Patterning centers, regulatory genes and extrinsic mechanisms controlling arealization of the neocortex

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The adult mammalian neocortex, the major region of the cerebral cortex, is divided into functionally specialized areas, defined by distinct architecture and axonal connections. Extrinsic influences, such as thalamocortical input, and genetic regulation, intrinsic to the dorsal telencephalon, control the gradual emergence of area-specific properties during development. Major recent advances in this field include: the first demonstration of the genetic regulation of arealization, implicating the transcription factors *Emx2* and *Pax6* in the direct control of area identities; and the demonstration of the potential role of the signaling protein, fibroblast growth factor 8, in the early patterning of arealization genes, such as *Emx2*.

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Abbreviations

A1	primary auditory area
bHLH	basic helix-loop-helix
BMPs	bone morphogenetic proteins
COUP-TF1	chick ovalbumin upstream transcription factor I
CP	cortical plate
dLG	dorsal lateral geniculate nucleus
eve	*even-skipped*
FGF	fibroblast growth factor
GABA	γ-amino butyric acid
IZ	intermediate zone
M1	primary motor area
MZ	marginal zone
NMDARs	*N*-methyl-D-aspartate receptors
PLC	phospholipase C
PP	preplate
S1	primary somatosensory area
Sey	*Small eye*
Shh	Sonic hedgehog
SP	subplate
TCAs	thalamocortical axons
V1	primary visual area
V2	secondary visual area
VP	ventroposterior nucleus
VZ	ventricular zone
Xt^J	*Extra-toes^J*

Introduction

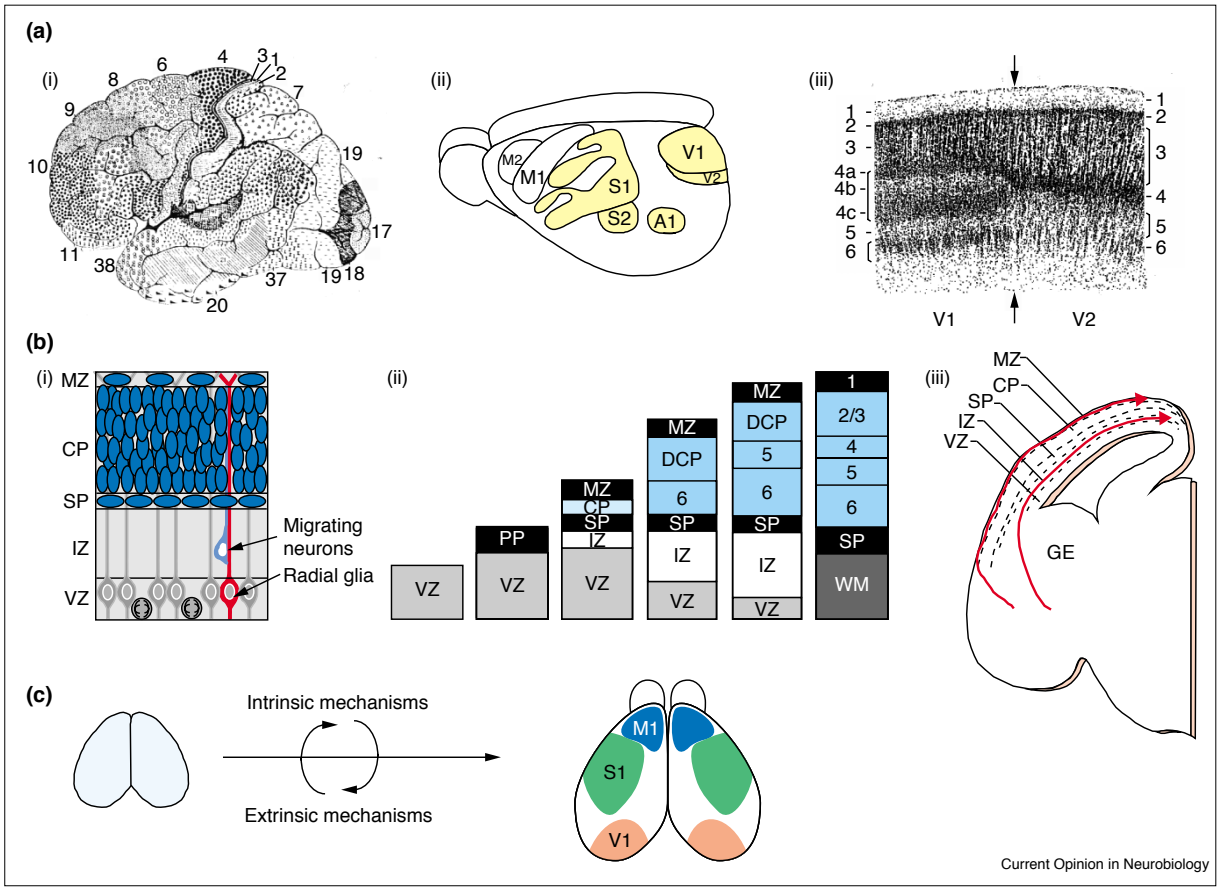
The neocortex, a dorsal telencephalic structure unique to mammals, is the largest region of the cerebral cortex and the one that exhibits the most substantial phylogenetic expansion and specialization [1,2]. The neocortex is also the most highly differentiated region of the cerebral cortex, having six major layers in its radial dimension. In its tangential dimension, the neocortex, like other cortical regions, is organized into subdivisions referred to as areas (Figure 1a). Areas are distinguished from one another by major differences in their cytoarchitecture and chemoarchitecture, and their input and output connections. The unique architecture and connections specific for each area determine, in large part, the functional specializations that characterize areas in the adult. In the adult, the transition from one neocortical area to another is not graded, but is often abrupt with sharp borders.

