Molecular logic of neocortical projection neuron specification, development and diversity

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Abstract

The sophisticated circuitry of the neocortex is assembled from a diverse repertoire of neuronal subtypes generated during development under precise molecular regulation. In recent years, several key controls over the specification and differentiation of neocortical projection neurons have been identified. This work provides substantial insight into the "molecular logic" underlying cortical development, increasingly supporting a model in which individual progenitor-stage and postmitotic regulators are embedded within highly-interconnected networks that gate sequential developmental decisions. Here, we provide an integrative account of the molecular controls that direct the progressive development and delineation of subtype and area identity of neocortical projection neurons.

Introduction

The mammalian neocortex is responsible for processing multiple modalities of sensory information, controlling motor output, and mediating higher-order cognitive functions. Its organization into only six histologically-distinct layers belies an extraordinary diversity of neuronal subtypes, which serve as building blocks for computationally-powerful neural circuitry. In recent years, tremendous progress has been made toward understanding the molecular events that control the development of these diverse types of neocortical neurons.

Two major classes of neurons, interneurons and projection neurons, populate the neocortex[1]. Interneurons connect locally within the neocortex, are largely inhibitory, and are generated by progenitors in the subpallial (ventral) proliferative zone of the telencephalon before migrating to the neocortex[2-4]. In contrast, projection neurons send axons to distant brain targets, are excitatory, and are generated by progenitors in the pallial (dorsal) proliferative zone[5,6]. Interneuron diversity and development have been reviewed elsewhere[7-9]; in this article, we focus exclusively on projection neurons.

Individual phenotypic characteristics, such as dendritic morphology, electrophysiological properties, or projection patterns, have been used in the past to systematically classify projection neurons[6,10-12]. Although these classification schemes have facilitated investigation of projection neuron development and function, a more comprehensive understanding of neuronal diversity will require integration of these and other phenotypic data, including transcriptomic and epigenomic profiles[13]. Here, we group neurons primarily by the target of their axons (Box 1), both because hodology is centrally related to function, and because establishment of appropriate projections requires successful stepwise execution of elaborate developmental programs.

Projection neurons progressively acquire subtype and area identities, and their developmental trajectories can be followed along three distinct axes: time, subtype differentiation, and area differentiation. Most work to date has addressed each of these axes separately, providing descriptive analyses of individual molecular controls acting either in

progenitors or in postmitotic neurons. However, it is becoming increasingly clear that specification of subtype and area identity, as well as the timing of specification decisions, are both interrelated and interdependent. In this review, we address transcriptional mechanisms controlling specification of projection neuron subtype and area identity in mice. We first examine molecular programs acting in progenitors to establish fate-restricted lineages and to impart positional information, and then analyze those acting in postmitotic neurons to direct extension of axons to appropriate targets and to refine initially promiscuous patterns of gene expression and connectivity. At each stage, we consider how genetic programs operate to establish boundaries in n-dimensional "identity space" between distinct projection neuron subtypes and between distinct cortical areas.

Progenitor specification

Progenitor diversity and corticogenesis—Early in development, the telencephalic wall is composed of undifferentiated neuroepithelial cells (Figure 1A). As these progenitors proliferate and expand in number, some begin to differentiate into radial glia (RG), establishing the ventricular zone (VZ) [14]. RG, in turn, give rise to additional progenitor classes, including outer radial glia (oRG) and intermediate progenitors (IP), which together form the subventricular zone (SVZ) [15,16].

Each of these progenitor populations has distinct morphological properties and follows a specific pattern of cell division. RG span the thickness of cortex from the ventricular (apical) surface to the pial (basal) surface, and are used as a scaffold by newly-born neurons as they migrate into cortex[17]. They primarily divide asymmetrically to self-renew, while also giving rise to oRG, IP, or neurons[18,19]. oRG are also unipolar, but can be distinguished from RG by their lack of an apical process[20-22]. They were first characterized in the outer SVZ of developing human cortex[20] and, until recently, were thought to be present only in gyrencephalic animals[21]. However, a small population also exists in the SVZ of rodents[22,23], undergoing asymmetric divisions to self-renew and generate neurons. IP have a multipolar morphology and, unlike RG and oRG, are not anchored to either the apical or basal cortical surface. They act primarily as transit-amplifying cells, undergoing limited proliferative divisions, and more often dividing symmetrically to produce two neurons[14,15,24-26]. A fourth class of progenitors, the short neural precursors (SNP), reside in the VZ, but they have a basal process that does not reach the pia. In other respects, SNP appear similar to IP, suggesting that they might represent RG in the process of becoming IP[26].

Neocortical progenitors begin to produce excitatory projection neurons around embryonic day (E) 10.5 in mice[27,28]. The earliest-born neurons migrate away from the ventricular surface to segregate from progenitors and form the preplate[29,30]. Later-born neurons migrate into the preplate, splitting it into the marginal zone and subplate, and establishing the cortical plate between the two[31]. Throughout the rest of corticogenesis, newly-born neurons migrate into the cortical plate, organizing themselves in an "inside-out" fashion (Figure 1B), such that early-born neurons populate deeper neocortical layers (VI, then V), and late-born neurons migrate past them to progressively populate more superficial layers (IV, then II/III).

Progenitor lineage commitment—In aggregate, neocortical progenitors generate different projection neuron subtypes in sequential waves; however, the lineages leading from progenitor cells to specific neuronal subtypes, and the molecular mechanisms that determine the fixed order in which neuronal subtypes are generated, remain largely unknown.

One widely-followed model of progenitor lineage commitment proposes that a single lineage of progenitors generates all subtypes of projection neurons, and that the competence of a given progenitor to generate specific subtypes becomes progressively limited over the

course of development. In support of this model, early-stage progenitors transplanted into late-stage cortex are capable of producing all subtypes, but late-stage progenitors transplanted into early-stage cortex are competent only to produce superficial-layer subtypes[32-34]. In addition, retroviral lineage tracing experiments show that single progenitors labeled early in corticogenesis are competent to produce neurons of all layers[35-37], whereas progenitors labeled later in corticogenesis primarily give rise to progeny residing in superficial layers[38]. *In vitro* studies of both primary dissociated and embryonic stem (ES) cell-derived cortical progenitors indicate that they are capable of autonomously recapitulating the sequential generation of neuronal subtypes characteristic of corticogenesis *in vivo*[39-42]. Although these various approaches demonstrate a narrowing of competence in the overall progenitor population over time, they do not show that every progenitor is initially capable of producing all subtype fates. These findings would also be consistent with changing relative abundance of different lineage-committed progenitor populations.

An alternative model of progenitor diversification proposes that independent, fate-restricted lineages of progenitors generate specific neuronal subtypes. Early evidence for this model came from the observation that a number of subtype-specific transcription factors are expressed in progenitors earlier in development, suggesting that different subsets of progenitors may be committed to generating particular classes of projection neurons. For example, *Fezf2* (*Fez family zinc finger 2;* formerly *Fezl*) is sparsely expressed in the proliferative zones primarily during deep-layer neurogenesis, and is specific postmitotically to corticofugal projection neurons (CFuPN) [43-47]. Conversely, *Cux1* and *Cux2* (*cut-like homeobox 1 and 2*) are expressed in the VZ and SVZ primarily during superficial-layer neurogenesis, and are specific postmitotically to callosal projection neurons (CPN) and other superficial-layer neurons[48-50]. Direct evidence for the existence of partially lineage-committed progenitors in the neocortex derives from recent genetic fate mapping experiments using mice that express Cre-ERT2 under the control of the endogenous *Cux2* promoter[51]. This work demonstrated that a subset of progenitors that is present from the earliest stages of corticogenesis exclusively produces CPN and other superficial-layer neuron subtypes (Figure 2A). While deep-layer neurons are being generated, *Cux2*-positive progenitors mainly undergo proliferative divisions, expanding as a population while producing only a limited number of neurons. Later, they switch to a neurogenic mode of division and generate superficial-layer neurons. Although the authors suggest that early-born neurons derived from the *Cux2* lineage become deep-layer CPN, a large number of all *Cux2* fate-mapped cells in deep-layers are interneurons, significantly complicating interpretation of single marker expression analysis[51]. Further investigation using additional and independent genetic lineage tracing approaches are likely to uncover additional complexity in cortical progenitor lineage relationships.

