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Distinct Actions of *Emx1*, *Emx2*, and *Pax6* in Regulating the Specification of Areas in the Developing Neocortex

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The mammalian neocortex is organized into subdivisions referred to as areas that are distinguished from one another by differences in architecture, axonal connections, and function. The transcription factors EMX1, EMX2, and PAX6 have been proposed to regulate arealization. *Emx1* and *Emx2* are expressed by progenitor cells in a low rostrolateral to high caudomedial gradient across the embryonic neocortex, and *Pax6* is expressed in a high rostrolateral to low caudomedial gradient. Recent evidence has suggested that EMX2 and PAX6 have a role in the genetic regulation of arealization. Here we use a panel of seven genes (*Cad6*, *Cad8*, *Id2*, *RZRβ*, *p75*, *EphA7*, and *ephrin-A5*) representative of a broad range of proteins as complementary markers of positional identity to obtain a more thorough assessment of the suggested roles for EMX2 and PAX6 in arealization, and in addition to assess the proposed but untested role for EMX1 in arealization. Orderly changes in the size and positioning of domains of marker expression in *Emx2* and *Pax6* mutants strongly imply that rostrolateral areas (motor and somatosensory) are expanded, whereas caudomedial areas (visual) are reduced in *Emx2* mutants and that opposite effects occur in *Pax6* mutants, consistent with their opposing gradients of expression. In contrast, patterns of marker expression, as well as the distribution of area-specific thalamocortical projections, appear normal in *Emx1* mutants, indicating that they do not exhibit changes in arealization. This lack of a defined role for EMX1 in arealization is supported by our finding of similar shifts in patterns of marker expression in *Emx1; Emx2* double mutants as in *Emx2* mutants. Thus, our findings indicate that EMX2 and PAX6 regulate, in opposing manners, arealization of the neocortex and impart positional identity to cortical cells, whereas EMX1 appears not to have a role in this process.

Key words: area identity; cadherins; corticogenesis; EphA7; ephrin-A5; Id2; p75; *patterning mechanisms; positional identity;* RORβ; RZRβ; *thalamocortical projections; transcription factors*

The neocortex, a dorsal telencephalic structure unique to mammals, is the largest region of the cerebral cortex and is responsible for sensory perception, cognition, and volitional control of movements. In its tangential dimension, the neocortex is organized into subdivisions referred to as areas. Areas are distinguished from one another by major differences in their architecture and axonal connections, which in large part determine the functional specializations that characterize areas in the adult. It has been assumed that the specification and differentiation of neocortical areas during development are controlled by an interplay between genetic regulation intrinsic to the neocortex and extrinsic influences arising from outside the neocortex (Rakic, 1988; O'Leary, 1989).

Until recently, most experimental evidence has implicated extrinsic mechanisms in arealization, in particular the influences of thalamocortical axons (TCAs), the major afferent projections to the cortex that define the modality of the primary sensory areas (Chenn et al., 1997). Evidence for the genetic regulation of arealization has only begun to emerge over the last 3 years (Monuki and Walsh, 2001; Ragsdale and Grove, 2001; O'Leary and Nakagawa, 2002). The initial evidence was indirect and limited to descriptions of graded or restricted patterns of gene expression across the ventricular zone or the cortical plate before TCAs enter the neocortex (Donoghue and Rakic, 1999; Mackarehtschian et al., 1999; Miyashita-Lin et al., 1999; Nakagawa et al., 1999). Analyses of *Gbx2* and *Mash1* mutant mice, which fail to develop a TCA projection (Miyashita-Lin et al., 1999; Tuttle et al., 1999), have shown that these differential patterns of gene expression are established and maintained in the embryonic neocortex independent of TCA input (Miyashita-Lin et al., 1999; Nakagawa et al., 1999) and are therefore likely controlled by mechanisms intrinsic to the dorsal telencephalon.

The closely related homeodomain transcription factors EMX1 and EMX2, and the paired-box domain transcription factor PAX6, have been proposed to be genetic regulators of arealization (O'Leary et al., 1994). *Emx1* and *Emx2* are expressed in a low rostrolateral to high caudomedial gradient (Gulisano et al., 1996; Mallamaci et al., 1998) and *Pax6* in a high rostrolateral to low caudomedial gradient (Stoykova and Gruss, 1994) across the embryonic neocortex. The expression of *Emx2* and PAX6 is restricted primarily to cortical progenitors cells, whereas *Emx1* is expressed in both progenitors and their postmitotic progeny. If involved in arealization, EMX1 and EMX2 should preferentially impart caudal and medial area identities (such as visual), whereas PAX6 should preferentially impart rostral and lateral area identities (such as motor and somatosensory).

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Recent studies have presented evidence for a role for EMX2 (Bishop et al., 2000; Mallamaci et al., 2000a) and PAX6 (Bishop et al., 2000) in arealization by analyzing *Emx2* and *Pax6 Small eye* (*Sey*/*Sey)* mutant mice. Because *Emx2* and *Pax6* mutants die on the day of birth, before areas become anatomically and functionally distinct, these analyses relied mainly on the use of genes expressed in differential patterns across the neocortex as putative molecular markers of area identity. Changes in marker expression and patterns of area-specific TCA projections suggested that rostrolateral areas are expanded, whereas caudomedial areas are reduced in *Emx2* mutants. The analysis of the *Pax6* mutants was limited to the patterned expression of *Cad6* and *Cad8* (Bishop et al., 2000), which exhibit changes opposite to those in *Emx2* mutants, suggesting that rostrolateral areas are reduced and caudomedial areas are expanded in the *Pax6* mutants.

Because the interpretations in these studies were based on very limited markers, not only in number but also in the classes of genes represented and in their patterns of expression, we have performed a more thorough marker analysis of arealization in *Emx2* and *Pax6* mutants, using a battery of seven complementary markers representative of a broad range of genes. These genes encode a broad range of proteins, including cell adhesion molecules (*Cad6* and *Cad8*), an HLH transcription factor (*Id2*), an orphan nuclear receptor (*RZRβ*, also termed *RORβ*), and a neurotrophin receptor (*p75*), as well as receptors and ligands involved in axon guidance and cell migration (*EphA7* and *ephrin-A5*). This more extensive analysis is especially critical for substantiating the role of PAX6 in arealization, because *Pax6* mutants lack a TCA projection, and therefore the area-specific patterning of this projection cannot be studied. In addition, we analyze sectioned material that reveals more complex and informative expression patterns than observed in the previous whole-mount *in situ* analysis.

The similarities in the graded expression of *Emx1* and *Emx2*, and their close sequence homologies, have prompted the proposal that EMX1 also may regulate arealization of the neocortex (Cecchi and Boncinelli, 2000). Therefore, we have also addressed its role in arealization by analyzing in *Emx1* mutant mice the patterned expression of the set of seven gene markers, as well as area-specific TCA projections. To our surprise, the *Emx1* mutants do not exhibit defects in arealization. To assess whether this may be attributable to *Emx2* compensating for the loss of *Emx1*, we performed a marker analysis of *Emx1; Emx2* double mutants. Our findings indicate that EMX2 and PAX6 disproportionately regulate arealization of the neocortex in opposing manners, whereas EMX1 has no apparent role in this process.

