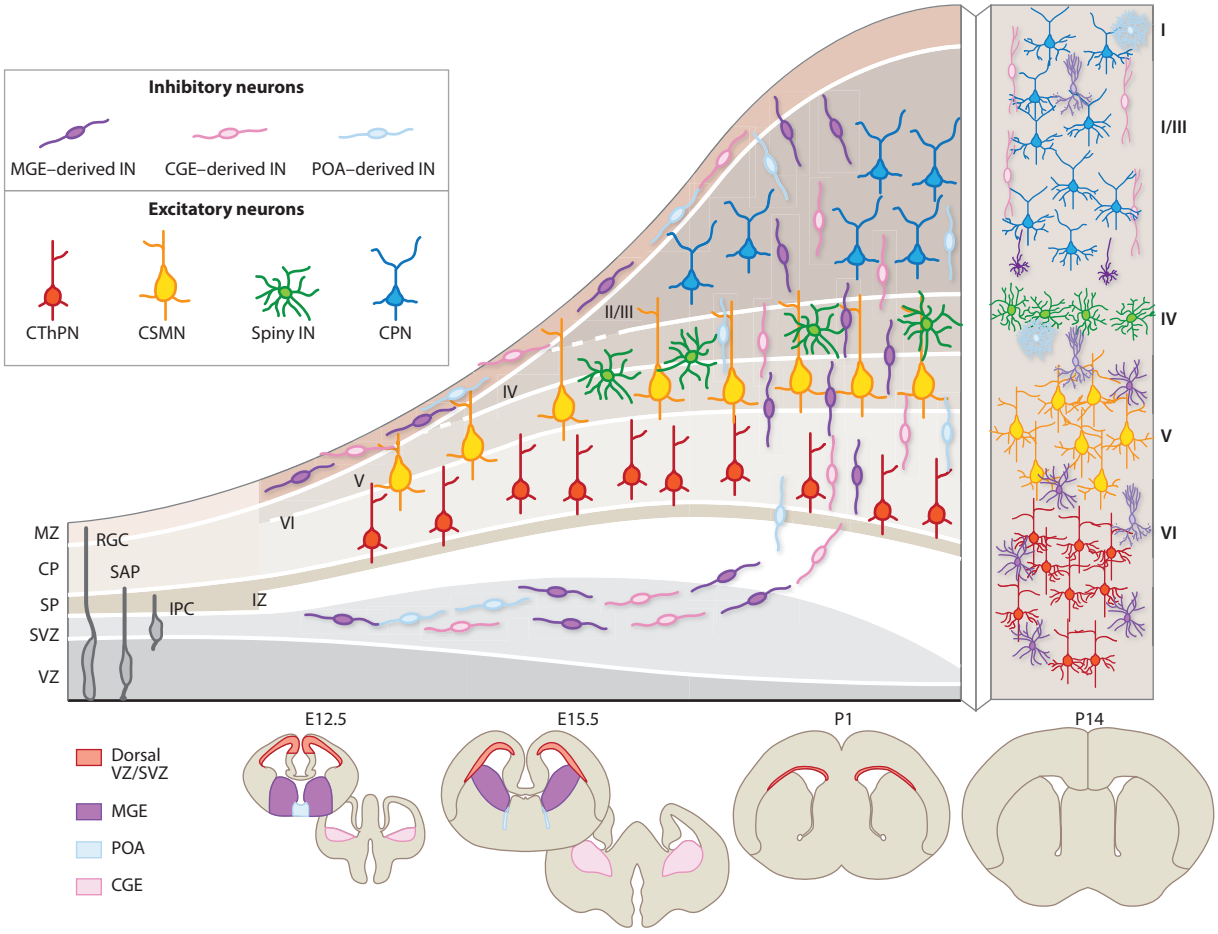

Figure 3

Developmental origin and distribution in the neocortex of projection neuron (PN) and interneuron (IN) subtypes. Excitatory neurons originate from progenitors in the dorsal telencephalon, and cortical inhibitory INs derive from progenitors in the ventral telencephalon [mainly the medial and ganglionic eminences (MGE), the caudal ganglionic eminence (CGE), and the preoptic area (POA)]. Over time, these germinal zones give rise to a diversity of neuronal subtypes, both PNs and INs, that acquire distinct laminar addresses in the neocortex. After reaching the cortex, INs migrate tangentially in streams located above [marginal zone (MZ)] and below [subventricular zone (SVZ)] the cortical plate (CP), before switching to a mode of radial migration to invade the CP. By the end of neurogenesis, PN and IN classes coexist at specific locations in the cortical layers and begin to wire into the local cortical microcircuit. Roman numerals refer to the six cortical layers. Abbreviations: CPN, callosal projection neuron; CSMN, corticospinal motor neuron; CThPN, corticothalamic; E, embryonic; IPC, intermediate precursor cell; IZ, intermediate zone; P, postnatal; RGC, radial glial cell; SAP, subapical progenitor; SP, subplate; VZ, ventricular zone.

In addition to comprising NE cells, RGCs, and IPCs, the cortex contains other progenitor classes, such as the subapical progenitors (SAPs), which also contribute to the expansion of the progenitor pools during neurogenesis (Pilz et al. 2013; for an extensive review of cortical progenitors, see Taverna et al. 2014) (**Figure 3**). The existence of such a variety of cortical progenitor types and their dynamic, although distinct, contributions to the generation of PNs suggest that progenitors fated to distinct PN lineages may already be present within the germinal zones, something that is still hotly debated.