A number of different models, ranging from strict sequential progression through competence states to immediate single-lineage commitment, can be entertained on the basis of current evidence. Of these, the sequential competence model (Figure 2B) seems least consistent with current experimental data. This model predicts that lineage-committed progenitors should not be present from the onset of corticogenesis, but *Cux2*-positive RG can be observed as early as E10.5[51]. Although it is clear that at least two distinct lineages exist, it is not known whether they are entirely mutually exclusive (Figure 2C), or whether some progenitors join the *Cux2*-positive lineage after generating deep-layer neuronal subtypes, therefore changing their competence state (Figure 2D). This question could be experimentally addressed by identifying a gene expressed only by *Cux2*-negative progenitors (potentially *Fezf2*) and generating a *Flp* knock-in line, in order to simultaneously fate-map deep-layer and superficial-layer neurons. Importantly, the *Cux2*-positive and the *Cux2*-negative lineages each include multiple projection neuron subtypes, and it remains to be determined whether further fate-restricted sub-lineages emerge in

progenitors (Figure 2E) or whether some fate specification decisions are resolved entirely postmitotically.

For the sake of simplicity, we have assumed here that all superficial-layer neurons are generated by *Cux2*-positive progenitors, and that *Cux2*-negative progenitors comprise a single lineage, but these remain open questions. Moreover, evidence for lineage-committed progenitors does not rule out the existence of multipotential progenitors. We anticipate that additional layers of complexity will emerge as these issues are more thoroughly investigated.

Positional information in progenitors—Neocortical arealization is initiated by expression of morphogens and signaling molecules from patterning centers at the borders of the neocortical primordium[52]. Beginning at E9.5 in mice, fibroblast growth factors FGF8 and FGF17 are secreted rostromedially by the commissural plate[53-56], while caudomedially, *Wnt* and *Bmp* family members are secreted from the cortical hem, and, laterally, the *Wnt* antagonist SFRP2 and several *Egf* family members are secreted from the antihem[57,58]. Of these morphogens, only *Fgf8* has been shown to function as a true organizer of area identity. Increasing *Fgf8* expression by *in utero* electroporation causes rostromedial areas of cortex to expand caudally[59,60]; conversely, reduced *Fgf8* expression in hypomorphic mutants causes caudal areas of cortex to expand rostrally, as does antagonism by overexpression of the cytoplasmic domain of its receptor *Fgfr3c*[61,62]. In addition, very early expression of *Fgf8* from an ectopic caudal or midlateral source can cause a complete duplication of the cortical area map[63].

Together, these diffusible factors induce graded expression of transcription factors in VZ progenitors, which in turn control the relative size and position of cortical areas (Figure 3A). *Pax6* (*paired box 6*) and *Emx2* (*empty spiracles homolog 2*) are expressed in the VZ in reciprocal rostrolateral to caudomedial gradients[64,65], whereas *Sp8* (*trans-acting transcription factor 8)* and *Couptf1* (chicken ovalbumin upstream promoter transcription factor 1) are expressed in reciprocal rostromedial to caudolateral gradients[66-69]. Because of the orthogonal orientation of these two pairs of gradients, relative expression levels of these four transcription factors (and possibly others, yet to be identified) can theoretically define any set of cortical coordinates, such that each postmitotic projection neuron might emerge from the ventricular zone poised to acquire a specific area identity.

Strong caudal expression of *Emx2* and *Couptf1* promotes specification of sensory areas. In *Nestin-Emx2* transgenic mice, *Emx2* is expressed more uniformly throughout the ventricular zone, leading to an increase in the size of visual cortex, and a concomitant size decrease and rostrolateral shift of somatosensory and motor areas. In the absence of one allele of *Emx2*, in contrast, motor areas expand, and sensory areas shift caudomedially[70]. Similarly, in *Couptf1* conditional null mice, motor areas expand dramatically to occupy a large portion of cortex, while sensory areas are displaced to a narrow occipital band that contains compressed, but properly-configured, sensory representations[71].

Rostrally, expression of *Pax6* and *Sp8* drives specification of motor identity. Both *Sp8* and *Pax6* conditional null mice, as well as *Pax6*^sey/sey ("small eye") hypomorphic mutants, exhibit a drastic loss of motor areas, although interpretation of these findings are complicated by a simultaneous decrease in the overall size of cortex[72-74]. Gain- and loss-of-function *in utero* electroporation experiments, however, independently support a role for *Sp8* in cortical area identity, both by cell-autonomous repression of *Couptf1* in neocortical progenitors and indirectly by induction of *Fgf8*[67,75].

Although manipulation of these transcription factor gradients is sufficient to change the size and position of cortical areas (Figure 3B), neuronal identity within the ectopically-located areas is largely established normally. Each respecified area expresses molecular markers that are appropriate to its new identity, attracts modality-specific thalamocortical input, and extends projections to correct targets. Taken together, these results suggest that progenitor-based controls establish a coordinate system of positional information that anchors area identity to specific rostrocaudal and mediolateral positions. This fate map in radial glia[76] and intermediate progenitors[77] (also known as the "proto-map") must then be transmitted to their neuronal progeny to be interpreted and executed by a second network of transcription factors that direct postmitotic acquisition of area identity.

Postmitotic subtype specification

Although neocortical projection neurons are generated by partially fate-restricted progenitors, postmitotic controls are also necessary to specify the precise subtype identities of newly-born neurons. Over the past decade, high-throughput efforts to define laminar- and subtype-specific gene expression patterns in the neocortex[45,78-83] have led to the identification of an increasing number of molecular controls over subtype development.

Delineation of SCPN and CThPN subtype identity—Subcerebral projection neurons (SCPN) and corticothalamic projection neurons (CThPN) are closely-related corticofugal projection neuron (CFuPN) subtypes that reside in the deep layers of the neocortex and are sequentially generated early in corticogenesis. Substantial plasticity exists in the specification of CFuPN into either SCPN or CThPN, and each population can expand at the expense of the other in the absence of critical controls (Figure 4).

The zinc finger transcription factor *Fezf2* is critical for specification of SCPN. It is expressed by a subset of ventricular zone (VZ) progenitors while deep cortical layers are being generated, and also by postmitotic CFuPN, although it is not known whether *Fezf2* functions primarily in progenitors or postmitotically. *Fezf2* is expressed at high levels by SCPN, and at lower levels by CThPN and SP neurons[43-47,84], and in *Fezf2* null mice the large pyramidal neurons that normally define layer V are entirely absent. Even more strikingly, expression of SCPN-specific genes is lost, and no cortical neurons project to the brainstem and spinal cord[46,84]. Instead, expression of *Tbr1*, a transcription factor critical for CThPN development[85,86], expands into presumptive layer V[46], and many of these *Tbr1*-expressing neurons project to thalamus[86], indicating that some SCPN are fate-converted to CThPN (while other SCPN are fate-converted to CPN, as discussed below). Thus, *Fezf2* specifies SCPN identity, at least in part by repressing CThPN identity.