MATERIALS AND METHODS

Mice. Single-mutant *Emx1* and *Emx2* mice used for this study were generated from heterozygous breeding pairs, which allowed for a direct comparison of gene expression patterns between +/+, +/−, and −/− littermates for each mutant studied. *Emx2* mice were maintained on a C57/BL6 background, and *Emx1* mice were maintained on a 129/Sv background. To generate *Emx1; Emx2* double mutant embryos, *Emx1*+/− mice were crossed with *Emx2*+/− mice. Offspring were genotyped and *Emx1*+/−; *Emx2*+/− and *Emx1*−/−; *Emx2*+/− mice were bred with each other to obtain double mutant embryos and their littermates. *Emx2* (Pelligrini et al., 1996), *Emx1* (Qiu et al., 1996), and *Emx1*; *Emx2* double mutant mice were genotyped by PCR as described. PAX6 mutants were obtained from mating heterozygous *Sey* mice (Hill et al., 1991) maintained on a C57BL/6J × DBA/2J background. *Sey* mice were genotyped by eye morphology: *Sey*/*Sey* mice lack eyes, and +/*Sey* mice have a pronounced reduction in the external size of the eye and the lens at embryonic day (E) 18.5 (Hill et al., 1991). Because *Emx2*−/−, *Sey*/*Sey, and Emx1*−/−; *Emx2*−/− mice die within a few hours after birth, embryos were removed and fixed at E18.5, just before birth, which for these mouse strains occurs at approximately E19.0. Although *Emx1* null mice live to adulthood and are fertile (Qiu et al., 1996), we used newborn [postnatal day (P) 0] *Emx1* mice to facilitate comparisons of gene expression patterns between the three mutants analyzed in this study. Analyses were done blinded to genotype. Midday of the day of vaginal plug detection was considered E0.5, and the first 24 hr after birth is termed P0. Animal care was in accordance with institutional guidelines.

In situ *hybridization.* For *in situ* hybridization on sections, brains were fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), cryoprotected with 30% sucrose in PFA/PB, and cut at 20 μm in the sagittal plane on a cryostat. *In situ* hybridization using ^{35}S-labeled riboprobes and counterstaining with bisbenzimide were performed as described previously (Liu et al., 2000). The following riboprobes were synthesized from cDNA templates: *Cad6* (from S. Mah and C. Kintner); *Cad8*; *Id2* (from M. Israel), *RZRβ* (from M. Becker-Andre, Serono Pharmaceutical Research Institute, Plan-Les-Ouates, Switzerland), *p75* (from K.-F. Lee, Salk Institute, LaJolla, CA), *EphA7* (from A. Brown and G. Lemke, Salk Institute, LaJolla, CA) and *ephrin-A5.* Whole-mount *in situ* hybridization of the intact forebrain and midbrain was performed with digoxygenin-labeled riboprobes for *Cad6* and *Cad8* using a modification of the protocol of Henrique et al. (1995), described in Nakagawa et al. (1999).

Retrograde axon labeling. Newborn mice were perfused transcardially with 4% PFA in PB, and their brains were removed and postfixed overnight. Thalamocortical projection neurons were retrogradely labeled using crystals of the lipophilic dyes 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes, Eugene, OR) and 4-[4-(dihexadecylamino)stryryl]-*N*-methylpyridinium iodide (DiA) (Molecular Probes) (Honig and Hume, 1989a,b) placed into the neocortex of *Emx1*+/+, +/−, and −/− littermates. In the same cortical hemisphere, a single small crystal of DiI was placed at a site that in a wild-type mouse would be near the center of primary visual area, and a single small crystal of DiA was placed at a site that in a wild-type mouse would be near the center of the primary somatosensory area. Care was taken to equate crystal size and placement between littermate sets of brains. In addition, we attempted to place the DiA crystal in the cortical plate and not involve the underlying subplate, the intracortical pathway of TCAs, to avoid the possible labeling of dorsal lateral geniculate thalamic nucleus (dLG) axons en route to the primary visual area. Brains were stored for 2 weeks at 30°C in fixative, which was sufficient time to completely fill thalamocortical projection neurons. The brains were then cut sagitally at 100 μm on a vibratome. Sections were counterstained with bisbenzimide, and every section through the thalamus and cortical crystal sites was serially mounted. Sections were analyzed and photographed with a Nikon upright fluorescence microscope using RITC (DiI), FITC (DiA) and UV (bisbenzimide) filter cubes. Before sectioning, the dorsal surface of the cortical hemisphere was photographed to document the locations of the DiI and DiA crystal placements; the placement sites and their sizes were further documented in the sections.

RESULTS

In the major component of this study, we examined in wild-type mice, in *Pax6*, *Emx2* and *Emx1* mutant mice, and in *Emx1; Emx2* double mutant mice, the expression of seven genes (*Cad8*, *Cad6*, *Id2*, *p75*, *RZRβ*, *ephrin-A5*, and *EphA7*) that in wild-type mice exhibit restricted or differential patterns of expression across the embryonic neocortex (see introductory remarks). Therefore, these genes are useful as molecular markers of position-dependent or areal properties of neocortical cells. Our analyses were done on E18.5 (hours before birth) or P0, the day *Emx2* and *Pax6* mutants die. Figure 1 presents in a schematic manner the graded expression of *Emx1*, *Emx2*, and *Pax6* and summarizes our predictions and findings from loss-of-function mutant analyses. For simplicity, we use the terms motor, auditory, somatosensory, and visual areas to describe the areal expression patterns that we observed.

Genes with expression patterns restricted to one area have yet to be identified (Liu et al., 2000), but the layer-specific expression domains of several of the genes examined here approximate the locations of cortical areas or boundaries between them; citations