At the core of the problem lies the longstanding question of whether the stereotyped production of neurons is the result of (*a*) a progressive competence-restriction mechanism, in which progenitors progressively and predictably restrict the potential neuronal outcomes that they can generate, and/or (*b*) a predetermined fate-restriction model, by which progenitors are precommitted to generate distinct classes of PNs.

In the early 1990s, milestone experiments by McConnell's group (Desai & McConnell 2000, Frantz & McConnell 1996, McConnell & Kaznowski 1991) began to address these questions by interrogating the fate potential of cortical progenitors in heterochronic transplantation paradigms. These studies demonstrated that early cortical progenitors are multipotent, whereas late cortical progenitors, even when exposed to a younger environment, are unable to produce the earlier PN fates (Frantz & McConnell 1996). The work provided clear evidence for a temporal, progressive restriction of progenitor fate potential. This was confirmed by elegant lineage fate-mapping analysis using sparse retroviral infection of VZ progenitors, which showed that when a single progenitor is labeled early in corticogenesis, it can give rise to neurons of all layers (Luskin et al. 1988, Walsh & Cepko 1993).

Ex vivo studies by Temple's group (Shen et al. 2006) further supported this model by showing that cultured multipotent progenitors sequentially give rise to early-born, deep-layer neurons and later-born, upper-layer neurons, although observing the birth of all lineages in vitro from the same single progenitor has been challenging. Thus, considerable evidence has accumulated in the past 15 years for a model in which cortical progenitors undergo changes in fate potential over time, probably mediated by a change in the length of the cell cycle and the number of divisions each progenitor undergoes before terminal differentiation (Calegari & Huttner 2003, Calegari et al. 2005, Pilaz et al. 2009).