In addition to being a "master" regulator of SCPN development, *Fezf2* also functions in the specification of CFuPN identity more broadly. CThPN and SP neurons appear disorganized in *Fezf2* null mice, and a number of CThPN-specific genes, including DARPP-32, *Grg4*, and *Foxp2*, fail to be expressed[46,84]. These findings suggest that low-level *Fezf2* expression by CThPN and SP neurons is necessary for precise differentiation of these populations. Furthermore, misexpression of *Fezf2* by *in utero* electroporation causes layer II/III CPN to redirect their axons toward a broad set of subcortical targets, including the thalamus, brainstem, and spinal cord[46,47,87,88]. Taken together, these data indicate that *Fezf2* instructs CFuPN identity, and not SCPN identity alone.

A second transcription factor, *Ctip2* (*COUPTF-interacting protein 2*), functions downstream of *Fezf2* to control appropriate differentiation of SCPN. Although SCPN are still born and migrate normally to layer V in the absence of *Ctip2*, they exhibit striking defects in axon outgrowth, fasciculation, and pathfinding. Most critically, SCPN axons fail to reach the spinal cord, as they become misrouted and defasciculated in the midbrain, only rarely

reaching pons, and never reaching the pyramidal decussation[45]. Although activation of *Ctip2* by *Fezf2* is critical for SCPN development, several transcriptional controls over CPN, CThPN, and SP development (including *Satb2*[89,90], *Sox5*[91], and *Couptf1*[92]) operate at least in part by repressing *Ctip2* expression, indicating that *Ctip2* is a critical target for transcriptional regulation during neocortical projection neuron development.

Tbr1 (*T-box brain 1*) acts in opposition to *Fezf2* and *Ctip2* to specify CThPN identity. It is expressed postmitotically by CThPN and SP neurons, and at lower levels by Cajal-Retzius cells and CPN[85,86]. In the absence of *Tbr1*, the subplate is not morphologically discernible, and subplate-specific genes fail to be expressed[85]. Similarly, early-born neurons that would normally develop into CThPN express aberrantly high levels of *Fezf2* and *Ctip2*, as well as several other SCPN-specific genes, and extend axons toward subcerebral targets instead of the thalamus[85,86,93]. *Tbr1* directly binds to highly-conserved regulatory regions to repress expression of *Fezf2*, therefore functioning, at least in part, by preventing SCPN specification[86,93].

Temporal control over CFuPN subtype generation—CFuPN subtypes are generated in temporally-overlapping waves and share the same core developmental program; however, specific controls direct the sequential generation of SP neurons, CThPN, and SCPN, ensuring precise acquisition of molecular identity by each subtype.

The transcription factor *Sox5* (*SRY-box containing 5*) controls the orderly emergence of CFuPN subtypes by repressing high-level expression of SCPN genes, including *Fezf2* and *Ctip2*, until generation of subplate neurons and CThPN is complete[91,94,95]. *Sox5* directly represses *Fezf2* by binding an enhancer element required for *Fezf2* expression in the forebrain[95]. In *Sox5*^{−/−} mice, subplate neurons express inappropriately high levels of CTIP2, take an abnormal laminar position in superficial cortical layers, and project to the cerebral peduncle[91]. In addition, FOG2 and CTIP2, normally specific to CThPN and SCPN, respectively, are co-expressed by a single population of neurons with mixed SCPN/CThPN character, indicating imprecise differentiation[94]. Loss of *Sox5* results in widespread CFuPN pathfinding defects, including extensive defasciculation of SCPN axons in the midbrain, and formation of an accessory subcerebral tract that projects through the external capsule[91]. Corticothalamic projections are also severely compromised, as reported by *Golli*-GFP and *Fezf2*-GFP transgenic labeling, as well as pancortical *Emx1-Cre;CAG-Cat-GFP* labeling[94].

Couptf1 suppresses SCPN identity in the latest-born, most superficially-located CThPN. In the absence of *Couptf1*, layer VIa neurons in somatosensory cortex become "motorized," expressing aberrantly high levels of CTIP2 and *Fezf2*, but maintaining expression of TBR1. Although more deep-layer neurons project subcerebrally in *Couptf1* conditional nulls, only the axons of SCPN prematurely generated at E12.5 and located in layer VIa are able to reach the spinal cord. Axons of SCPN generated at E13.5 and located in layer V, in contrast, aberrantly terminate in pons before entering the spinal cord[92]. In a general sense, repression of SCPN subtype identity by *Couptf1* represents an additional aspect of its function repressing motor identity in favor of sensory identity.

Delineation of CFuPN and CPN subtype identity—CFuPN share a developmental boundary with callosal projection neurons (CPN), and especially with deep-layer CPN, which are generated during the same temporal window, and reside intermingled with CFuPN in layers V and VI. From the time CFuPN and CPN axons exit the cortical plate, they follow dramatically divergent trajectories, either away from cortex or toward the midline[96]. Accordingly, some critical controls over CFuPN and CPN development function largely by repressing molecular programs that would instruct differentiation toward the alternate fate (Figure 4).

As described above, *Fezf2* functions centrally to specify CFuPN identity, which requires suppression of CPN fate. *Fezf2* overexpression *in vivo* is sufficient to redirect the axons of superficial-layer CPN toward subcortical targets[46,87,88]. In the absence of *Fezf2*, neurons expressing alkaline phosphatase from the *Fezf2* locus extend axons across the corpus callosum. In addition, more neurons in layer V display electrophysiological characteristics typical of CPN and express CPN-specific genes, suggesting that many SCPN are fate-converted to CPN[87]. Interestingly, these neurons appear to take on a deep-layer CPN identity, expressing broad CPN identity genes, such as *Satb2* and *Lpl*, but not expressing genes specific to superficial-layer CPN, such as *Inhba* and *Limch1*[50,97].

The transcription factor *Satb2* (*special AT-rich sequence binding protein 2*) is critical for CPN specification and concomitant repression of CFuPN fate. *Satb2* is expressed at high levels by CPN, and likely also by associative neurons, in all layers of cortex[89,90]. In the absence of *Satb2*, almost no axons cross through the corpus callosum, even though establishment of the midline appears normal. Instead, neurons expressing *LacZ* from the *Satb2* locus project toward the brainstem and spinal cord[89,98]. Expression of several genes characteristic of CPN, including *Cdh10*, *Dkk3*, *Sip1*, and *Cux1*, is lost or severely reduced in *Satb2* null mice. Conversely, superficial-layer neurons in these mice express high levels of *Ctip2*, as well as a number of other genes characteristic of SCPN, including *Clim1*, *Cdh13*, and *Grb14*. *Satb2* operates by directly repressing *Ctip2*, rather than by upstream control of *Fezf2*, and, consequently, *Satb2* null CPN are not fully fate-converted to SCPN[89,90]. Recently, the transcriptional co-regulator *Ski* (*ski sarcoma viral oncogene homolog*) has been shown to be a critical component of the repressor complex recruited by *Satb2* to initiate HDAC1-dependent chromatin remodeling, and $Ski^{-/-}$ mice largely phenocopy $Satb2^{-/-}$ mice[99].

Epistasic analysis of subtype specification—In several instances, transcription factors that specify subtype identity have been shown to repress each other directly, raising the possibility that inhibiting differentiation programs for alternate fates, rather than actively specifying a particular fate, might be their primary function. Under this model, simultaneous deletion of two competing transcription factors, such as *Tbr1* and *Fezf2* or *Satb2* and *Ctip2*, might partially restore proper subtype specification. Indeed, formation of the corticospinal tract (lost in $Fezf2^{-/-}$) is partially rescued in $Tbr1^{-/-};Fezf2^{-/-}$ mice, although projections to the thalamus (lost in $Tbr1^{-/-}$) are still completely absent[86]. Similarly, formation of the corpus callosum (lost in $Satb2^{-/-}$) is partially rescued in $Satb2^{-/-};Ctip2^{-/-}$ mice[98]. These results suggest that downstream programs are able to direct some neurons to differentiate appropriately, even in the absence of important specification controls, as long as competing controls are not active.