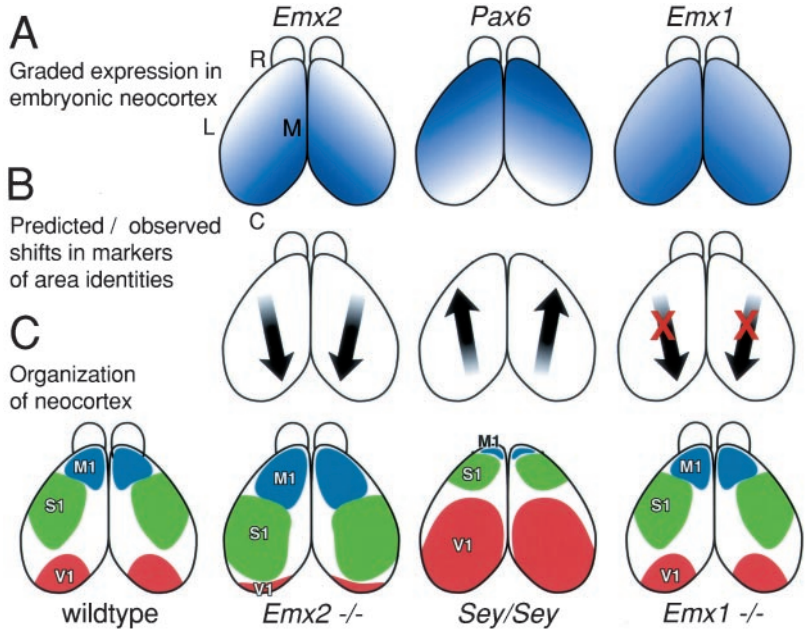

Figure 1. Hypotheses, predicted results, and interpretations of analyses in this study. Diagrams are of dorsal views of the mouse neocortex. *A*, Graded expression patterns of the transcription factors *Emx2*, *Pax6*, and *Emx1* across the embryonic neocortex. *Emx2* and *Emx1* are expressed in a high caudomedial to low rostrolateral gradient, whereas *Pax6* is expressed in an opposing gradient. *B*, *Arrows* indicate the direction of the predicted shifts in markers of area identity in *Emx2*, *Pax6* (*Sey*/*Sey*), and *Emx1* loss-of-function mutants, if these genes are involved in regulating arealization of the neocortex. The predicted shifts are observed in *Emx2* and *Pax6* mutants but not in *Emx1* mutants (indicated by *red X* marks). *C*, Organization of the mouse neocortex into areas predicted by our findings. These diagrams are not intended to show the exact sizes and shapes of the primary neocortical areas but rather to depict the disproportionate changes in area size and positioning, or no changes, in arealization in the different mutants. These predicted organizations suggested by our analyses of gene markers and area-specific thalamocortical projections are limited because the *Emx2* and *Pax6* mutants die on the day of birth, before areas become anatomically and functionally distinct, and thalamocortical projections do not develop in *Pax6* mutants. For simplicity, only the primary visual (*V1*), motor (*M1*), and somatosensory (*S1*) areas are shown. *C*, Caudal; *L*, lateral; *M*, medial; *R*, rostral; *Sey*, small eye mutant.

are provided for instances in which these relationships have been corroborated in previous studies. However, even genes with restricted or graded expression patterns that do not directly relate to a specific cortical area or a boundary between areas can be used as markers for position-dependent molecular properties of neocortical cells that differ across the tangential or "areal" extent of the neocortex and presumably are part of a repertoire of molecular markers that define the area identity and characteristics of neocortical cells. Expansions and contractions of the expression domains in the mutant neocortex, in particular if the changes are consistent across the set of markers, are evidence for changes in the position-dependent molecular properties of neocortical cells.

Areal patterns of gene expression in the neocortex are altered in opposing manners in *Emx2* and *Pax6* mutants

In the first part of this study, we examine in *Emx2* and *Pax6* mutant mice the expression of seven genes that in wild-type mice are expressed in restricted or graded patterns that relate to the organization of the neocortex into areas. To facilitate this comparison, we have divided the seven genes into three groups (*Cad8*, *Cad6*, and *Id2*; *RZRβ* and *p75*; *ephrin-A5* and *EphA7*) on the basis of how effectively their expression patterns complement or corroborate one another. The expression of these genes is also layer-specific, and for most of them, their areal expression patterns differ between layers. Although *Emx2* mutants exhibit minor lamination defects (Mallamaci et al., 2000b), at a qualitative level of analysis, the changes in areal patterning of the marker genes appears to be consistent across layers; it is difficult to comment on this issue in the *Pax6* mutants because of the significant defects in lamination (Caric et al., 1997).

Emx2 and *Pax6* mutants: *Cad8*, *Cad6*, and *Id2*

As we showed recently using whole-mount *in situ* analysis (Bishop et al., 2000), the rostral domain of *Cad8* expression observed in wild-type cortex exhibits a caudal expansion in *Emx2* mutants and a rostral restriction in *Pax6* mutants (Fig. 2*A–D*). However, these whole-mount *in situ* analyses of the cortical hemisphere reveal only the superficial rostral expression domain of *Cad8*, which corresponds to the high level of expression in layers 2/3 of the motor area (Suzuki et al., 1997; Inoue et al., 1998). Therefore, we have confirmed and extended the finding of Bishop et al. (2000) by doing an analysis of the more complex, and revealing, expression pattern of *Cad8* obtained by *in situ* hybridization on sections from E18.5 wild-type and mutant cortex.

In wild-type mice, *Cad8* is broadly expressed across the neocortex within layer 5; however, throughout rostral neocortex, *Cad8* is also highly expressed in layers 2/3 (Suzuki et al., 1997; Nakagawa et al., 1999) (Fig. 2*A′*,*C′*). The caudal boundary of this superficial expression domain (i.e., layers 2/3) has been reported to correspond to the boundary between motor and somatosensory areas (Suzuki et al., 1997). In *Emx2* mutants, this superficial rostral expression domain is expanded, and its caudal boundary is shifted caudally (Fig. 2, compare *A′*, *B′*). An opposite and dramatic change is observed in *Pax6* mutants in which the superficial rostral expression domain is absent (Fig. 2, compare *C′*, *D′*).

Similar to its rostral superficial expression domain, in caudal neocortex corresponding to the visual area, *Cad8* is also expressed in layers 2/3 and deep to them, whereas in contrast, expression in the somatosensory area positioned between the motor and visual areas is very low in the superficial layers and limited mainly to layer 5 (Suzuki et al., 1997; Nakagawa et al., 1999) (Fig. 2*A′*,*C′*). In *Emx2* mutants, the expression pattern characteristic of the somatosensory area also shifts caudally and is found within caudal neocortex in the location normally occupied by the caudal superficial *Cad8* expression domain characteristic of the visual area, which appears to be absent (Fig. 2*B′*).

Figure 3 illustrates the expression of *Id2* and *Cad6* in *Emx2* and *Pax6* mutants and their wild-type littermates. In wild-type mice, both genes are broadly expressed across the neocortex, but each exhibits differential laminar patterns of expression characterized by abrupt changes in expression that are layer specific. *Id2* exhibits strong expression in layer 5 in intermediate and caudal parts of the neocortex but weak expression in rostral parts. The change in layer 5 from strong to weak expression occurs abruptly at a position that corresponds to the boundary between motor and somatosensory areas (Rubenstein et al., 1999). This boundary between strong and weak layer 5 expression is shifted caudally in