More recent genetic fate-mapping studies have further probed the fate potential of cortical progenitors to determine whether progenitors fate-committed to produce distinct classes of PNs do exist. Results have been mixed. One study showed that progenitors expressing the transcription factor (TF) Cux2 (known to be a marker of CPNs and selected inhibitory INs of layer II/III) largely produce CPNs of layer II/III. In this study, the authors used a *Cux2*-CreERT2 knock-in line to fate-map cortical progenitors of the early VZ and found that a large proportion of these progenitors give rise to upper-layer PNs. *Cux2*-Cre-positive progenitors are present in the VZ as early as E10.5, and they divide, rather than differentiate, when deep-layer CFuPNs are normally produced. Notably, when forced to differentiate during the window of production of deep-layer neurons, such progenitors still generate upper-layer neurons, suggesting that their fate commitment is intrinsically determined and independent of temporal restrictions (Franco et al. 2012). These results provide evidence that the existence of progenitor subpopulations prefated to generate specific subtypes of PNs may be a component of the logic that builds PN diversity during corticogenesis. Although this may be an exciting additional strategy to diversify progenitor behavior and regulate complex lineage determination decisions, two subsequent studies failed to identify fate-restricted cortical progenitors. Guo and colleagues (2013) lineage fate-mapped progenitors using the same *Cux2*-CreERT2 reporter line used by Franco and colleagues (2012) and, in parallel work, a transgenic bacterial artificial chromosome (BAC) line driving *CreERT2* expression from the *Fezf2* promoter (*Fezf2*-CreERT2). They demonstrated the existence of multipotent progenitors able to generate not only different classes of PNs but also glia, as detected in P1 cortex. It is possible that these results are partially confounded, because BAC transgenic lines often do not precisely reproduce the temporally and spatially regulated expression of the endogenous locus and may not be precise enough for this type of lineage fate mapping. In addition, genetic background of the strain employed plays a role in this process, making it harder to compare directly the outcomes of the two studies (Eckler et al. 2015, Gil-Sanz et al. 2015).

Support for the progressively restricted generation of cortical PN subtypes from multipotent progenitors also emerged from an elegant mosaic analysis with double markers (MADM) study of neocortical neurogenesis (Gao et al. 2014). By using the *Emx1*-CreERT2 and *Nestin*-CreERT2 lines, independently, in the MADM system, the authors provided quantitative clonal analysis of RGC fate potential with single-cell resolution. The data indicate that clonally related neurons derived from the same RGC during early neurogenesis (from E10 to E13) span all cortical layers. Clones generating neurons of a single layer were only found when RGCs were labeled at late stages of neurogenesis. Although these data strongly point toward a model of progressive restriction of progenitor fate, it is possible that more rare progenitors prefated to form only selected types of PNs exist but were not detected. It is worth noting that the two models are not necessarily mutually exclusive, as the cortical VZ may be a mosaic of progenitors with different fate potentials.

In the near future, integrated approaches utilizing mosaic clonal analysis (using modified retroviruses or the MADM system) and molecular fate-mapping strategies employing multiple knock-in Cre lines (e.g., *Cux2*-CreERT2, *Fezf2*-CreERT2) should help clarify the complexity of regulatory strategies used by progenitors to generate PN diversity.

Birth of Cortical Interneurons: Shared Strategies and Main Differences

In contrast to the progenitors of PNs, which are adjacent to the developing cortex, progenitors of cortical INs are spatially segregated in distinct regions of the ventral telencephalon distal to the cortex. Currently, it is unclear whether distinct types of progenitor cells of the ganglionic eminences (GEs) possess distinct lineage potential, and therefore strategies to generate IN diversity are a matter of intense investigation.

In contrast to PNs, cortical INs are generated outside of the developing cortex, from ventrally located progenitors within the medial and caudal GEs (MGE and CGE, respectively) (Anderson et al. 1997, Tamamaki et al. 1997) and the preoptic area (POA) (Gelman et al. 2009) (**Figure 3**). From these ventral structures, INs migrate extensively to reach the developing cortex. There, they migrate tangentially through the SVZ and the dorsally located marginal zone, before invading the cortical plate (reviewed in Guo & Anton 2014) (**Figure 2**).