It has been assumed that the specification and differentiation of neocortical areas is controlled by interplays between genetic — intrinsic — and epigenetic — extrinsic — mechanisms [3–5], but, until recently, most experimental evidence has implicated extrinsic mechanisms, in particular the influence of thalamocortical axons (TCAs) [6]. Only in the last two years has compelling evidence for the genetic regulation of arealization begun to emerge, including the first demonstration of regulatory genes that control area specification [7••,8••] and evidence for patterning centers and signaling molecules that may set up the initial patterning of these genes [9••]. These and other important advances have spawned numerous review articles on topics of cortical development [10–14]. Here, we provide an update and synthesis of this dynamic field.

Corticogenesis

During development, the areas of the neocortex differentiate within an earlier, more uniform structure, comprised of postmitotic neurons and termed the cortical plate (CP) (Figure 1b,c). Most neocortical neurons, including all projection neurons, are generated within the ventricular zone (VZ) of the dorsal aspect of the lateral ventricle. The first postmitotic neurons accumulate on the top of the VZ, forming the preplate (PP), positioned just beneath the pial surface. Neurons subsequently generated in the VZ migrate along radial glia, aggregate within the PP, and form the CP, which splits the PP into a superficial marginal zone (MZ) and a deep subplate (SP). The CP gradually differentiates in a deep to superficial pattern, forming layers 6 through 2 of the adult neocortex [6]. The MZ contains Cajal-Retzius neurons that express reelin, a large secreted protein required for the proper radial migration of CP neurons and their formation of layers. Considerable progress has been made towards understanding the mechanisms of radial migration of neocortical neurons, including reelin's mode of action, its receptors and other components of its signaling pathway [15–17]. The SP contains local and long-distance projection neurons, proposed to serve a number of critical roles in cortical development, among them the pioneering of the internal capsule and the formation of major input and output projections paths between the cortex and the rest of the central nervous system [6,18]. A surprising finding,

Figure 1



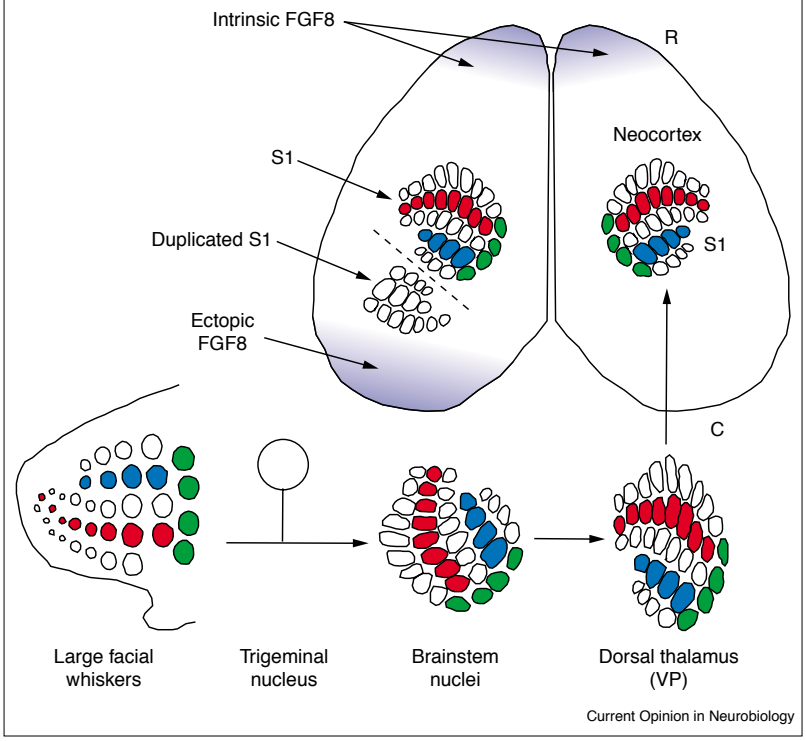
Basics of neocortical development. **(a)** Organization of the neocortex into areas. (i) Areas of the human cerebral cortex as defined by Brodman. Adapted with permission from [98]. The lateral surface of the human cerebral hemisphere shows a number of cytoarchitectonically distinct areas, such as V1 (area 17) at the caudal pole, S1 (area 3) in the middle, and, just rostral to it, M1 (area 4). Subsequent analyses confirmed that these areas are also functionally and connectionally distinct. (ii) Selected areas of the rat neocortex, showing A1, V1, V2, S1, S2 (the secondary somatosensory area), as well as M1and M2 (the secondary motor area). Adapted with permission from [1]. (iii) An example of abrupt borders between areas. The cytoarchitectonic border between V1 and V2 is shown (by arrows) in an eight month human fetus, using a Nissl stain. Each area has six primary layers, but their architecture and sublayering differs. Reproduced with permission from [98]. **(b)** Generation, migration, and lamination of neocortical neurons. (i) Most neocortical neurons, and all glutaminergic projection neurons, are generated in the neocortical VZ. Most CP neurons migrate radially on radial glial fibers from the VZ, though the intermediate zone (IZ) and aggregate in the CP. (ii) Layer development in the rodent neocortex. The first neurons generated in the VZ aggregate on top of it and form the PP, which is later split into MZ and SP by the later-generated CP neurons. CP neurons are generated in an inside-out fashion, and layers differentiate from the CP in the same pattern: earlier-born neurons form the deeper layers, whereas later-born neurons migrate past them and form the more superficial layers. WM, white matter. (iii) Most neo cortical interneurons (e.g. GABAergic interneurons) are generated in the ganglionic eminences (GE), and migrate tangentially through the IZ and MZ to distribute across the neocortex. **(c)** Development of neocortical areas. Initially: the CP lacks the many features that distinguish areas in the adult. Area-specific features gradually differentiate within it, by a process controlled by mechanisms both intrinsic and extrinsic to the neocortex.

first described a few years ago, is that, at least in rodents, the majority of cortical interneurons are not generated in the cortical VZ, but ventral to it, within the lateral and medial ganglionic eminences [19–21]. The tangential migration of postmitotic interneurons from these external germinal zones into and throughout the neocortex occurs along multiple paths and is directed in part by members of the Slit and Semaphorin families of guidance molecules (Figure 1b) [22,23•].