Progressive refinement of subtype identity—Mature deep-layer neurons exhibit strikingly divergent patterns of gene expression and axonal projection, but some of these differences begin to emerge only after several days of postmitotic refinement. Newly postmitotic neurons often extensively co-express transcription factors that later become restricted to different subtypes[89,94,98,100-102]. For example, between E12.5 and E14.5, neurons in the cortical plate co-express high levels of CTIP2 and TBR1/FOG2, which resolve over time to SCPN and CThPN, respectively[94,101]. Similarly, at E13.5, deep-layer neurons briefly co-express CTIP2 and SATB2, which later become restricted to SCPN and CPN[89,98]. The period of time during which deep-layer neurons co-express multiple subtype controls might correspond to a particularly plastic state, when decisions regarding subtype identity are being crystallized. This initially widespread expression of incompatible subtype controls is intriguing, however, given recent evidence of fate commitment by progenitors[51].

We propose that the timing of fate specification decisions might be linked to biologically-meaningful decision points, favoring either commitment of progenitors or later resolution postmitotically (Box 2). CFuPN and deep-layer CPN begin to extend axons in different directions even as they migrate through the intermediate zone[103], and, therefore, specification into one of these two broad fates might need to occur in progenitors. In contrast, CThPN and SCPN axons travel through the internal capsule together for several days before their trajectories diverge[104]. This coincides with a period during which newly-postmigratory CFuPN transition from co-expressing high levels of TBR1 and CTIP2 to expressing either one or the other, potentially reflecting postmitotic commitment[101].

Projection neuron areal specialization

Postmitotic regulators transform continuous gradients of positional information inherited from progenitors into sharp areal boundaries, instruct the formation of sensory maps, and direct projection neurons to acquire areally-appropriate phenotypic characteristics (Figure 5). Two such controls, *Lmo4* and *Bhlhb5*, are expressed in complementary patterns and are critical for determining the precise placement of molecular boundaries between areas.

Lmo4 (*Lim domain only 4*) is a transcription factor that is expressed postmitotically in motor cortex and higher-order sensory areas, but excluded from primary somatosensory, visual, and auditory cortex (Figure 5) [45,105,106]. Conditional loss of *Lmo4* function results in a modest rostral expansion of somatosensory-specific genes, at the expense of motor-specific genes, although these defects do not suggest a dramatic failure of area identity acquisition[107]. In addition, the boundaries of individual barrels become blurred, and the vibrissal barrel field is slightly narrowed overall[107,108]. *Lmo4* controls several aspects of area-specific output connectivity in motor cortex, including extension of caudal collaterals by some CPN and SCPN (backward projection neurons; BPN), as well as the ratio of brainstem- to spinal cord-projecting SCPN in rostral motor cortex[106].

The basic helix-loop-helix transcription factor *Bhlhb5* (*BHLH domain-containing, class B5*) is initially expressed in a high-caudomedial to low-rostrolateral gradient in the cortical plate, but its expression becomes progressively restricted to primary sensory areas (somatosensory, visual, and auditory). In the absence of *Bhlhb5*, molecular area identity is extensively disrupted in somatosensory and caudal motor cortex, and area-specific genes, including *Lmo4*, are aberrantly expressed. Although the position and configuration of the barrel field are unchanged, thalamocortical input appears more diffuse, and the cytoarchitectural organization of vibrissal barrels is only faintly discernible[109]. *Bhlhb5*, therefore, centrally contributes to the emergence of hallmark somatosensory cortex-specific features, including appropriate gene expression and precise cellular organization of vibrissal barrel fields.

A number of additional transcriptional regulators contribute to postmitotic acquisition of area identity. *Tbr1*, discussed above as a critical control over CThPN subtype identity, also contributes to area identity acquisition. It is expressed most highly in rostral areas of cortex, and, in the absence of *Tbr1* function, genes typically expressed in caudal regions of cortex expand rostrally[85]. Notably, abnormalities in gene expression are not limited to layer VI[85], suggesting that transient or low-level *Tbr1* expression in superficial layers is also instructive for area identity. The homeodomain transcription factor *Otx1* (*orthodenticle homolog 1*) is necessary for the establishment of area-specific connectivity by SCPN. It is present in the cytosol of VZ progenitors and, at later stages, in the nuclei of CThPN and SCPN, with nuclear translocation taking place during the first postnatal week[110]. Although expression of *Otx1* is uniform across cortical areas, loss-of-function selectively affects SCPN in visual cortex, which inappropriately maintain their spinal projections, adopting a final connectivity pattern normally specific to SCPN in motor cortex[110,111]. Lastly, *Couptf1*, already discussed as an important control over arealization at the progenitor level, is also expressed

postmitotically[92]. It is not known whether *Couptf1* acts to regulate the development of area-specific gene expression and projection patterns solely by its functions in progenitors, or whether it also has continued functions in postmitotic neurons.

This emerging understanding of the expression and function of key postmitotic regulators is beginning to illuminate the molecular logic underlying area identity acquisition. For example, the division of cortex into two broad domains defined by *Bhlhb5* and *Lmo4* suggests that a common program controls primary sensory area development, whereas an opposing program governs acquisition of features shared by other areas, including higher-order sensory areas and motor areas. Recent evidence indicates that emergence of distinct gene expression profiles in primary and higher-order sensory areas requires thalamocortical input[112], suggesting that extrinsic factors are critical for later stages of cortical area patterning. Overall, only a small number of postmitotic controls over area identity acquisition have been identified, and further important regulators likely remain to be discovered.

Integrating subtype and area identity

Early neuroanatomists first classified the neocortex into areas on the basis of regional variation in laminar morphology, cell density, and thickness[113,114]. These cytoarchitectural differences reflect whether an area is specialized for input, output, or integration, and arise from adjustments in the relative proportion of neurons instructed to differentiate into CThPN, SCPN, layer IV granular neurons (GN), or CPN. Therefore, areal specialization requires not only establishment of specific input and output connectivity, but also production of specific ratios of projection neuron subtypes.

Recent reports suggest that some transcription factors coordinate regulation of subtype and area specification. As discussed above, *Tbr1* and *Couptf1* are both important regulators of CFuPN specification, and also promote motor and sensory area identity, respectively[85,86,92,93]. Similarly, the transcription factor *Ap2γ* (*activating enhancer binding protein 2 gamma*) controls how many superficial-layer CPN are generated in an area-specific manner by regulating the number of *Tbr2*-positive IP in occipital cortex during the later stages of corticogenesis[115]. These findings provide initial mechanistic insight for earlier reports that progenitor cell cycle dynamics differ across cortical areas in primates[116].

Deciphering neocortical evolution

Over the course of cortical evolution, radial and tangential expansion have been accompanied by neuronal diversification and regional specialization, allowing both for increased sophistication of cortical circuitry and for the emergence of a growing repertoire of functionally-specialized areas[117]. It has been proposed that the dorsal pallium of ancestral amniotes, like that of modern-day sauropsids, possessed only subcortically-projecting neurons[118] and was divided into two major functional areas[119]. Although the mammalian lineage has retained this basic organization, it has also incorporated additional neuronal subtypes specialized for receiving and processing input, as well as for intra- and interhemispheric integration[120]. Further, specific cognitive tasks have been compartmentalized into well-defined primary, secondary, and higher-order motor and sensory areas.