Much like dorsally located PN progenitors, IN progenitors can be classified into several subtypes: RGC-like cells, IPCs, and SAPs (reviewed in Taverna et al. 2014). Unlike dorsal progenitors, which produce only neurons of the neocortex, progenitors of the ventral germinal zones also produce classes of neurons destined to other brain regions (e.g., the striatum and the hippocampus) and subsets of oligodendrocytes that do not reach the cortex (Qi et al. 2002). These data provide a first level of evidence that these progenitors are likely extremely heterogeneous.

It is unclear whether distinct types of progenitor cells with distinct lineage potentials exist in the ventral telencephalon, and the strategies used to generate IN diversity are being investigated. Genetic fate-mapping and transplantation studies thus far support a model in which the anatomical location of progenitors and the timing of neurogenesis have predictive value for the type of IN produced (Xu et al. 2004). Specifically, PV-positive FS chandelier cells and basket cells, SST-positive nonspiking INs, and SST/CR-positive Martinotti cells (Marín et al. 2012, Vitalis & Rossier 2011) are born from progenitors of the MGE during early neurogenesis (between E10 and E13). The CGE instead produces CR- and VIP-expressing bipolar and double bouquet INs and rapidly adapting reelin-positive INs with multipolar morphology (Rudy et al. 2011). Finally, the POA, which accounts for only 10% of all cortical INs, gives rise to a small but highly diverse group of INs, including multipolar NPY-, basket PV-, and SST-expressing INs (Gelman et al. 2009).

The MGE, CGE, and POA germinal zones express specific TF codes that define them molecularly (Flames et al. 2007). These TFs are not bare markers. Instead, they play key roles in the balanced production of specific IN pools. For example, conditional inactivation of Nkx2.1 (which primarily labels progenitors in the MGE) between E9.5 and E12.5 compromises the generation of FS PV- and SST-expressing INs and increases the generation of adapting and late-spiking CR- and VIP-expressing INs (Butt et al. 2008). Molecular markers that define subregions within the three main subdivisions are also known. Within the MGE, for example, Nkx6.2 specifically labels the ventral MGE, and lineage fate-mapping studies show that this region preferentially produces Martinotti SST/CR-expressing INs (Fogarty et al. 2007, Sousa et al. 2009). These data root the idea that progenitors are diverse in molecular terms.

Distinct germinal zones give rise to INs of distinct identities. However, fate-mapping experiments have also shown that the migratory behavior within the cortex of INs produced in different germinal zones is distinct. MGE-derived IN subtypes migrate to layers II–VI, following the same inside-out pattern of laminar distribution as PNs (Marín et al. 2012, Xu et al. 2004). Specifically, in agreement with their early development, PV- and SST-expressing INs preferentially occupy the deep cortical layers (Xu et al. 2008). By contrast, CGE-derived INs preferentially populate the most superficial layers, independently of their time of birth (Miyoshi et al. 2010). These data suggest that the intrinsic mechanisms controlling spatial distribution of INs in the cortex and their integration within the columnar microcircuits do not universally apply to every subtype and that these decisions might be intimately dependent on IN class-specific identity, rather than simply day of birth.

Interestingly, even INs generated within the same germinal zone behave differently. The POA contains at least two small progenitor domains, one expressing *Nkx5.1* and the second *Dbx1* (Gelman et al. 2009, 2011). Genetic tracing of cells derived from *Nkx5.1*$^+$ progenitors indicates that they are distributed primarily in the superficial layers, predominantly express NPY, and show adapting firing properties (Gelman et al. 2009). By contrast, the *Dbx1* domain generates a wider spectrum of INs that populate the deep layers and are distinct from one another based on a large variety of molecular and electrophysiological traits (Gelman et al. 2011). It is unlikely, yet conceivable, that this small pool of progenitors is heterogenous and contains multiple progenitors with distinct prefated identities; alternatively, the same progenitors could give rise to multiple classes of cortical INs, possibly following a temporal sequence.

Several questions remain unanswered. Do individual progenitors give rise to multiple IN subtypes? Do INs that belong to the same lineage behave similarly? Are there prespecified subtypes of progenitors committed to specific populations?