Differentiation of areas

The CP lacks the many features that distinguish areas in the adult, even after all the neurons have been generated and layers begin to differentiate within it. The sharp architectural borders clearly evident between many areas in the adult are lacking in the CP; instead the architecture of the CP is uniform across its tangential extent. Also absent are the restricted, area-specific distributions of distinct types of projection neurons, characteristic of the

Figure 2



Development of barrels in the somatosensory area. Vibrissae-related maps are found in the trigeminal complex of the brainstem, which receives input from the trigeminal ganglion. These brainstem nuclei in turn project to the contralateral VP nucleus of the dorsal thalamus. VP axons project to S1. Note the preservation of topography shown in different colors. When FGF8 is ectopically expressed in the caudal part of the neocortical primordium by *in vivo* electroporation, a partial duplication of the S1 barrel-field is observed [9••]. Barrel formation is dependent upon an orderly TCA input from the VP [45]. Therefore, the ectopic source of FGF8 is expected to influence the areal targeting of TCAs, such that those arising from the appropriate subset of barreloids in the VP duplicate their orderly projections to two discrete sites in the CP. C: caudal; R: rostral.

functional specializations of different cortical areas in adults. Instead, early cortical projection neurons have widespread distributions in parts of areas, and even whole areas, in which they are not found in the adult. Their restricted adult distributions arise through the elimination of functionally inappropriate axon segments and branches [24]. This mechanism is used to generate the characteristic patterning of callosal, intracortical, and subcortical projections of the mammalian neocortex. However, areal differences in the initial distribution of projection neurons do appear to exist. For example, the adult primary motor area (M1) has a higher density of layer 5 corticospinal neurons than does the primary somatosensory area (S1); this difference is evident, albeit not to the same degree as in the adult, even prior to the phase of axon elimination [25•].

Role of thalamocortical axon projections in arealization

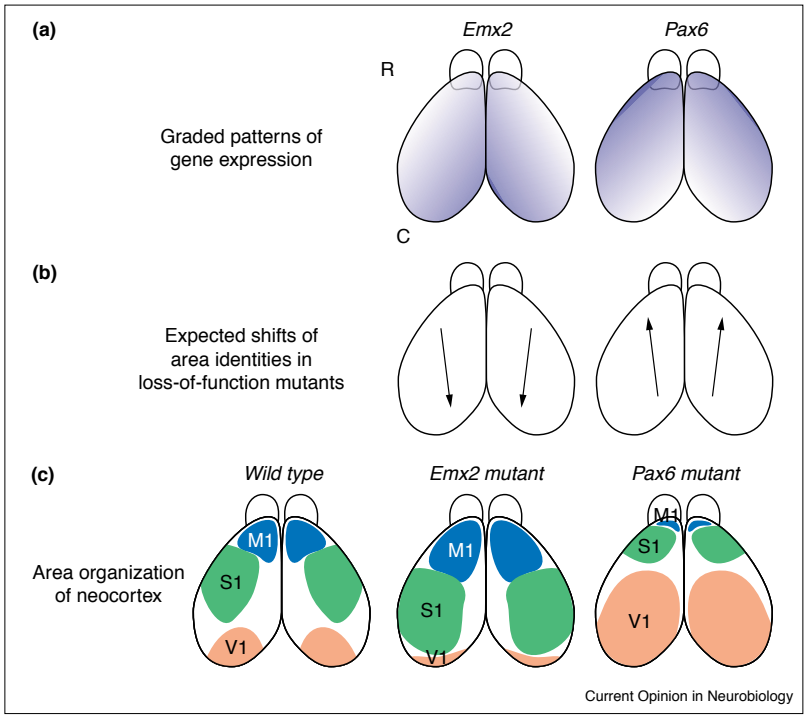
TCAs originating in the principal sensory nuclei of the dorsal thalamus form the major input to the neocortex and relay visual, auditory and somatic sensations to the primary sensory areas of the neocortex, in an area-specific manner. Because TCAs are the sole source of modality-specific sensory information to the neocortex, clearly, the functional specializations of the primary sensory areas are defined by, and dependent upon, TCA input. Numerous studies have shown that the differentiation of anatomical features that distinguish cortical areas depend to a large extent upon TCA input [4–6]. Consistent with this role, the TCA projection exhibits area-specificity throughout its development, and the gradual differentiation of areas within the CP parallels the elaboration of the TCA projection [6]. In addition, peripheral manipulations and transplantation experiments have demonstrated that the CP exhibits considerable plasticity during the development of area-specific features and that, in many respects, diverse parts of the CP initially have similar developmental potentials [3,6]. Again, TCA input has been implicated as a major influence controlling this plasticity.

The role of TCAs in shaping cortical architecture is not limited to these later events. Recent *in vitro* evidence suggests that TCAs release a diffusible mitogenic activity, which promotes the production of both glia and neurons by the cortical VZ [26•]. If a similar mechanism operates *in vivo*, such an early influence of TCAs on corticogenesis could contribute to area-specific differences in cytoarchitecture that become evident later in development. For example, depending on the timing of this mitogenic influence, TCAs may differentially control, across the VZ, the generation of neurons of a specific laminar fate.

Molecular control of area-specific thalamocortical projections

Although considerable recent progress has defined the molecular control of TCA pathfinding from the dorsal

Figure 3



Graded expression of patterns of *Emx2* and *Pax6* in the neocortical VZ and the prediction of areal shift in the absence of these regulatory genes. **(a)** *Emx2* is expressed in a high caudomedial to low rostrolateral manner, whereas *Pax6* expression shows an opposite gradient. **(b)** If these regulatory genes play roles in specifying the positional information of neuroepithelial cells, in the absence of *Emx2*, rostral areas, such as motor areas, should expand, and caudal areas, such as visual areas, should shrink. *Pax6* mutation is expected to cause an opposite effect. **(c)** Analyses of gene expression patterns and the areal targeting of the TCA projection support this hypothesis [7••,8••]. C: caudal; R: rostral.

thalamus to the neocortex [27–32], a similar characterization of the area-specific targeting of TCAs within the neocortex has lagged behind. As in the retinotectal system [33], area-specific TCA targeting is likely primarily controlled by guidance molecules, but it can also be influenced by neural activity, because blockade of neural activity results in aberrant areal targeting of TCAs [34]. SP neurons and their axons have also been implicated in area-specific TCA targeting [18,35], but their role and its molecular basis are vague. In addition, lesion studies indicate that relationships between the neocortex and dorsal thalamic nuclei appear to be plastic early in development. For example, in the marsupial, *Monodelphis domestica*, large lesions that remove one-third to three-quarters of the developing neocortex prior to the arrival of TCAs, result in an orderly compression of area-specific TCA projections [36].