Expansion of cortical thickness and elaboration of projection neuron diversity in mammals were facilitated by the appearance of IP, which are not present in sauropsids[121]. These transit-amplifying cells establish a supplementary progenitor compartment, the subventricular zone (SVZ), that contributes neurons to all layers of the neocortex, exponentially increasing the neurogenic capacity of the cortical germinal zone[26]. In primates

and other mammals with well-developed cortices, such as ferrets, the SVZ is distinguished by its internal organization into an inner and an outer subcompartment (inner and outer SVZ; ISVZ and OSVZ) [21]. Although mice do not possess a compartmentalized SVZ, oRG-like cells are nonetheless present, suggesting that the well-organized primate OSVZ represents an expansion of a preexisting population of progenitors[22]. The emergence of the SVZ likely enabled the elaboration of the six-layered mammalian neocortex[23], as well as the the ability to produce new cortical neuron subtypes, including CPN.

CPN are an evolutionary innovation of placental mammals and have become the most abundant and diverse class of cortical projection neurons in eutherians. There has been considerable expansion and diversification of this population in rodents, and even more in primates[118,122]. CPN located in different layers, and even in different sublaminae, have remarkably different patterns of gene expression, suggesting that subpopulations of CPN have diverged to acquire specialized functions[50]. Consistent with the hypothesis that CPN were derived from pre-existing corticofugal populations[123], repression of CFuPN programs by *Satb2* is absolutely necessary for the emergence of callosal projections[89,90,98]. Further molecular controls over the differentiation of individual CPN subpopulations remain to be identified.

The area plan of ancestral amniotes is thought to have consisted of a sensorimotor area immediately adjacent to a primary visual area, as in many modern sauropsids[119]. In placental mammals, the ancestral sensorimotor area has diverged into distinct primary somatosensory and motor areas[124], while V1 and S1 have become tangentially separated by the addition of a host of novel secondary and higher-order sensory areas[125]. These territories are all marked by expression of *Lmo4*, suggesting that the complementary expression patterns of *Bhlhb5* and *Lmo4*[106,109,126] reflect the distinct evolutionarily origins of these two portions of the mammalian area plan. Interestingly, *Lmo4* may be further specialized in humans, as it is expressed differentially between the right and left hemispheres of human embryos, and may mediate some aspects of left-right asymmetry between the two cortical hemispheres[127].

In summary, our current knowledge supports a model in which, over the course of evolution, a growing number of transcription factors was progressively recruited to control cortical development, gradually adding layers of neuronal diversity and areal specialization to a simpler ancestral framework.

Perspectives

Implications for disease and repair—Studies of cortical development have uncovered important genetic determinants that might provide mechanistic insights into neurodegenerative disease. For instance, recent work on *hSOD1^G93A* mice, a model of amyotrophic lateral sclerosis (ALS), has shown that there is widespread degeneration not only of corticospinal motor neurons (CSMN), but of SCPN more broadly, as identified by expression of subtype-specific developmental control genes[45,128]. Therefore, degeneration of SCPN across multiple cortical areas might be a significant source of non-motor ALS symptoms in humans. Future work could seek to identify developmentally-specified determinants of SCPN susceptibility to degeneration, and perhaps leverage this knowledge toward development of treatments for ALS.

The developmental history of neuron subpopulations may also provide insight into closely-related neurodegenerative diseases with distinct pathologies. Progressive loss of descending cortical motor output is a prominent feature of both primary lateral sclerosis (PLS) and hereditary spastic paraplegia (HSP), but SCPN are differentially affected in these two motor neuron diseases. In PLS, SCPN projecting to bulbar, cervical, thoracic, and lumbar segments

of the brainstem and spinal cord broadly degenerate, leading to generalized progressive weakness of voluntary muscles[129,130]. In HSP, in contrast, lumbar-projecting CSMN selectively degenerate, leading to difficulty walking[131,132]. The molecular basis for differential pathology of SCPN subpopulations in these diseases is not known, but is likely related to genetic determinants of SCPN located in distinct cortical regions that target specific rostro-caudal segments of the brainstem and spinal cord.

A more sophisticated understanding of the molecular controls that direct subtype-specific neuronal differentiation could also enable novel strategies for nervous system repair. In fact, lessons from development have already been adapted to *in vitro* systems, using morphogen signaling to guide the differentiation of ES cells into neocortical progenitors[40-42,133]. Progenitors derived using these protocols generate heterogeneous neuron populations that can project axons to a range of targets and integrate into host cortical circuits when transplanted[41,134]. Future work might direct differentiation of these ES cell-derived progenitors into large quantities of a specific neuronal subtype by leveraging recently-identified developmental controls. "Master" regulators such as *Fezf2*, which is sufficient both to program[46,47,87] and reprogram[88,135,136] neuronal subtype identity *in vivo*, are particularly promising candidates for instructing ES cell differentiation *in vitro*.

Future directions—In recent years, several important controls over the specification and differentiation of long-distance neocortical projection neurons have been identified. Although neurons that extend axons to the contralateral hemisphere, to thalamus, or to subcerebral targets have been extensively studied, much less is known about specific molecular controls or markers of associative cortical neuron subtypes, including intracortical projection neurons and layer IV GN. Furthermore, considerable uninvestigated diversity exists among neurons that project to the same target. Some subpopulations, such as CSMN that target different spinal segments[137], are defined areally and have been identified based on hodology, while others, such as CPN in different sublaminae, are known only by gene expression[50]. It will be of great interest to identify genetic determinants responsible for engineering these additional levels of complexity.

Current models of cortical development are restricted to a handful of regulators, which account for a limited subset of key nodes within a broader regulatory network that is likely to be considerably more complex. In future studies, large-scale cell type-specific proteomic and genomic approaches should make it possible to analyze network dynamics, rather than epistatic relationships between pairs of genes. New methods for genome-wide methylation mapping might enable investigation of changes in the epigenetic landscape that accompany lineage commitment decisions and progressive specification of neuronal identity. In addition, relationships between transcription factors and effectors that determine the terminal differentiated state of a neuron, such as cell adhesion molecules and axon guidance receptors, are mostly unknown. Such a comprehensive understanding of developmental mechanisms might provide insights necessary to overcome barriers to the programming and reprogramming of specific cortical neuron types.

Biographies

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Jeffrey D. Macklis' laboratory is focused on the development, diversity, selective degeneration, regeneration, and directed differentiation of projection neuron subtypes in the cerebral cortex. His lab studies neocortical projection neuron differentiation; cortical progenitor biology; induction of neurogenesis within murine neocortex; directed cortical neuron subtype differentiation from neural progenitors and pluripotent cells, functional repair of brain and spinal cord circuitry, and connections between development and degeneration of specific neuron types. He is a Professor of Stem Cell and Regenerative Biology at Harvard University, Cambridge, Massachusetts, Professor of Neurology [Neuroscience] at Harvard Medical School, Boston, Massachusetts, and was founding Program Head of Neuroscience in the Harvard Stem Cell Institute. He attended the Massachusetts Institute of Technology (M.I.T.), Cambridge, Massachusetts, Harvard Medical School, and graduate school at M.I.T. within the Harvard–M.I.T. Division of Health Sciences and Technology. He trained in developmental neuroscience with Richard Sidman. Until 2011, he was Director of the Massachusetts General Hospital–Harvard Medical School Center for Nervous System Repair.

Glossary

Hodology	The path followed by axons to reach their targets.
Neuroepithelial cells	Neuroectodermal progenitors that are the main proliferative cell type of the early neocortex. They later differentiate into radial glial cells.
Gyrencephalic	Having a folded cerebral cortex, with gyri (ridges) and sulci (furrows).
Lineage	The shared ancestry of cells that can be traced back to a common progenitor through sequential cell divisions.