Recent studies have investigated the clonal relationship between INs generated from the same progenitor (Brown et al. 2011, Ciceri et al. 2013). Although they used slightly different experimental approaches, both studies found that clonally related, MGE-derived INs tend to cluster in the cortex and that their horizontal distribution within the cortex is nonrandom (Brown et al. 2011, Ciceri et al. 2013). However, in one study, clones of neurons derived from Nkx2.1$^+$ progenitors labeled at E12.5 were found in both the deep and the superficial cortical layers, with most clusters containing INs of different identities. These data are consistent with a model of progressive fate restriction of progenitors, such that a single progenitor progressively gives rise, as development proceeds, to different types of INs that adopt distinct laminar addresses. By contrast, the second study found that marking Nkx2.1$^+$ progenitors at E11.5 and E14.5 labels clusters of INs largely segregated within either the deep or the superficial cortical layers, respectively (Brown et al. 2011, Ciceri et al. 2013). These data argue for the existence of prefated progenitors that produce specific IN lineages, which in turn acquire defined laminar distributions. As for the progenitors of PNs, it

is possible that these two models (fate-committed versus temporally restricted progenitors) coexist but apply to different progenitor pools.

To complement and clarify these initial results, it will be necessary to perform systematic clonal analysis of single IN progenitors with exquisite spatial and temporal resolution. The recently developed MADM technique is likely to provide a powerful genetic approach to mark the clonally related progeny of single progenitors. This technique could be combined with the use of viruses that tag each progenitor (and their progeny) with a unique identifying sequence, virtually eliminating the risk of analyzing mixed clones.

Although much work remains to be done, the current data point to a need for great diversification of progenitor fate during IN development. In this regard, it is interesting that IN germinal zones are located away from the cerebral cortex and that the domains that preferentially generate defined IN subclasses are molecularly distinct. This could be a strategy to enable finer, differential control of distinct progenitor pools, given that the cortex and the germinal zones are spatially separated. This strategy may have been necessary because INs destined to other brain regions, i.e., hippocampus and striatum, are produced within the same domains that generate the INs of the neocortex. This is a very distinct approach from that employed by dorsal progenitors, which maintain a spatially confined relationship to the neurons they generate. For both groups of neurons, the regulatory strategies used by progenitors to produce neuronal diversity require additional investigation.

Postmitotic Control of Neuronal Diversity

It is unquestionable that, mechanistically, key lineage determination decisions that establish PN and IN diversity occur at the progenitor stage. However, compelling emerging evidence indicates that regulatory events restricted to postmitotic early stages of development also contribute to establishing class-specific identities for both PNs and INs.

The discovery of several neuron subtype-specific TFs has led to the observation that many of these genes are specifically induced early postmitotically as neurons migrate away from the germinal zone. Mechanistically, reciprocal regulation between arrays of postmitotically expressed TFs progressively refines neuronal subtype identity during generation of some PN classes (Greig et al. 2013, Srinivasan et al. 2012). The TF Bcl11b, first discovered as a marker of ScPNs controlling axon fasciculation and extension (Arlotta et al. 2005), is specifically repressed by Satb2, a chromatin remodeling protein restricted to postmitotic CPNs (Alcamo et al. 2008, Britanova et al. 2008), and by its partner Ski (Baranek et al. 2012). In the absence of either Satb2 or Ski, *Bclb11* is ectopically expressed in CPNs, which in turn fail to develop connections through the CC (a key trait of CPNs) and, instead, extend axons ipsilaterally to the subcortical target (Alcamo et al. 2008, Baranek et al. 2012). In addition, several subtype-specific molecular markers of CPNs are not expressed in the absence of Satb2 (Alcamo et al. 2008, Britanova et al. 2008).