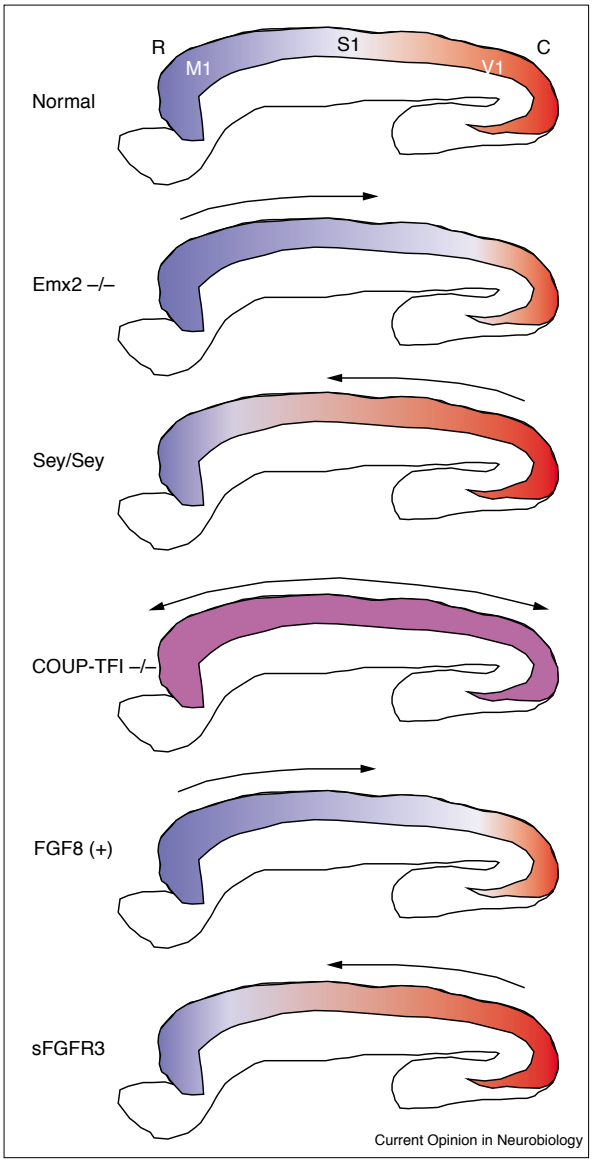
Members of the cadherin family of cell adhesion molecules may influence the development of area-specific TCA projections, because the principal sensory thalamic nuclei and their target primary sensory areas show matching expression of *cadherin-6*, *-8*, and *-11* [37–39]. Presently, the best molecular candidates for the molecular control of TCA pathfinding are members of the Eph family of receptor tyrosine kinases and their ephrin ligands [40]. Both Ephs and ephrins act as axon guidance molecules in many systems, including the retinotectal projection, whose topographic mapping resembles the mapping of TCA projections within the neocortex. In the rhesus monkey, primary (V1) and secondary (V2) visual areas form reciprocal connections with adjacent dorsal thalamic nuclei, the dorsal lateral geniculate (dLG) and pulvinar, respectively. Before TCA projections are established, *EphA3*, *A6* and *A7*, and *ephrin-A5* are expressed at higher levels in V1 than in V2. These three EphA receptors are also highly expressed in overlapping graded patterns in pulvinar and to a lesser extent in dLG, whereas *ephrin-A5* is highly expressed in the ventrolateral complex that projects to S1 rather than to visual areas [41•]. A similar theme is observed in mice: *ephrin-A5* is expressed in a medial to lateral gradient across S1, and *EphA4* is expressed in a matching gradient across the ventroposterior nucleus (VP), which provides TCA input to S1 [42•,43]. *In vitro*, ephrin-A5 repels VP axons. Surprisingly, however, in *ephrin-A5* knockout mice, VP axons properly target S1 and form an orderly map of the body, albeit one that is distorted [42•]. The *ephrin-A5* expression in S1 has also been suggested to influence, by repulsion, the targeting of axons from medial dorsal thalamic nuclei, which express *EphA5* and pass under S1 en route to their target areas in limbic cortex, a region that does not express *ephrin-A5* [44].

Neural activity and patterning of area-specific functional modules

Barrels

S1 of rodents is characterized by unique functional modules, termed barrels, comprised of layer 4 neurons aggregated around dense clusters of arborizations of TCAs that arise from the VP. Barrel patterns mirror the distribution

Figure 4



Area shifts in various experimental conditions. Schematic, sagittal view of the neocortex in neonatal mice, including three putative areas, M1, S1, and V1. The expression patterns of two hypothetical genes with high rostral to low caudal (purple) or high caudal to low rostral (red) gradient in the CP are also shown. In the *Emx2* knockout mice and *Pax6* mutant (*Sey*) mice, areas are shifted caudally and rostrally, respectively [7••,8••]. In *COUP-TFI* mutant mice, genes that normally show graded expression in the CP, including *Cad8*, *RORβ*, and *Id2*, are uniform [67•]. Overexpression of FGF8 in the rostral cortex at E11.5 results in the caudal shift of areas, similar to *Emx2* mutation, whereas expression of the soluble FGF receptor, sFGFR3, which should act as a dominant negative factor for FGF signaling, causes a rostral shift [9••]. C; caudal; R: rostral.

of large whiskers on the snout; this pattern is reiterated in specific brainstem nuclei and in the VP, where 'barreloids' correspond to barrels (Figure 2). The differentiation of barrels depends upon intact connections with the periphery [45]. Heterotopic transplantations show that other parts of embryonic rat neocortex (e.g. V1) can develop barrels when placed into S1 prior to normal barrel formation, indicating that barrels are not a genetically-specified feature unique to S1 [46]. The development of patterned neural projections has long been thought to depend upon patterned neural activity mediated, in part, by *N*-methyl-D-aspartate receptors (NMDARs) [47]. Indeed, application of an NMDAR antagonist to S1 during the critical period of barrel formation, at levels that block all glutamatergic responses (TCAs are glutamatergic), does not prevent the clustering of TCAs into a whisker-related pattern, but does result in errors in functional representation of whiskers in individual barrels [48,49]. Thus, a mechanism involving NMDA may control the segregation of TCAs into a whisker-related pattern, and the subsequent clustering of layer 4 neurons into barrels [45].