Competence	The differentiation potential of a cell, as determined by its intrinsic molecular state.
Fate mapping	Labeling a progenitor cell with a permanent and heritable mark to identify all its progeny.
Flp **knock-in line**	A mouse line in which expression of *Flp* recombinase is driven by the promoter of a gene of interest.
Morphogen	A secreted factor that can induce at least two different cell fates in a concentration-dependent manner by forming a gradient.
Fasciculation	Bundling together of axons that project to a common final or intermediate target through adhesive interactions.
Cajal-Retzius cells	Early-born cortical neurons that express the glycoprotein Reelin and reside in layer I.
Enhancer element	A short region of DNA, typically occupied by multiple transcription factors, that is sufficient to drive expression of a gene with temporal and/or cell-type specificity.
Chromatin remodeling	Changes in the three-dimensional structure of chromatin brought about by epigenetic modifications. These structural changes can result in either transcriptional activation or silencing of genes located in the involved chromatin segment.
Barrel	A cylindrical column of neurons in layer IV of the neocortex that receives and processes sensory input from a single whisker. The topographical organization of the barrels in cortex corresponds precisely to the arrangement of whisker follicles on the snout.

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Online summary

The sophisticated circuitry of the neocortex is assembled from a diverse repertoire of neuronal subtypes generated during development under precise molecular regulation, and forming distinct functional areas within the tangential expanse of the neocortex. This collection of specialized neurons is produced by a variety of progenitors with distinct morphological and molecular properties, and with distinct patterns of cell division.

The lineages leading from progenitor cells to specific neuronal subtypes, and the molecular mechanisms that determine the fixed order in which neuronal subtypes are generated, remain largely unknown. Recent work suggests that some subtypes of neurons are produced by lineage-committed progenitors, although a number of models of lineage commitment can be entertained on the basis of current evidence.

Area identity acquisition is initiated by diffusible factors released from the periphery of the neocortical domain, and subsequent induction of graded expression of arealizing transcription factors in ventricular zone progenitors. These progenitor-based controls establish a coordinate system of positional information that anchors area identity to specific rostrocaudal and mediolateral positions, which must then be transmitted to their neuronal progeny to be interpreted by a second network of transcription factors that direct postmitotic acquisition of area identity.

Projection neuron subtype identity is progressively established by extensive transcriptional cross-repression between genetic programs driving the development of one subtype of projection neuron and those driving the development of alternate subtypes. These competing regulators sort newly-postmitotic projection neurons into one of three broad subtype identities: corticothalamic, subcerebral, and callosal.

Postmitotic regulators, including *Lmo4* and *Bhlhb5*, transform continuous gradients of positional information inherited from progenitors into sharp areal boundaries, instruct the formation of sensory maps, and direct projection neurons to acquire areally-appropriate phenotypic characteristics.

Over the course of evolution, a growing number of transcription factors was progressively recruited to control cortical development, gradually adding layers of neuronal diversity and areal specialization to a simpler ancestral framework.

The emerging understanding of the expression and function of key molecular regulators is beginning to illuminate a molecular logic underlying subtype and area identity acquisition. We propose that the order- and dose-dependent nature of projection neuron identity specification can be formalized by analogy to first-order Boolean logic, with decision points represented by "molecular logic gates".

Box 1

Projection neuron diversity in the cerebral cortex

Projection neurons are classified broadly according to whether they extend axons within one cortical hemisphere (associative projection neurons), across the midline to the contralateral hemisphere (commissural projection neurons), or away from cortex (corticofugal projection neurons). Some neurons project to multiple targets and can therefore be classified into more than one broad class. Importantly, neurons of a given subtype residing in different cortical areas (motor, somatosensory, visual, and auditory) project to anatomically and functionally distinct targets[138].
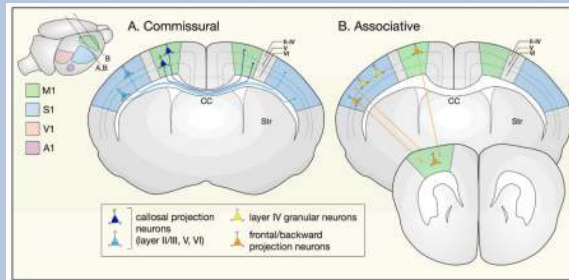
Commissural projection neurons, project to the contralateral cortical hemisphere. Most cross the midline through the corpus callosum (callosal projection neurons, CPN), while a smaller population crosses through the anterior commissure. CPN reside primarily in layers II/III (~80%), with fewer in layers V and VI (~20%), and extend axons to mirror-image locations in the same functional area of the contralateral hemisphere, enabling bilateral integration of modality-specific information.

Associative projection neurons, present in all layers of the neocortex, project within a single cortical hemisphere. This population includes short-distance intrahemispheric projection neurons, which extend axons within a single cortical column or to nearby cortical columns (such as layer IV granular neurons) and long-distance intrahemispheric projection neurons, which extend axons to adjacent or distant cortical areas (such as forward and backward projection neurons).
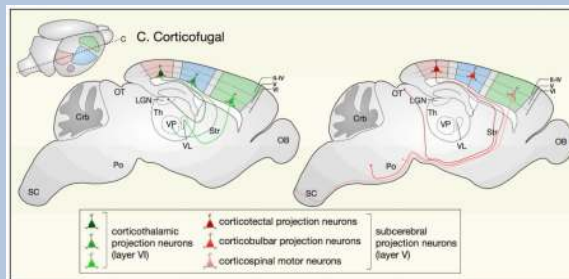
Corticofugal projection neurons (CFuPN), project away from cortex to subcortical targets and include corticothalamic projection neurons (CThPN), which reside in layer VI, and subcerebral projection neurons (SCPN), which reside in layer V.

CThPN extend axons to specific thalamic nuclei in an area-specific manner: motor cortex CThPN establish connections with the ventral lateral and ventral anterior nuclei, sensory cortex CThPN with the ventral posterior nucleus, and visual cortex CThPN with the lateral geniculate nucleus.

SCPN extend axons to different primary targets in the brainstem and spinal cord depending on their areal location. In general: motor cortex SCPN project to spinal cord (corticospinal motor neurons) and brainstem motor nuclei (cortico-brainstem motor neurons); somatosensory cortex SCPN to the trigeminal principal sensory nucleus and dorsal column medullary nuclei (corticobulbar projection neurons); and visual cortex SCPN to optic tectum (corticotectal projection neurons).
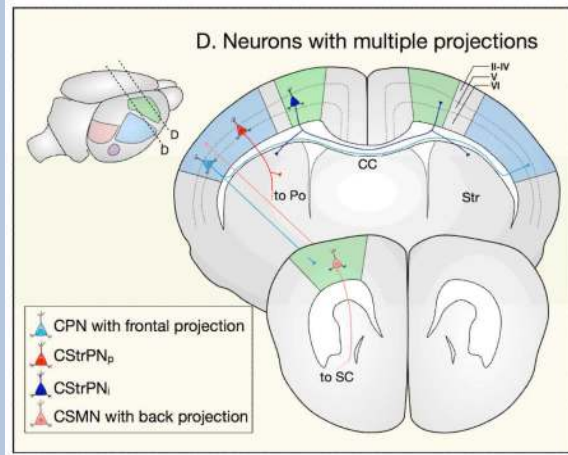
Neurons that send projections to multiple targets can sometimes be classified into more than one of the categories above. Examples include CPN with frontal projections, which extend axons to the contralateral hemisphere and to ipsilateral frontal cortex; SCPN with backward projections, which extend axons to subcerebral targets and to ipsilateral caudal cortex; and intratelencephalic corticostriatal projection neurons (CStrPNi), which extend projections to the contralateral hemisphere and to ipsilateral striatum{Sohur:2012ep}. Other neurons that project to multiple targets, such as pyramidal corticostriatal projection neurons (CStrPNp), can be classified into only one category.