Another example of TFs progressively refining neuronal subtype identity is provided by the TF Tbr1, which is expressed postmitotically in subplate neurons, CThPNs, and CPNs; in the absence of Tbr1, the subplate is no longer morphologically discernible and fails to express its specific markers, whereas CThPNs aberrantly express high levels of *Fezf2* and *Bcl11b*, causing the development of ectopic connectivity to subcerebral targets rather than the thalamus (Han et al. 2011, McKenna et al. 2011).

Notably, postmitotic decisions can also contribute to the establishment of appropriate cortical architecture and connectivity, by influencing both the temporal dynamics of PN generation and the regional (areal) distribution of specific classes of PNs. The TF Sox5 is required for the correct temporal sequence of generation of both subplate neurons and CFuPNs. In the absence of Sox5,

subplate neurons acquire molecular features of ScPNs (a fate normally generated more than 2 days later), and CThPN identity is compromised (Lai et al. 2008).

Other noteworthy TFs are Bhlhb5 and Lmo4 (both expressed only postmitotically), which regulate area-specific differentiation of CSMNs. In the absence of Bhlhb5, CSMNs from the caudal motor cortex are not properly specified and fail to connect to the spinal cord, whereas in the absence of Lmo4, CSMNs in the rostral motor cortex lack backward-projecting collaterals (Cederquist et al. 2013, Joshi et al. 2008).

Similar postmitotic specification strategies seem to also be in place during cortical IN differentiation. For example, the LIM-homeodomain protein Lhx6, which is specifically expressed early postmitotically in migratory INs derived from the MGE (*Nkx2.1* domain), is critical for multiple aspects of development of all MGE-derived cortical INs, including the migration, differentiation, and maturation of PV- and SST-expressing INs (Liodis et al. 2007, Neves et al. 2013). Interestingly, *Lhx6* is upstream of the genes encoding two other TFs, Sox6 and Satb1, which also act postmitotically to control the differentiation of MGE-derived INs (Azim et al. 2009, Batista-Brito et al. 2009, Close et al. 2012, Denaxa et al. 2012). In the absence of Sox6, a dramatic loss of *PV* and *SST* expression and a concomitant increase in *NPY* expression take place, accompanied by major physiological and behavioral abnormalities, such as severe epileptic encephalopathy (Azim et al. 2009, Batista-Brito et al. 2009). In the case of the TF Satb1, its postnatal conditional inactivation (at P1) affects the maturation of SST-expressing INs, compromising their connectivity and integration into cortical circuits (Close et al. 2012, Denaxa et al. 2012).

The growing evidence that aspects of class-specific neuronal identity are controlled postmitotically and that expression of some key TFs is maintained suggests the possibility that TFs actively control, at least in part, the maintenance of neuronal class-specific identity. This hypothesis remains untested; however, it is interesting that the class-specific identity of young PNs can be changed via direct lineage reprogramming, despite neurons being postmitotic. Forced, ectopic expression of a single TF, encoded by the selector gene *Fezf2* (Lodato et al. 2014a), can convert CPNs of layer II/III into neurons with molecular, electrophysiological, and connectivity features of CFuPNs (Rouaux & Arlotta 2013). Similarly, stellate excitatory INs of layer IV change their electrophysiological features and connectivity in response to Fezf2 (De la Rossa et al. 2013). Interestingly, the capacity of Fezf2 to instruct CFuPN identity declines dramatically with the age of neurons, as shown when targeted CPNs lose their ability to respond to Fezf2 and to reprogram by P21 (Rouaux & Arlotta 2013). The data support the hypothesis that, at least within a critical early window of nuclear plasticity, neurons of the cortex maintain their identity through mechanisms that are reversible and independent of the postmitotic nature of the cell.

Orchestrated Assembly of the Cortical Local Circuit

Despite many years of research and much progress in understanding aspects of the functional organization of the neocortex, little is currently known regarding the principles and mechanisms that wire its outstanding diversity of PN and IN classes into a stereotyped local microcircuit.