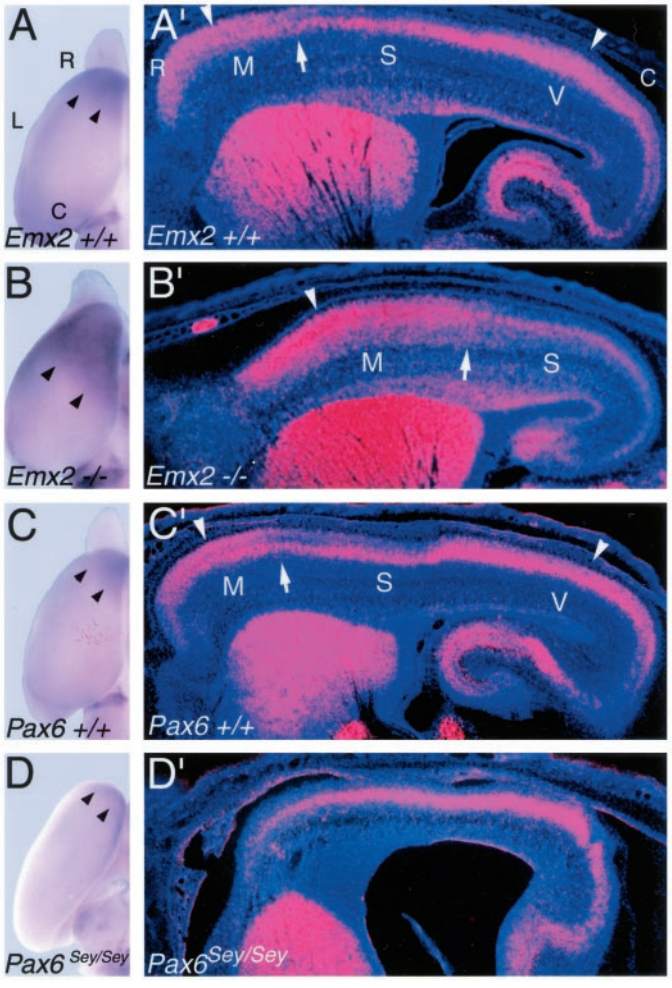

Figure 2. Opposing changes in the expression domains of the cadherin, *Cad8* in *Emx2*, and *Pax6* (*Sey*/*Sey*) mutants. *A–D*, Dorsal views of whole mounts of P0 cortical hemisphere of *Emx2* wild-type (+/+) (*A*), *Emx2* mutant (−/−) (*B*), *Pax6* wild-type (+/+) (*C*), and *Pax6* (*Sey*/*Sey*) mutant (*D*) processed for *in situ* hybridization using digoxygenin-labeled riboprobes for *Cad8*. *Arrowheads* mark the caudal limit of the rostral expression domain of *Cad8* in the superficial layers, which is characteristic of motor areas. *A′–D′*, Sagittal sections through E18.5 brains of mice of the corresponding genotypes as in *A–D*, processed for *in situ* hybridization using S^{35}-labeled riboprobes for *Cad8*. Sections were later counterstained with bisbenzimide. Sections are taken from similar mediolateral positions; rostral is to the *left* and dorsal is to the *top*. Each *panel* is a montage of single-exposure photos using dark-field illumination with a red filter to view the silver grains and with UV fluorescence to view the counterstain. Marked are the approximate locations of the motor (*M*), somatosensory (*S*), and visual (*V*) areas in the wild-type cortex and their shifted locations in the *Emx2*−/− cortex suggested by the expansion and caudal shift in patterns of *Cad8* expression, which are unique in each of these areas in wild-type mice. *Arrowheads* in *A′–C′* mark rostral and caudal expression domains in the superficial layers characteristic of motor and visual areas; in comparison, expression is substantially diminished in the superficial layers of the intervening somatosensory area. *Arrows* in *A′–C′* mark the presumed border between motor and somatosensory areas. The *Cad8* expression rostral to these *arrows* is the expression that is evident in the whole mounts shown in *A–C*. This superficial rostral expression domain characteristic of motor areas is essentially absent in *Pax6* (*Sey*/*Sey*) mutants (*D*, *D′*). The wild type and mutants in each pair are age-matched littermates. See Results for details. *C*, Caudal; *L*, lateral; *R*, rostral.

Emx2 mutants and rostrally in *Pax6* mutants. In addition, wild-type mice exhibit another differential expression pattern specific for layers 2/3. *Id2* is strongly expressed in layers 2/3 of rostral neocortex, but in intermediate parts of the neocortex (e.g., the somatosensory area), the expression level declines rapidly to low or nondetectable levels characteristic of caudal neocortex (i.e., the visual area). The rostral domain of strong expression in layers 2/3 expands caudally in *Emx2* mutants, leaving only the extreme caudal pole of the cortical hemisphere with a low level of *Id2* expression. In *Pax6* mutants, the domain of strong expression characteristic of layers 2/3 of rostral neocortex appears to be absent throughout the neocortex.

In wild-type mice, *Cad6* is expressed prominently in dorsal and lateral parts of the neocortex corresponding to somatosensory and auditory areas and exhibits a domain of low expression, especially deficient in layers 4 and 5, in extreme rostral neocortex (Suzuki et al., 1997; Nakagawa et al., 1999). In *Emx2* mutants, the domain of low *Cad6* expression normally confined to extreme rostral neocortex is greatly expanded and covers virtually the rostral half of the neocortex. In contrast, this domain is essentially absent in *Pax6* mutants, and high levels of *Cad6* expression continue to the rostral pole of the neocortex.

These data on changes in the expression of *Cad8*, *Cad6*, and *Id2* are consistent with an expansion of rostral neocortical areas and a reduction of caudal areas in *Emx2* mutants and a contraction of rostral neocortical areas and an expansion of caudal areas in *Pax6* mutants.

Emx2 and *Pax6* mutants: *RZRβ* and *p75*

Figure 4 illustrates the expression of *RZRβ* (*RORβ*) and *p75* at E18.5 in *Emx2* and *Pax6* mutants and their wild-type littermates. *RZRβ* and *p75* exhibit strongly graded patterns of expression across the neocortex, but in opposing directions (Mackarehtschian et al., 1999; Rubenstein et al., 1999). In wild-type mice, *RZRβ* exhibits a graded expression within layer 4 that extends across most of the rostrocaudal extent of the neocortex; expression is strong rostrally and progressively declines to very low levels near the extreme caudal pole of the neocortex. In addition to this reported graded expression within layer 4 (Rubenstein et al., 1999), we find that *RZRβ* is also differentially expressed within layer 5, with a domain of high expression restricted to the rostral pole of the neocortex that exhibits a rapid decline of expression to reach very low levels of expression at about the midpoint of the neocortex; expression is very low or nondetectable in the caudal half of the neocortex.

Each of these layer-specific graded expression patterns of *RZRβ* exhibit opposing changes in *Emx2* and *Pax6* mutants. In *Emx2* mutants, *RZRβ* is highly expressed in layer 4 across virtually the entire neocortex, and the domain of *RZRβ* layer 5 expression characteristic of rostral neocortex is substantially expanded and exhibits high expression throughout intermediate parts of the neocortex and well into caudal neocortex. In *Pax6* mutants, we observe the opposite changes in expression, with virtually no expression detected in any layers in the caudal half of the neocortex.