Now, through the use of mouse genetics, the effects of activity blockade on TCA patterning and postsynaptic components of barrels have been dissociated. In mice in which the NMDAR1 subunit gene was deleted specifically from excitatory cortical neurons [50•], TCAs corresponding to large whiskers segregated into a whisker-related pattern in S1. However, layer 4 neurons did not aggregate into barrels. Relatively comparable findings are described in mice deficient for either phospholipase C(PLC)-β1 or for the metabotropic glutamate receptor, mGluR5, indicating that TCA glutamatergic neurotransmission triggers a signaling mechanism involving PLC-β1 [51•]. Thus, neurotransmission by TCAs is not required for their coarse clustering into a whisker-related pattern, but is likely to be essential for the refinement of their patterning and the activation of a postsynaptic signaling cascade that results in the aggregation of layer 4 neurons into barrels.

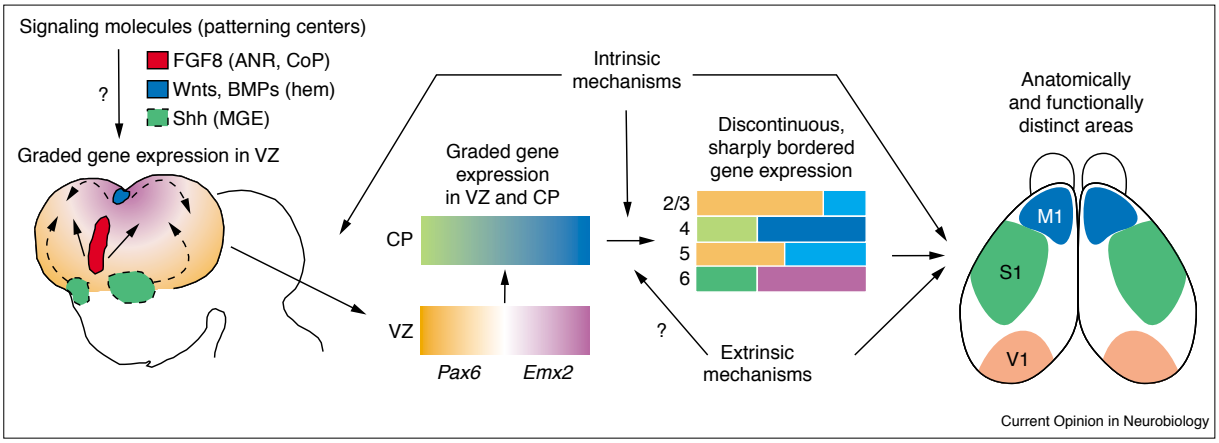
Ocular dominance columns

Similarly, the segregation of TCAs from the dLG into ocular dominance columns in layer 4 of V1 has also long been thought to be due to an activity-dependent sorting process [47]. This dogma has been challenged by new findings in the ferret, which show that dLG TCAs are in segregated ocular dominance columns, days after they innervate layer 4 (see review by Crowley and Katz, this issue). These early columns are unaffected by experimentally induced imbalances in retinal activity, implying that their initial formation is activity-independent. Therefore, they may develop by directly targeting subsets of dLG axons to particular locales in cortical layer 4 or by sorting, on the basis of molecular differences between subsets of axons [52•].

Differential gene expression intrinsic to the neocortex

Evidence for the genetic control of arealization has only been obtained in the past few years. Initially, this evidence was indirect and limited to descriptions of genes — including those encoding transcription factors and nuclear

Figure 5



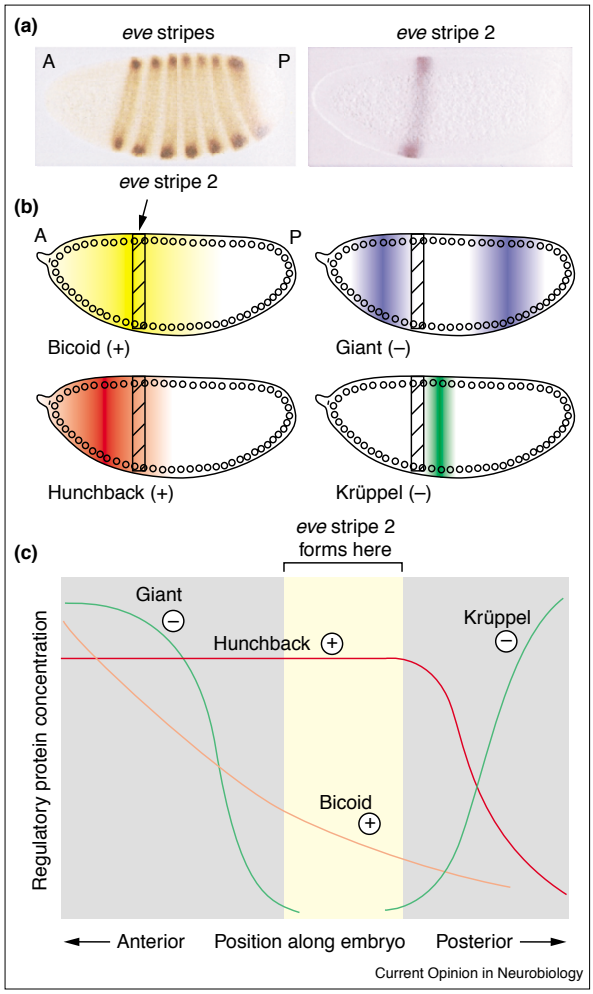
A current view of the specification and differentiation of neocortical areas. The initial, tangential gradients of regulatory genes in the VZ may be set up by signaling molecules secreted from putative patterning centers: FGF8, from the anterior neural ridge (ANR) and the CoP (commissural plate); Wnts and BMPs from the cortical hem; and Shh from the medial ganglionic eminence (MGE). The positional information contained in such gradients is likely to be inherited because of the radial migration of most CP neurons. The gradients of gene expression in the CP are often converted into sharp borders, paralleling the formation of anatomically and functionally distinct areas in the adult. The same genes show different expression patterns in different layers (such as the green and purple genes in layer 2/3 and 6), suggesting that area-specific regulation of such genes is modulated by layer-specific properties. Although the initial establishment of the graded gene expression in the CP is probably controlled by mechanisms intrinsic to the telencephalon, and later steps more likely involve extrinsic influences, such as those mediated by the incoming TCAs, the precise roles of each of the mechanisms are not well understood.