Although intuitively one might expect that the number of elements in a given system, in this case the number of neurons present in the neocortex, reflects the complexity of the computational functions performed, it is unclear whether beyond numbers, specific classes of neurons play critical roles. From a computational standpoint, the neocortex is capable of creating innumerable nuances in behavior using only two opposing forces: excitation and inhibition. It is possible that one strategy to achieve such complexity from this simplicity involves the generation of a circuit in which synaptic contacts are not generated randomly, but rather PN and IN subclasses connect with each other following specific rules.

Understanding the principle driving connectivity of PN and IN diversity in the neocortex requires technologies that can provide sufficiently fine resolution of synaptic connectivity while also allowing for class-specific identification of the neurons present in the circuit. The advent of improved methods to map synaptic connections with second-generation viral tracers combined with genetically modified mouse lines that restrict labeling to defined classes of INs and PNs (Huang & Zeng 2013) holds promise for generating a precise connectivity map of the local microcircuits. Although this fine map is not yet available, the neuron type specificity of excitatory and inhibitory connections has been interrogated in several electrophysiological studies, some of which also took advantage of optogenetic tools to directly monitor in vivo neuronal activity and the real-time effect of circuit manipulation on specific behaviors (Cardin et al. 2010).

Pioneer electrophysiological studies investigating the computational role of PNs within excitatory circuits (i.e., PN-PN circuits) have shown that different PN classes have highly selective synaptic interconnectivity even within the same local circuits (Brown & Hestrin 2009). The pattern of connectivity shown by different classes of neighboring PNs reflects the identity of both the pre- and post-synaptic cell types, as demonstrated by simultaneous whole-cell recording of multiple PN types within layer V (Morishima et al. 2011). In the visual cortex, for example, corticocortical neurons show a significantly higher preference for connections with their neighboring CTPNs than with each other (Brown & Hestrin 2009). Paired recording of retrogradely labeled ScPNs (i.e., corticopontine neurons) also shows preferential connectivity in the frontal cortex: These neurons make more excitatory inputs onto cells that share the same long-range axonal target than onto those that project ipsilaterally (Morishima et al. 2011). Together, these results support a model in which the specific identity of PNs influences the nature of the local excitatory subnetworks.

The circuit organization of inhibitory networks in the neocortex (i.e., PN-IN networks) is not well mapped, and despite the great degree of specificity shown by INs in targeting subcellular components of their PN partners (soma versus dendrites or axon initial segment), the general principles underlying IN synaptic connectivity are still elusive. A general model of promiscuous inhibitory connectivity (a blanket of inhibition) has been proposed (Fino & Yuste 2011), but many studies also support a rather fine-scale specificity of synaptic connectivity for cortical INs. For example, using paired intracellular recording and photostimulation-evoked synaptic currents analyses, the FS-INs of layer II/III were found to preferentially connect with excitatory PNs in the same layers that have established reciprocal synapses, rather than with PNs that are not reciprocally connected (Yoshimura & Callaway 2005).

Similarly, whole-cell recording from layer V inhibitory INs has shown that they form synapses with neighboring PNs, and participate in intralaminar and interlaminar subnetworks, in a PN-subtype-dependent manner (Otsuka & Kawaguchi 2009). Studies in the prefrontal and entorhinal cortex also support the theory that the choice of postsynaptic PN target by inhibitory INs and the properties of their synaptic connections depends on the identity of the PN partners (Lee et al. 2014, Varga et al. 2010).

Interestingly, recent studies suggest that synaptic interconnectivity between different classes of INs (e.g., IN-IN connections) also exhibits a high degree of specificity. For example, employing optogenetic manipulation in combination with single-cell recording, two independent groups have shown that VIP-expressing INs primarily suppress the activity of SST-expressing INs and a small fraction of the PV-expressing INs, which in turn directly inhibit the inputs and outputs of PNs. Therefore, VIP-expressing INs provide disinhibitory, regulated control of PN activity (Lee et al. 2013, Pi et al. 2013).