In wild-type neocortex, *p75* expression is confined to the subplate and layer 6 of caudal neocortex and tapers off sharply to nondetectable levels of expression rostrally (Mackarehtschian et al., 1999). In *Emx2* mutants, the domain of *p75* expression is contracted and shifted caudally. The changes in *p75* expression are more impressive in the *Pax6* mutants, in which the domain of

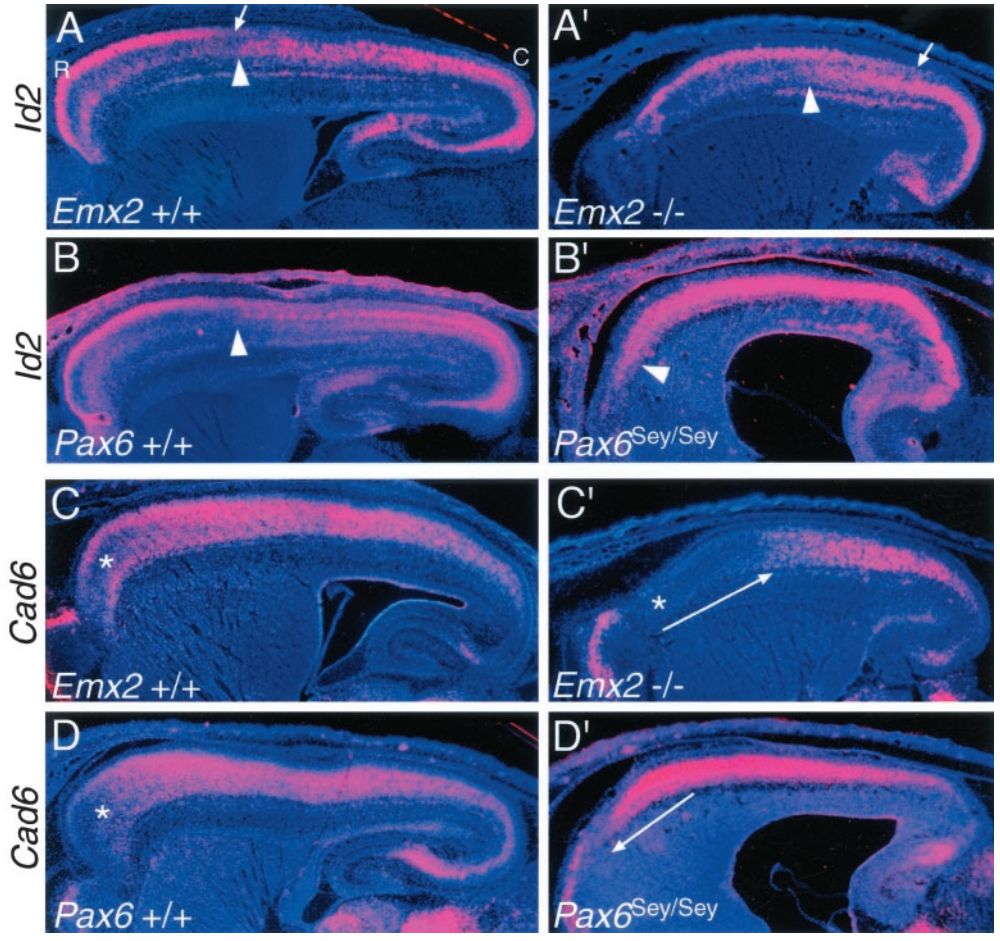

Figure 3. *Id2* and *Cad6*, used as markers of rostral neocortical areas, show opposing shifts in *Emx2* and *Pax6* mutants. *In situ* hybridizations on sagittal sections through the forebrain of E18.5 mice using S^{35}-labeled riboprobes for the HLH transcription factor, *Id2* (*A–B′*) or the cadherin, *Cad6* (*C–D′*), and counterstained with bisbenzimide are shown. Sections are from *Emx2* wild-type (+/+) and mutant (−/−) littermates or *Pax6* wild-type (+/+) and mutant (*Sey*/*Sey*) littermates and are taken from similar medial–lateral positions. Each *panel* is a montage of single-exposure photos using dark-field illumination with a red filter to view the silver grains and UV fluorescence to view the counterstain. *Id2* exhibits a graded expression in superficial layers of rostral areas in wild-type mice; the *arrows* in *A* and *A′* mark the position where the expression declines to very low levels. The *arrowheads* in *A–B′* mark the transition from low to high expression reported in layer 5; this transition corresponds to the border between motor and somatosensory areas. The *asterisks* in *C*, *C′*, and *D* mark a domain of low *Cad6* expression normally characteristic of far rostral neocortex. This domain of low expression expands and shifts caudally in *Emx2* mutants, whereas in *Pax6* mutants it shifts rostrally and essentially disappears (*C′*, *D′*, *long arrows*). See Results for details. *C*, Caudal; *R*, rostral.

p75 expression expands rostrally to such an extent that it covers virtually the entire rostrocaudal axis of the neocortex.

These data on changes in the expression of *RZRβ* and *p75* are consistent with an expansion of rostral neocortical areas and reduction of caudal areas in *Emx2* mutants and contraction of rostral neocortical areas and expansion of caudal areas in *Pax6* mutants.

Emx2 and *Pax6* mutants: *ephrin-A5* and *EphA7*

Figure 5 illustrates the expression of *ephrin-A5* and *EphA7* at E18.5 in *Emx2* and *Pax6* mutants and their wild-type littermates. In wild-type mice, *ephrin-A5* is expressed most highly in dorsolateral parts of the neocortex that correspond to the somatosensory area (Mackarehtschian et al., 1999). The domain of high *ephrin-A5* expression is shifted and restricted caudally in *Emx2* mutant neocortex, whereas in *Pax6* mutants, the domain of high *ephrin-A5* expression is shifted and restricted rostrally. In wild-type mice, *EphA7* is expressed throughout the neocortex but at much lower levels in intermediate parts of the neocortex corresponding to the somatosensory area (Rubenstein et al., 1999). In *Emx2* mutants, this domain of lower *EphA7* expression is reduced in extent and shifted caudally, whereas in *Pax6* mutants a domain of lower *EphA7* expression similar to that observed in wild type is not evident, but a small domain of reduced expression is apparent and restricted to a more rostral position. These data on changes in the expression of *ephrin-A5* and *EphA7* are consistent with an expansion of rostral neocortical areas and reduction of caudal areas in *Emx2* mutants and contraction of rostral neocortical areas and expansion of caudal areas in *Pax6* mutants.

Areal patterns of gene expression in the neocortex of *Emx1* mutants resemble those in wild-type mice

The changes in the patterns of gene markers described above indicate that EMX2 and PAX6 are involved in regulating the areal patterning of molecular correlates of neocortical arealization. In the second part of this study, we have performed a set of analyses to determine whether EMX1 may also confer position-dependent or area-specific properties to neocortical cells. First, we have used the same panel of seven genes as markers for potential changes in arealization of *Emx1* mutant neocortex by doing a comparison of gene expression patterns in *Emx1*+/+, +/−, and −/− littermates. As illustrated in Figures 6 and 7, we observe no significant alterations in the areal expression patterns of the panel of seven genes analyzed (data from the *Emx1* heterozygous mice are not shown).