CC, corpus callosum; Crb, cerebellum; LGN, lateral geniculate nucleus of thalamus;OB, olfactory bulb; OT, optic tectum; Po, pons; SC, spinal cord; Th, thalamus; VL, ventral lateral nucleus of thalamus; VP, ventral posterior nucleus of thalamus.

Box 2

Toward a molecular logic of neocortical development
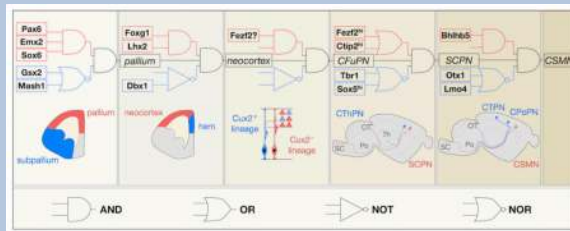
We propose that the order- and dose-dependent nature of projection neuron identity specification can be formalized using first-order Boolean logic, with decision points represented by "molecular logic gates". Below, we illustrate this approach to schematizing the developmental trajectories of specific projection neuron subtypes, using SCPN as an archetypal population (see figure).

The neocortical domain is established by transcription factors that act combinatorially to repress subpallial programs (such as *Pax6*, *Emx2*, and *Sox6*) [73,139-144] and cortical hem programs (such as *Lhx2* and *Foxg1*) [145-147]. Subsequently, neocortical progenitors are further specified into at least two partially fate-restricted lineages by yet unidentified molecular controls. Progenitors that are *Cux2*-negative, and possibly *Fezf2*-positive, generate CFuPN, while progenitors that are *Cux2*-positive generate CPN and other neurons in superficial layers[46,51].

CFuPN become committed to a specific subtype at a decision point gated by cross-repression between *Fezf2*, which directs SCPN specification, and *Tbr1* and *Sox5*, which direct CThPN specification[46,47,84,86,87,91,93,94]. Once SCPN are specified, *Ctip2* promotes subsequent differentiation steps, including axon outgrowth, fasciculation, and targeting[45]. Additional controls instruct further specialization of SCPN subpopulations, including collateralization and pruning decisions (*e.g., Otx1*[110]; *Lmo4*[110]).

Each sequential decision point described above is gated by the coordinated activity of multiple transcriptional regulators and chromatin-modifying proteins, which direct extensive changes in the transcriptional and epigenetic state of a cell. Although we have only considered regulation of subtype specification, other aspects of neuronal development, such as area specification and migration, proceed in parallel, orchestrated by partially-intersecting molecular programs.
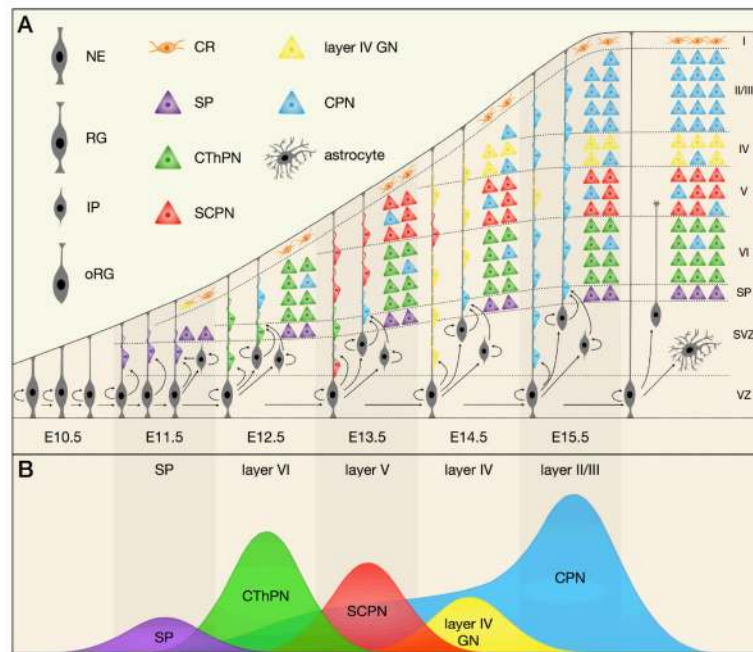
Figure 1. Neocortical projection neurons are generated in an "inside-out" fashion by diverse progenitor types in the VZ and SVZ
This schematic depicts the sequential generation of neocortical projection neuron subtypes and their migration to appropriate layers over the course of mouse embryonic development. (a) Radial glia (RG) in the ventricular zone (VZ) begin to produce projection neurons around E11.5. At the same time, RG generate intermediate progenitors (IP) and outer radial glia (oRG), which establish the subventricular zone (SVZ) and act as transit-amplifying cells to increase neuronal production. After neurogenesis is complete, neural progenitors transition to a gliogenic mode, generating astrocytes and oligodendrocytes. Cajal-Retzius (CR) cells primarily migrate into neocortical layer I from non-cortical locations, while other projection neurons are born in the neocortical VZ / SVZ and migrate along radial glial processes to reach their final laminar destinations. (b) Distinct projection neuron subtypes are born in sequential waves over the course of neurogenesis. The peak birth of subplate (SP) neurons occurs around embryonic day (E) 11.5, with the peak birth of corticothalamic projection neurons (CThPN) and subcerebral projection neurons (SCPN) occuring at E12.5 and E13.5, respectively. Layer IV granular neurons (GN) are born around E14.5. Some callosal projection neurons (CPN) are born starting at E12.5, and those CPN born concurrently with CThPN and SCPN also migrate to deep layers. Most CPN are born between E14.5 and E16.5, and these late-born CPN migrate to superficial cortical layers. Peak sizes are proportional to the approximate number of neurons of each subtype born on each day.
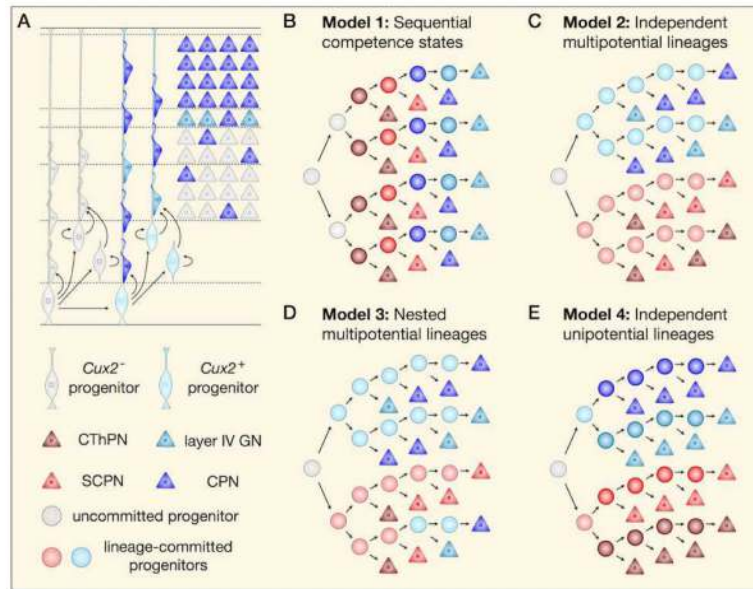NE, neuroepithelial cell; WM, white matter