Although the selectivity of connections within neuronal circuits seems to be intimately linked to the identity of pre- and post-synaptic partners, the cellular and molecular mechanisms

underlying pairing selectivity are largely unknown. Some lines of evidence seem to suggest that the final organization of cortical microcircuits is controlled by events occurring well before completion of neuronal differentiation, during development. For example, PN identity affects the radial distribution of cortical INs during development; misplaced PNs lead to aberrant radial migration of INs and thus dysfunctional local circuits. Importantly, this effect depends on the class-specific identity of the PNs involved (Lodato et al. 2011).

In line with this finding, recent data on the lamination of CGE-derived INs are compatible with the idea that the class-specific identity of the future PN synaptic partner is crucial in determining the final laminar location of an IN and its positioning into a circuit. MGE- and CGE-derived IN subtypes display distinct migratory behaviors once they have reached the cortex. MGE-derived IN subtypes are found in layers II–VI, following the same inside-out pattern of laminar distribution as PNs, whereas CGE-derived INs are more abundant in layers II–IV and preferentially populate the most superficial layers, independent of their time of generation (Miyoshi et al. 2010). These results cannot fit a model in which time of birth determines the position of INs into layers and highlight the existence of more finely modulated mechanisms, possibly related to the class-specific identity of the PNs present in each layer.

Along these lines, emerging data suggest that the identity of PNs may also have an impact on glia. PNs in different layers display distinct myelin distribution profiles along their axons, suggesting an effect of PN identity on the behavior of oligodendrocytes (Tomassy et al. 2014). For example, a novel pattern of myelination, termed intermittent myelin, is found along the axons of PNs in layer II/III, but not on PNs in layers V and VI, which are both more heavily myelinated and display canonical profiles of myelin distribution. This pattern may reflect idiosyncratic interactions between different types of PNs and oligodendrocytes. Recent years have seen a surge of examples supporting an emerging model in which lineage decisions of developing PNs affect the behavior of other cells in the cortex, both neurons and glia, to ultimately shape working circuits, allow cortical diversification, and sustain complex behavior.

CLOSING REMARKS

The study of cortical development and function has fascinated neuroscientists for centuries. In particular, as appreciation has grown over the years for the unparalleled diversity of neuronal subtypes that populate the cortex, questions have surfaced about the principles that shape this cellular diversity during development, the mechanisms that maintain this landscape of neurons unchanged for the life of the organism, and the rules that wire distinct neurons within complex circuits that subserve higher-order functions. Great progress has been made investigating individual aspects of the development, function, and plasticity of the neocortex. This has led to great insights into the tissue organization, cytoarchitecture, cellular composition, circuit assembly, and function of the neocortex. However, new technologies enable a somewhat novel conceptual, experimental approach to understanding cortical composition and function. Among others, methods to label, purify, and molecularly profile distinct neuronal populations have expanded our understanding of the molecular events that accompany the establishment of neuronal diversity and will continue to generate molecular insights in higher-throughput mode. Single-cell transcriptomics is generating interesting data on the diversity of cortical neurons, raising the possible existence of new neuronal classes (Zeisel et al. 2015). Next-generation viral tracing and optogenetic manipulation of precisely defined neurons and circuits now provide a new level of resolution in studying the structure-function relationships that drive cortical functions (Cardin et al. 2010, Osakada & Callaway 2013). Finally, novel three-dimensional imaging techniques allow for a global view of neuronal diversity and connectivity that was not previously feasible (Chen et al. 2015, Renier et al.

2014). These advances enable an integrated, system-level approach that considers multiple aspects of the biology of neurons and circuits (from molecules to high-throughput maps of connectivity, neuron class-specific manipulation, and large-scale functional recording and imaging) to decode neocortical neuron diversity and function in development and disease.

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