Expression analysis of *Cad8* and *Cad6* was done using *in situ* hybridization on both whole mounts and sagittal sections of E18.5 in wild-type and *Emx1* mutants (Fig. 6). In whole mounts of wild-type brains, the expression domain of *Cad8* is restricted to rostral neocortex, which corresponds to the high level of expression in layers 2/3 of the motor area (Suzuki et al., 1997; Inoue et al., 1998). This rostral expression domain of *Cad8* is unaltered in *Emx1* mutants compared with wild type (Fig. 6*A*,*B*). In whole mounts of wild-type brains, *Cad6* is expressed in a domain in lateral neocortex corresponding to somatosensory and auditory areas (Suzuki et al., 1997; Inoue et al., 1998). This expression domain of *Cad6* appears unaltered

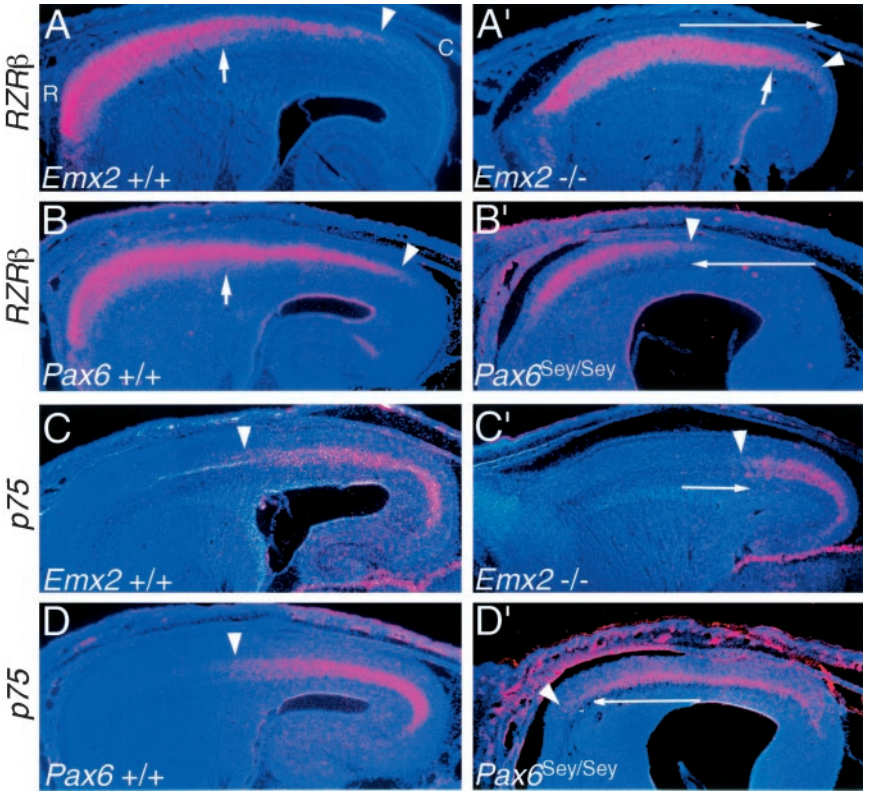

Figure 4. The complementary expression patterns of *RZRβ* and *p75* show opposing shifts in *Emx2* and *Pax6* mutants. *In situ* hybridizations on sagittal sections through the forebrain of E18.5 mice using S^{35}-labeled riboprobes for either the nuclear receptor *RZRβ* (*A–B′*) or the neurotrophin receptor *p75* (*C–D′*) and counterstained with bisbenzimide are shown. Sections are from *Emx2* wild-type (+/+) and mutant (−/−) littermates or *Pax6* wild-type (+/+) and mutant (*Sey*/*Sey*) littermates and are taken from similar medial–lateral positions. Each *panel* is a montage of single-exposure photos using dark-field illumination with a red filter to view the silver grains and UV fluorescence to view the counterstain. *RZRβ* shows two distinct high rostral to low caudal gradients across the wild-type neocortex (*A*, *B*), a gradient in superficial layers that extends farther caudally (marked by *arrowheads*) than a gradient within the deeper layers (marked by *short arrows*). Both gradients of *RZRβ* expression expand caudally in *Emx2* mutants (*A′*) and constrict rostrally in *Pax6* mutants (*B′*). *p75* is expressed in the deep layers in roughly the caudal half of the wild-type neocortex (*C*, *D*). *p75* expression constricts caudally in *Emx2* mutants (*C′*) and expands rostrally in *Pax6* mutants (*D′*). The *arrowheads* mark the rostral limit of expression. The *long arrows* in *A′–D′* indicate the opposing shifts in expression in the mutants. See Results for details. *C*, Caudal; *R*, rostral.

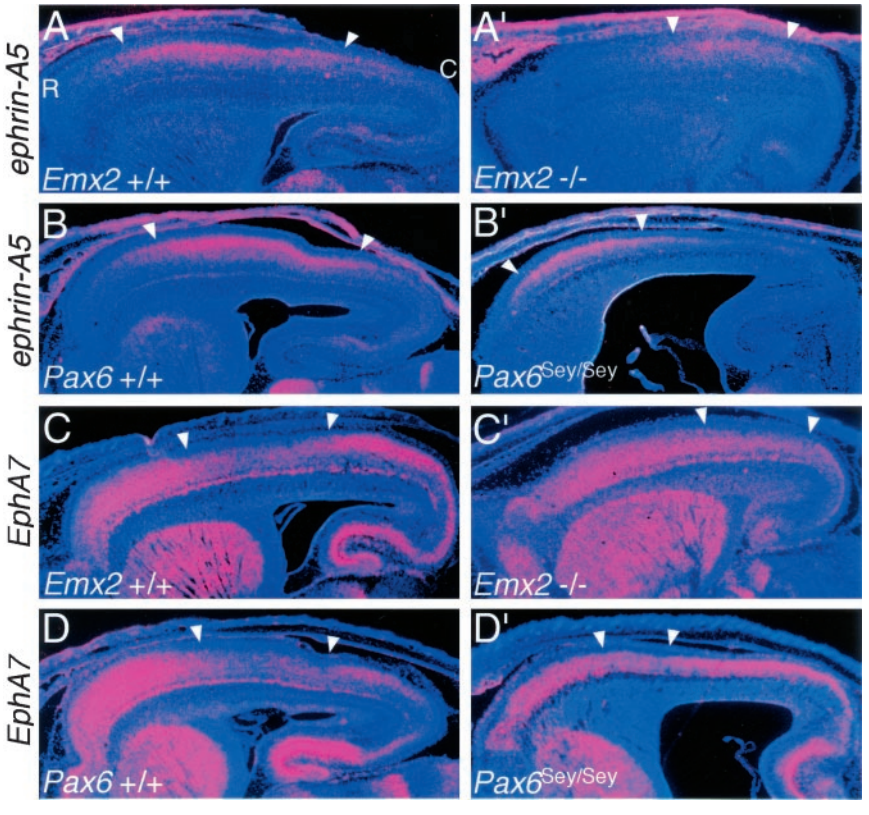

Figure 5. The complementary expression patterns of *ephrin-A5* and *EphA7*, used as markers of intermediate neocortical areas, show opposing shifts in *Emx2* and *Pax6* mutants. *In situ* hybridizations on sagittal sections through the forebrain of E18.5 mice using S^{35}-labeled riboprobes for the axon guidance ligand, *ephrin-A5*, and one of its receptors, *EphA7*, and counterstained with bisbenzimide are shown. Sections are from *Emx2* wild-type (+/+) and mutant (−/−) littermates (*A–B′*) or *Pax6* wild-type (+/+) and mutant (*Sey*/*Sey*) littermates (*C–D′*) and are taken from similar medial–lateral positions. Each *panel* is a montage of single-exposure photos using dark-field illumination with a red filter to view the silver grains and UV fluorescence to view the counterstain. In wild-type mice, *ephrin-A5* has high expression centered on the somatosensory area (*A*, *B*), whereas *EphA7* has low expression centered on the somatosensory area (*C*, *D*). These domains shift caudally in *Emx2* mutants (*A′–B′*) and rostrally in *Pax6* mutants (*C′–D′*). *Arrowheads* mark the domains of high *ephrin-A5* or low *EphA7* expression. See Results for details. *C*, Caudal; *R*, rostral.

in *Emx1* mutants compared with wild type (Fig. 6*C*,*D*). Expression analyses show that the more complex expression patterns observed in sagittal sections are also similar between wild-type and *Emx1* mutants for both *Cad8* (Fig. 6*A′*,*B′*) and *Cad6* (data not shown). Expression analyses of *Id2*, *RZRβ*, *p75*, *ephrin-A5*, and *EphA7*, on sagittal sections also reveal very similar patterns in the cortex of *Emx1* mutants compared with wild type (Fig. 7). Taken together, these findings indicate that molecular markers of neocortical areas are expressed normally in *Emx1* mutant neocortex.