receptors, cell adhesion molecules, and axon guidance receptors and ligands — expressed in graded or restricted patterns within the VZ or CP prior to TCAs entering the neocortex [43,53–56]. The proposal that these differential patterns of gene expression are established and maintained in the neocortex independent of TCA input has been directly shown by two studies using *Gbx2* and *Mash-1* mutant mice [55,56]. Expression of *Gbx2*, a homeodomain transcription factor, and *Mash-1*, a basic helix-loop-helix (bHLH) transcription factor, is sparse or non-detectable in neocortex and mice deficient for either one fail to develop a TCA projection [30,56]. The differential expression of other genes examined was unchanged in mutants compared to wildtype littermates, indicating that these other patterns are established by mechanisms independent of TCAs and likely intrinsic to the dorsal telencephalon.

However, the conclusions from these studies are limited because *Gbx2* and *Mash-1* mutants die at birth, before any area-specific patterns of architecture or connections become evident. In addition, many genes that exhibit an early strongly graded expression, independent of TCA input (e.g. chick ovalbumin upstream transcription factor I [*COUP-TF1*]), postnatally develop complex, discontinuous expression patterns coincident with area-specific TCA projections and architecture [57•]. Thus, although the early differential expression of many genes is independent of TCA input, TCAs may influence their later expression. Indeed, TCAs influence, or are even required for, the expression of the α1 subunit of the γ-amino butyric acid ($GABA_A$) receptor [58] and the dopamine D3 receptor [59] in early postnatal rat S1.

The degree to which TCAs influence cortical gene expression, especially regulatory genes that, in turn, control downstream genes and potentially modulate the differentiation of areas, remains an open and important question. A thorough analysis of this issue requires the production of mice that are postnatally viable, but fail to form a TCA projection. A complementary approach would be to generate mice, in which the area-specific targeting of TCAs, or the extent of neocortex innervated by TCAs from a given principal sensory nucleus, is altered by genetic manipulations of dorsal thalamus. This could be accomplished by ectopically expressing either receptors that control area-specific TCA targeting, or regulatory genes that determine the nuclei-specific properties of dorsal thalamic neurons. As discussed above, such guidance receptors have not been definitely identified, but EphAs are candidates. Several regulatory genes including the LIM-homeodomain transcription factors, *Lhx2* and *Lhx9*, as well as *Gbx2*, and the bHLH transcription factor *Neurogenin2*, are expressed throughout development in distinct, but overlapping patterns in subsets of dorsal thalamic nuclei [60•]. On the basis of the functions of these genes in other systems [61–64], they are good candidates to act in a combinatorial manner to control the specification and differentiation of nuclei-specific properties of dorsal thalamic neurons, including their area-specific targeting. *Lhx2* is especially intriguing, because it is most

Figure 6



Combinatorial action of graded repressors and enhancers can generate patterns of gene expression with sharp borders. **(a)** A *Drosophila* embryo with seven stripes of *eve* expression is shown (left). *eve* is initially expressed at a low level in all nuclei of the syncytium, and the striped expression pattern develops gradually. When a piece of *eve* regulatory region is fused to a *LacZ* reporter and introduced to the embryo, β-galactosidase is expressed precisely in the position of the second of the seven *eve* stripes (right). **(b)** The non-uniform distribution of four regulatory proteins, Bicoid, Giant, Hunchback, and Krüppel, and the position of eve stripe 2 (hatched band; arrow) are shown on schematics of an embryo. **(c)** These four regulators of transcription act in combination to define the expression pattern of eve in the second stripe. Bicoid and Hunchback proteins activate *eve* in a broad domain, and the anterior and posterior borders of eve expression are formed through repression by Giant and Krüppel, respectively. In embryos that lack Krüppel, eve stripe 2 expands posteriorly. A similar patterning mechanism may operate in the mammalian neocortex to form sharp borders of gene expression. Adapted with permission from [99].

highly expressed in the medial geniculate nucleus of dorsal thalamus [60•] and in its target, the primary auditory area (A1) [55]. Thus, *Lhx2* may independently regulate the matching expression of molecules in dorsal thalamus and neocortex that control the targeting of TCAs to the auditory area.

Genetic regulation of area identity

Genes that regulate arealization presumably confer area identities to cortical cells and regulate the expression of axon guidance molecules that control the area-specific targeting of TCAs. Two genes proposed to regulate arealization are the homeodomain transcription factor *Emx2* and the paired-box transcription factor *Pax6* [3]. *Emx2* is expressed in a low rostrolateral to high caudo-medial gradient [65] and *Pax6* in a high rostrolateral to low caudomedial gradient [66] across the VZ of the embryonic neocortex. If involved in arealization, *Emx2* should preferentially impart caudal and medial area identities, whereas *Pax6* should impart rostral and lateral identities (Figure 3).