Figure 2. Models of deep-layer and superficial-layer projection neuron production by distinct progenitor lineages
(a) Fate-mapping experiments have established that most superficial-layer (commissural and associative) projection neurons derive from *Cux2*-positive progenitors, while deep-layer (corticofugal) neurons derive from *Cux2*-negative progenitors. Several models have been proposed to describe how this process occurs. (b) The "sequential competence states" model suggests that individual progenitors are able to produce a single neuronal subtype at a time as they progress through a series of competence windows, and that fate-restricted lineages do not exist. Although this model has been refuted, the precise structure of lineage trees during corticogenesis remains unknown. It is possible that progenitors commit to independent lineages before the onset of neurogenesis (c), or that some progenitors first give rise to neurons of one lineage and later commit to a different lineage (d). Similarly, progenitors might be multipotential, giving rise to more than one type of neuron (c and d), or become progressively fate restricted until they are unipotential (e).
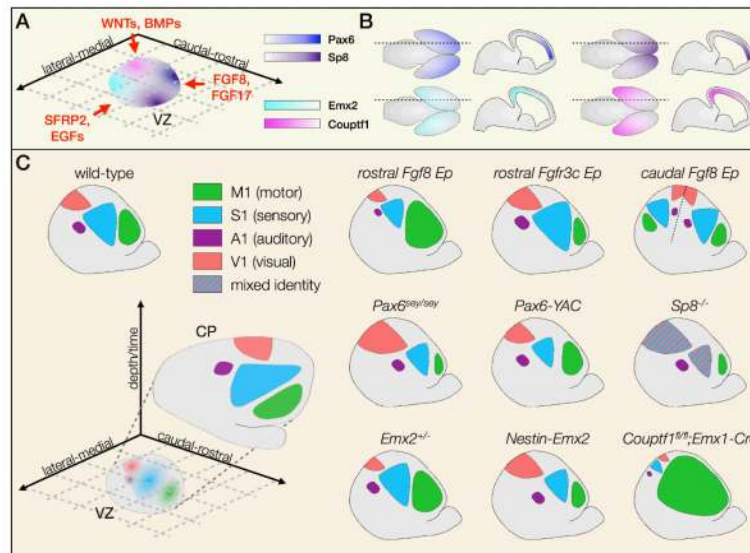
Figure 3. Transcription factors in the VZ establish an area identity fate map
(a) Arealization of the cerebral cortex is initiated by diffusible morphogens and signaling molecules secreted from opposing sides of the neocortical periphery (left panel). These signals induce expression of complementary and orthogonal transcription factor gradients such as *Pax6*/*Emx2* and *Sp8*/*Couptf1*, seen in a schematized flatmount view of the ventricular zone (VZ) (b) *Pax6* is expressed most highly rostrolaterally, in opposition to *Emx2*, which is expressed most highly caudomedially. Similarly, *Sp8* is expressed most highly rostromedially, in opposition to *Couptf1*, which is expressed most highly caudolaterally. Gradients are shown in wholemount (left) and sagittal (right) views for each. (c) Progenitors located at different medio-lateral and rostro-caudal coordinates express specific levels of these transcription factors, which combinatorially establish a fate map of cortical areas in the ventricular zone. This fate map is later translated into a definitive area map in the cortical plate (CP), shown in flatmount view (left panel). Manipulation of morphogen signaling or VZ transcription factor expression results in dramatic changes in the size and position of cortical areas (right panel). Hatching indicates mixed area identity. A1, primary auditory cortex; Ep, electroporation; M1, primary motor cortex; S1, primary somatosensory cortex; sey/sey, *small eye* hypomorphic mutant; V1, primary visual cortex; YAC, yeast artificial chromosome
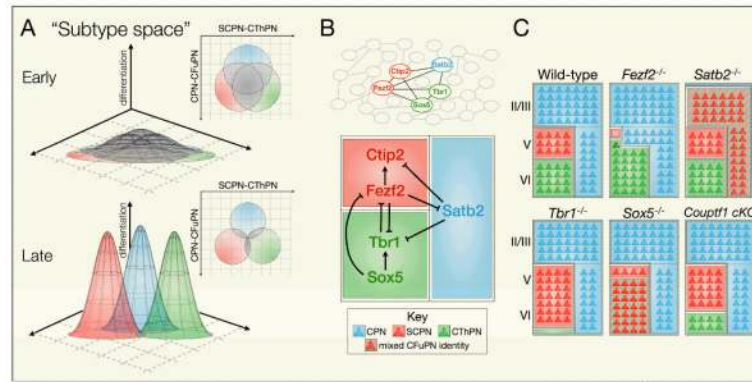
Figure 4. Competing molecular programs direct differentiation of newly-postmitotic projection neurons into one of three broad subtype identities

(a) The subtype identities of postmitotic projection neurons are depicted within a theoretical n-dimensional "subtype space" in which individual subtype identities (as defined by gene expression, morphology, dendritic structure, projection patterns, physiology, and other characteristics) occupy distinct coordinates. Boundaries between these identities, preventing neurons of one subtype from taking on characteristics of another subtype, are established by the action of cross-repressive molecular controls. One boundary exists between neurons specified as SCPN and those specified as CThPN, and another exists between CFuPN (SCPN/CThPN) and CPN. Early in corticogenesis, undifferentiated neurons have largely overlapping subtype identities (top). As development proceeds, neurons differentiate and subtypes become more distinct from each other (bottom).

(b) Known molecular controls represent key nodes of an elaborate transcriptional network, only beginning to be elucidated (top). Arrows indicate known cases of genetic or transcriptional activation or repression, and further interactions and molecular controls remain to be identified (bottom). (c) Changes in expression of these key regulators can cause boundaries between subtypes to shift, with neurons partially or completely acquiring features characteristic of other subtypes. In some mutants, neurons acquire CFuPN identity generally, rather than a well-defined CThPN or SCPN identity. The boundaries between CFuPN and deep-layer or superficial-layer CPN may shift independently of one another, represented by the dashed line between deep-layer and superficial-layer CPN.

CFuPN, corticofugal projection neurons; CPN, callosal projection neurons; CThPN, corticothalamic projection neurons; SCPN, subcerebral projection neurons
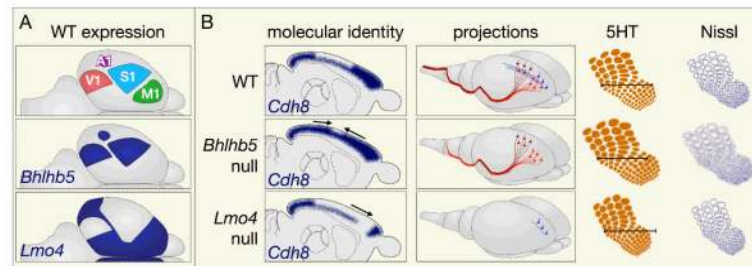
Figure 5. Postmitotic regulators set up sharp gene expression boundaries between cortical areas and direct area-specific phenotypic differentiation of projection neurons

Loss of *Bhlhb5* or *Lmo4* function affects multiple aspects of postmitotic area identity acquisition, including gene expression, projection patterns, and cellular organization in the S1 barrel field. a) On postnatal day 7, *Bhlhb5* is expressed in S1, A1, and V1, whereas *Lmo4* is expressed in M1 and excluded from primary sensory areas. b) In the absence of *Bhlhb5* (middle row), molecular identity of sensory areas is compromised; for example, *Cdh8* expression expands into S1, from which it is normally excluded. Areally-determined projection patterns change, as CSMN in caudal motor cortex fail to reach the spinal cord. Thalamocortical axons (shown by serotonin (5-HT) immunostaining) innervate a wider area of S1 in *Bhlhb5$^{-/-}$*, with indistinct cortical barrels (shown by Nissl staining). Conversely, in the absence of *Lmo4* (bottom row), molecular identity of motor areas is compromised, and motor expression of *Cdh8* and other genes is reduced. Neurons in motor cortex are inappropriately specified, and fail to send backward collaterals. Thalamocortical axons innervate a narrower area in *Lmo4* conditional null mutants, although cortical cytoarchitecture has not been investigated by Nissl staining.

WT, wild-type; V1, primary visual cortex; A1, primary auditory cortex; S1, primary sensory cortex; M1, primary motor cortex