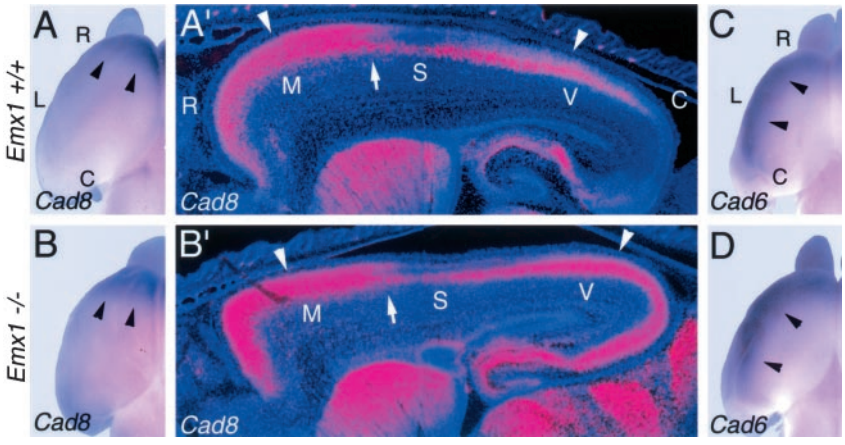

Figure 6. Patterned expression of *Cad8* and *Cad6* appears normal in *Emx1* mutants. *A–D*, Dorsal views of whole mounts of E18.5 cortical hemispheres of *Emx1* wild-type (+/+) and mutant (−/−) mice processed for *in situ* hybridization using digoxygenin-labeled riboprobes for *Cad8* (*A*, *B*) or *Cad6* (*C*, *D*). *Arrowheads* in *A* and *B* mark the caudal limit of the rostral *Cad8* expression domain characteristic of motor areas and mark the lateral expression domain of *Cad6* in *C* and *D*. *A′*, *B′*, Sagittal sections through E18.5 brains of *Emx1* wild-type (+/+) and mutant (−/−) mice processed for *in situ* hybridization using S^{35}-labeled riboprobes for *Cad8.* Sections were later counterstained with bisbenzimide. Sections are taken from similar medial–lateral positions; rostral is to the *left* and dorsal is to the *top*. Each *panel* is a montage of single-exposure photos using dark-field illumination with a red filter to view the silver grains and UV fluorescence to view the counterstain. Marked are the approximate locations of the motor (*M*), somatosensory (*S*), and visual (*V*) areas suggested by the patterns of *Cad8* expression, which are unique in each of these areas in wild-type mice. *Arrowheads* in *A′–B′* mark rostral and caudal expression domains in the superficial layers characteristic of motor and visual areas; in comparison, expression is substantially diminished in the superficial layers of the intervening somatosensory area. Arrows in *A′–B′* mark the presumed border between motor and somatosensory areas. The *Cad8* expression rostral to these *arrows* is the expression that is evident in the whole mounts shown in *A* and *B*. The wild-type and mutants in each pair are age-matched littermates. See Results for details. *C*, Caudal; *L*, lateral; *R*, rostral.