Emx2 and Pax6

The role of Emx2 in arealization has been tested by using molecular markers of area identity and labeling TCAs in mice deficient for *Emx2* [7••,8••] . Changes in the patterns of gene expression suggest that rostral–lateral areas are expanded, whereas caudal–medial areas are reduced in the mutant. Alterations in the organization of area-specific TCA projections are consistent with this interpretation. Retrograde labeling from the neocortex of *Emx2* mutants indicates an orderly expansion and a caudal shift of the topographic projection of the VP, indicative of an expansion of S1 and a caudal shift of its border, together with a contraction of V1. Bishop *et al.* [7••] used the same molecular markers to also analyze *Small eye (Sey)* mutants, a naturally occurring, non-functional mutation of *Pax6*. The gene expression patterns in *Pax6* mutants exhibit the opposite changes to those in *Emx2* mutants, suggesting that rostral–lateral areas are reduced and caudal–medial areas are expanded. Unfortunately, both *Emx2* and *Pax6 (Sey/Sey)* mutant mice die soon after birth, thus the adult anatomical and functional organization of the mutant cortex cannot be studied. Nonetheless, these findings indicate that arealization of the neocortex is altered in opposing manners in *Emx2* and *Pax6* mutant mice, as predicted from the countergradients of expression of *Emx2* and *Pax6* within the neocortical VZ (Figure 3). Thus, Emx2 and Pax6 cooperate to regulate arealization of the neocortex and likely to confer area identity to cortical cells.

COUP-TF1

A similar analysis of arealization has been carried out in mice deficient for *COUP-TF1* [67•], an orphan nuclear receptor that has a high caudal to low rostral graded expression across the neocortex, and is expressed in VZ, SP and CP [57•]. Thus, if *COUP-TF1* regulates arealization, one would predict changes in area identities similar to those observed in the *Emx2* mutants. About 1–2% of *COUP-TF1* null mice live until three weeks of age. However, analyses of areal differentiation at later postnatal ages is compromised by a substantial reduction in the number of TCAs that reach the neocortex, the failure

of TCAs to invade the CP, and a massive loss of layer 4 and SP neurons [68]. Zhou *et al.* [67•] report that, in *COUP-TF1* mutants, VP neurons can be retrogradely labeled from the cortical position that would normally develop as the visual area. However, it is difficult to relate these findings to changes in arealization for the reasons noted above. In addition, *COUP-TF1* is highly expressed in dorsal thalamus [57•,69], suggesting that the defects observed in *COUP-TF1* mutants could be autonomous to TCA projection neurons. The marker analyses of [67•] suggest that COUP-TF1 is critical for regulating arealization, but may be secondary to Emx2 and Pax6. With the exception of *Emx2* and *Pax6*, molecular markers lost their normally restricted expression patterns in the *COUP-TF1* mutants. Instead, they were uniformly expressed across the rostral–caudal extent of the neocortex (Figure 4). This finding differs from that in *Emx2* and *Pax6* mutants, in which the differential expression patterns of marker genes was largely retained but expanded or contracted (Figure 3). In contrast, *Emx2* and *Pax6* show normal graded patterns of expression in the *COUP-TF1* mutants. Thus, *COUP-TF1* mutants have an apparent loss of areal specificity, with the exception of *Emx2* and *Pax6*. A parsimonious explanation for these findings is that COUP-TF1 does not regulate arealization *per se*, but is downstream to regulatory genes, such as *Emx2* and *Pax6*, that control arealization, and is required for their proper action.

Interestingly the graded patterns of gene expression observed in the neocortex often extend beyond it. For example, the graded expression of *Emx2* continues to increase through the various hippocampal fields. This suggests a relationship between arealization of the neocortex and other regions of the cortex. Analyses of *Emx2* mutants shows that Emx2 is required for the normal growth and differentiation of hippocampal fields [70–71], but not for imparting area identities to their constituent neurons [72].

Signaling molecules that may initiate cortical patterning

Recent studies have begun to define candidate patterning centers and their signaling molecules that act early in development to establish and maintain the graded expression of regulatory genes across the neocortical VZ [7••,73,74] (Figure 5). Several secreted proteins, including fibroblast growth factor (FGF) 8, Sonic hedgehog (Shh), bone morphogenetic proteins (BMPs), and Wnts, cooperate to establish other developmental fields, such as the limb buds [75,76]. FGF8 is produced in the anterior neural ridge, located at the rostral perimeter of the prospective neocortex. Later, its expression domain extends caudally along the dorsal midline towards the diencephalon [77,78••]. BMPs (BMP2, 4, 5, 6, 7) and Wnts (Wnt2b, 3a, and 5a) are expressed in the cortical hem, a caudal midline structure adjacent to the hippocampal anlage [79,80]. *Shh* is expressed in ventral telencephalon [81]. Analyses of mutant mice and *in vivo* and *in vitro* overexpression experiments have suggested roles for several of these molecules in patterning the telencephalon (Shh [82–84], Wnt3a [9••,85], BMPs [86–88]).

FGF8 is a strong candidate for setting up the graded expression of *Emx2* (Figure 5). In embryonic chicks, a local ectopic source of FGF8 can repress the expression of *Emx2* [78••]. In mice, increasing or decreasing endogenous FGF8 *in utero* alters neocortical patterning [9••]. Overexpression of *FGF8* rostrally in the cortical primordium results in neocortical areas shifting caudally, similar to *Emx2* mutant mice [7••,8••], whereas diminishing the endogenous FGF8 signal results in areas shifting rostrally (Figure 4). These areal shifts are consistent with changes predicted by decreasing or increasing the graded expression of *Emx2* across the neocortical VZ, and are likely due to an effect of FGF8 on Emx2 levels.

A particularly intriguing finding shows that introducing an ectopic source of FGF8 caudally into the cortical primordium results in a partial duplication of the S1 barrel-field, as indicated by an ectopic, mirror-image, subset of barrels caudolateral to S1 [9••] (Figure 2). Because barrel formation depends upon an intact and orderly TCA input from the VP [45], the ectopic source of FGF8 must influence the areal targeting of TCAs. Thus, TCAs arising from the VP must send their orderly projections to two discrete sites, rather than to a singular site, in the CP. Because the TCA projection from the VP is also shifted caudally in *Emx2* mutant mice [7••], the barrel duplication observed in these mutants may be due to an ectopic, local FGF8 repression of *Emx2* expression. This, in turn, would alter the expression of guidance molecules that control the area-specific targeting of TCAs.

Other studies have provided insights into additional candidate regulators of *Emx2* expression. Ectopic expression of BMP4 in the dorsal telencephalon of embryonic chicks appears to enhance the expression of *Emx2*, either directly or through downregulation of FGF8 [88]. Expression of *Emx2* is also lost in the cortex of the *Extra-toes^J* (*Xt^J*) mutant mouse, which has a naturally occurring mutation of *Gli3*, a zinc-finger transcription factor widely expressed in the telencephalon; *Pax6* expression is maintained in the mutant [74,89]. However, it is unclear whether *Emx2* is normally regulated directly by Gli3, or by other molecules deficienct in *Xt^J* mutants [74,79,89].

Genetic fingerprinting of areas

How is the graded expression of regulatory genes, such as *Emx2* and *Pax6*, translated into downstream gene expression patterns with abrupt borders that relate to areas? Studies in *Drosophila* embryos have defined distinct mechanisms whereby transcription factors regulate the sharply bordered expression of downstream genes (Figure 6). For example, the graded distribution of a single regulatory protein, Dorsal, generates, through concentration-dependent differences in binding efficacy to gene promoter and repressor elements, expression patterns of downstream genes with sharp borders that align with the boundaries of different embryonic tissues [90]. In contrast, the sharply patterned expression of *even-skipped* (*eve*) stripe 2 gene is generated by the combined action of multiple activators and repressors of transcription

[91,92]. Similar mechanisms are being elucidated in the developing spinal cord, where Shh, secreted by the notocord and floorplate, represses or induces the expression of different classes of transcription factors in the VZ [93]. Initially, these transcription factors have graded, overlapping expression patterns that progressively sharpen through mutual repression. This mechanism generates different domains of progenitors, defined by their expression of unique subsets of transcription factors, which, in turn, generate unique classes of spinal interneurons and motor neurons.

Similar mechanisms probably operate in the neocortex to generate areas, although some differences occur, and others pathways are likely to exist (Figure 6). For example, at no time during neocortical neurogenesis are sharply bordered patterns of regulatory genes observed in the VZ; all reported have graded expression patterns. Initially, even expression in the CP is graded, but later, many genes acquire expression patterns with abrupt borders that, in some instances, appear to match the border between areas (e.g. [42•,94]). However, the expression of none of these genes is limited to a single area. To date, the only genetic marker restricted to one area is the H-2Z1 transgene, which marks the granular parts of mouse S1 [95]. H-2Z1 expression is not detected until after TCAs from the VP have invaded the S1 CP. *In vitro* explantation and *in vivo* heterotopic transplantation experiments indicate that the S1 expression of H-2Z1 is specified early in embryonic cortical development [95,96]. However, the maintenance of H-2Z1 expression *in vivo* requires TCA input, and the refinement in its expression pattern parallels the differentiation of the cytoarchitectural features characteristic of S1, a process driven by TCAs [95–97]. Thus, if the expression of H-2Z1 mimics that of an endogenous gene, this endogenous gene does not drive arealization, but instead is a product of it.

Therefore, a major unresolved issue concerns the extent to which areas are genetically distinct. A differential display PCR screen has addressed this issue by comparing RNA derived from rostral (motor areas) and caudal (visual areas) of E16 rat neocortex (prior to TCAs invading the neocortex). Over 10,000 bands were analyzed, and, of the 148 differentially expressed gene fragments, none were expressed in only rostral or caudal neocortex [57•]. Therefore, area-specific genes *per se* either do not exist or are very rare, at this developmental stage or even later. Thus, in terms of gene expression, a neocortical area is not defined by the expression of a specific set of genes restricted to that area. Instead, a neocortical area is defined by the expression of a unique subset of genes, each of which is also expressed in other areas.

However, the actual scenario is even more complex because each layer has a unique profile of gene expression. Each gene differentially expressed in the neocortex has different expression patterns in each layer. For example, *Id2*, an HLH transcription factor, has an abrupt border of expression in layer 5 that appears to correspond to the border between S1 and M1. However, in layers 2/3, *Id2* has a graded expression that continues across the tangential extent of the neocortex, being highest in rostral (motor), intermediate in S1, and lowest in caudal (visual) areas [94] (KB Bishop, DDM O'Leary, unpublished data). Thus, in the strictest sense, a 'protomap' of areas that imparts the same genetic area-identity to each progeny does not exist in the neocortical VZ, because this would require the same differential or areal gene expression patterns in multiple layers.

Conclusions

Major advances have been made in the past two years towards understanding the mechanisms that control the arealization of the neocortex. These advances include the first direct demonstration of the genetic regulation of arealization, which implicate roles for the transcription factors Emx2 and Pax6, and the signaling molecule, FGF8. To date, this evidence is largely limited to alterations in patterns of gene expression and area-specific TCA projections in neonatal mice. Thus, it will be important to assess the impact of manipulating these genes on the mature functional and anatomical organization of the neocortex.

Although recent progress has been heady, important questions remain. How is a neocortical area defined in terms of gene expression, and how does differential gene expression impart unique area properties? How are differences in the expression of the same gene between layers within an area explained, within the context of the genetic specification of areas? An understanding of these issues will require identifying arealization genes that complement *Emx2* and *Pax6*, defining the interaction between these genes, determining their downstream targets, and describing the mechanisms by which patterned gene expression is regulated. An issue intertwined with these is the degree to which TCAs influence cortical gene expression, especially regulatory genes that, in turn, can control the expression of a broad set of downstream genes and potentially modulate the differentiation of areas. A full understanding of arealization will also require a detailed characterization of the early patterning centers and the action of the signaling molecules that they secrete. These findings will be critical for understanding the interplay between intrinsic and extrinsic mechanisms in the control of arealization, and the degree to which this process is plastic.

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