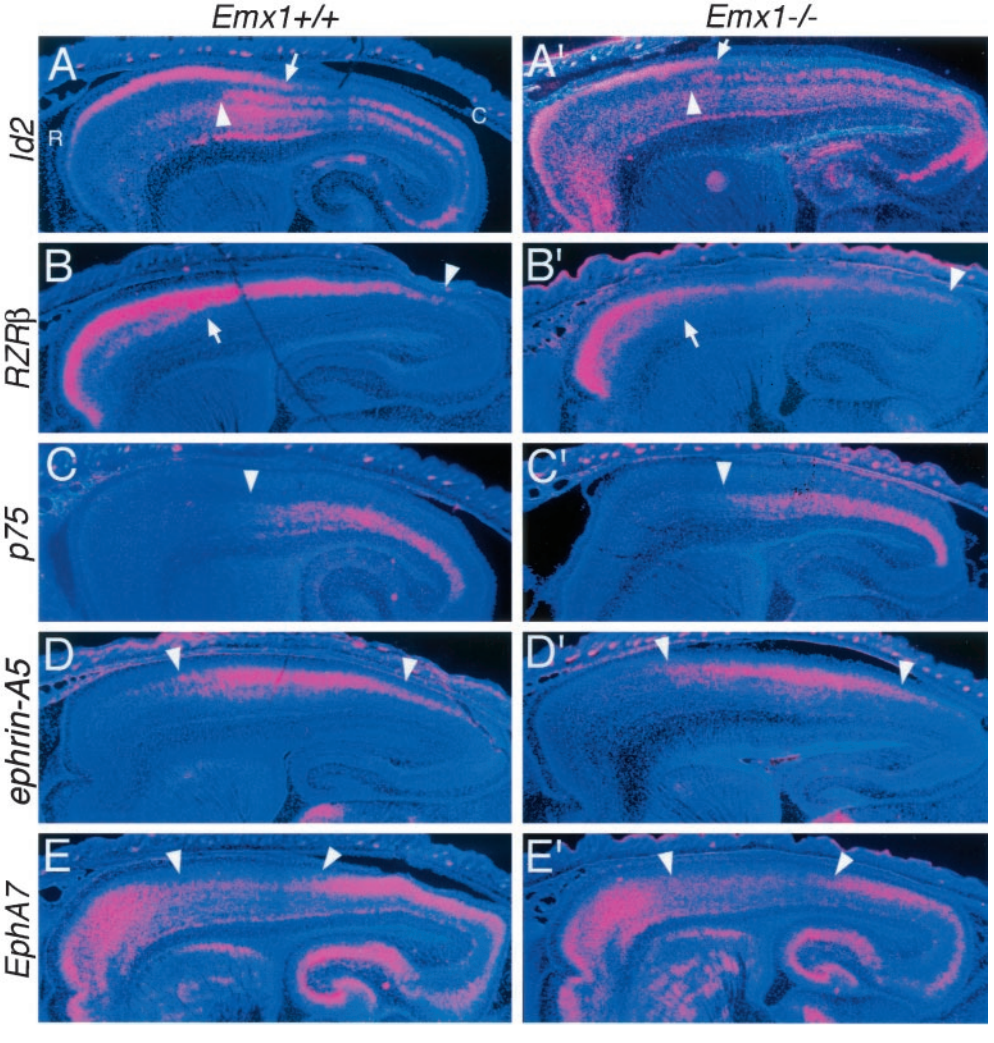

Figure 7. The expression domains of genes that mark rostral, caudal, or intermediate areas of the neocortex appear normal in *Emx1* mutants. *In situ* hybridizations on sagittal sections through the forebrain of E18.5 *Emx1* wild-type (+/+) and mutant (−/−) littermates using S^{35}-labeled riboprobes for *Id2*, *RZRβ*, *p75*, *ephrin-A5*, and *EphA7* and counterstained with bisbenzimide are shown. Sections are taken from similar medial–lateral positions. Each *panel* is a montage of single-exposure photos using dark-field illumination using a red filter to view the silver grains and UV fluorescence to view the counterstain. *Id2* exhibits a graded expression in superficial layers of rostral areas in wild-type mice; the *arrows* in *A* and *A′* mark the position where the expression declines to very low levels. The *arrowheads* in *A–B′* mark the transition from low to high expression reported in layer 5; this transition corresponds to the border between motor and somatosensory areas. *RZRβ* is expressed in two distinct high rostral to low caudal gradients across the neocortex (*B*, *B′*); a gradient in superficial layers that extends farther caudally (marked by *arrowheads*) than a gradient within the deeper layers (marked by *short arrows*). *p75* is expressed in the deep layers in roughly the caudal half of the neocortex (*C*, *C′*). The *arrowheads* mark the rostral limit of expression. *ephrin-A5* has high expression centered on the somatosensory area (*D*, *D′*), whereas *EphA7* has low expression centered on the somatosensory area (*E*, *E′*). *Arrowheads* mark the domains of high *ephrin-A5* or low *EphA7* expression. Overall, the areal expression patterns of this panel of marker genes are similar in wild-type and mutant neocortex. See Results for details. *C*, Caudal; *R*, rostral.

Area-specific thalamocortical projections appear normal in *Emx1* mutants

As an additional assessment of the potential role for EMX1 in regulating neocortical area identity, we used DiI and DiA as retrograde axon tracers to assess area-specific thalamocortical projections in newborn *Emx1* mutant mice and their wild-type littermates. In wild-type mice, placements of DiI crystals confined to the cortical plate of occipital cortex, the location of the primary

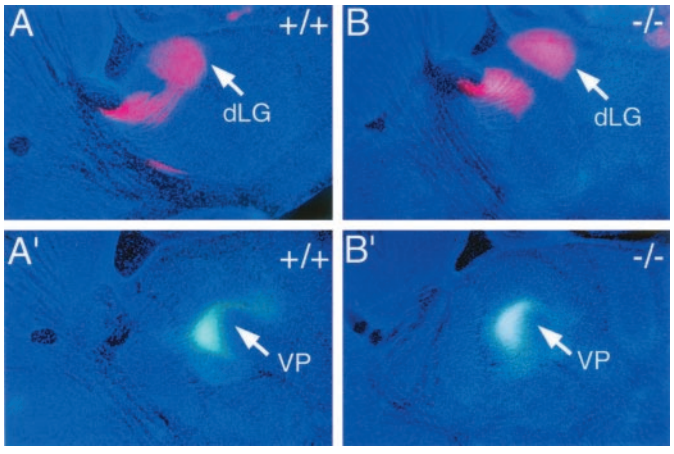

Figure 8. Area-specific thalamocortical projections appear normal in *Emx1* mutant mice. Sagittal sections through P0 *Emx1* wild type (+/+) and mutant (−/−) brains showing retrograde DiI (*red*) and DiA (*green-bluish*) labeling and bisbenzimide counterstain (*dark blue*). Rostral is to the *left* and dorsal is to the *top* in all *panels*. *A*, *B*, An injection of DiI into visual (occipital) cortex retrogradely labels neurons in the dorsal lateral geniculate nucleus (*dLG*) in both *Emx1*+/+ and *Emx1*−/− mice. *C*, *D*, An injection of DiA into somatosensory (parietal) cortex of the same set of brains retrogradely labels neurons in the ventroposterior thalamic nucleus (*VP*) in both *Emx1*+/+ and *Emx1*−/− mice. Each pair of sections is from the same brain; the dLG-labeled sections are lateral to those with the VP labeling. See Results for details.

visual area, retrogradely label neurons in the dLG (Fig. 8*A*). Placement of DiI crystals at the same site in *Emx1* mutants results in a distribution of retrogradely labeled neurons (Fig. 8*B*) similar to that observed in wild type (Fig. 8*A*). In wild-type mice, placements of DiA crystals confined to the cortical plate of parietal cortex, the location of the somatosensory area, retrogradely label neurons in the ventroposterior thalamic nucleus (Fig. 8*A*′, *VP*). Placement of DiA crystals at the same site in *Emx1* mutants results in a distribution of retrogradely labeled neurons (Fig. 8*B*′) similar to that observed in wild-type mice (Fig. 8*A*′). Thus, retrograde labeling from somatosensory and visual areas of the neocortex indicates that thalamocortical connections in *Emx1* mutants exhibit normal area-specific patterns of connections. Taken together, our analyses using molecular markers and axon tracing suggest that the *Emx1* null mutation does not affect arealization in embryonic or neonatal mice.

Areal patterns of gene expression in the neocortex of *Emx* double mutants resemble those in *Emx2* single mutants

The coincident graded expression of *Emx1* and *Emx2* in the cortical ventricular zone, and their high sequence homology, suggests the possibility that the lack of an arealization phenotype in the *Emx1* mutants may be caused by *Emx2* compensating for the loss of *Emx1*. To investigate this possibility, we examined the expression patterns of the panel of seven gene markers in sagittal sections through *Emx1*−/−; *Emx2*−/− double mutant and wild-type (i.e., *Emx1*+/−; *Emx2*+/+) littermates at E18.5. Because *Emx* double mutants lack a TCA projection (K. M. Bishop, S. Garel, J. L. R. Rubenstein, and D. D. M. O'Leary, unpublished observations), we were not able to examine the areal patterning of this projection.

In Figure 9, we illustrate the expression patterns for four of the seven genes analyzed (*Cad6*, *p75*, *ephrin-A5*, and *EphA7*), and for ease of comparison, we include panels showing the expression of these genes in *Emx2* single mutant neocortex. The findings from these four markers are representative of the findings from the full panel of seven markers. In the areal dimension, the expression domains of each of these markers are shifted caudally in *Emx1; Emx2* double mutants compared with wild type. Because the tangential extent of the neocortex is substantially reduced in the *Emx1; Emx2* double mutants compared with wild-type mice (by ~50% in its surface area), one needs to be somewhat cautious in interpreting patterns of gene expression. However, the relative shift and positioning of the expression domain of each of these marker genes in the *Emx1; Emx2* double mutants appears to be similar to that in the *Emx2* single mutants. Thus, the lack of a defective arealization phenotype in *Emx1* mutants is not caused by *Emx2* compensating for the loss of *Emx1*. Therefore, these findings support the conclusion from our analyses of the *Emx1* mutants that EMX1 has no apparent role in regulating arealization of the neocortex.

DISCUSSION

Defining roles for *Emx1*, *Emx2*, and *Pax6* in arealization

We have presented evidence that strengthens the claim that EMX2 and PAX6 regulate arealization of the developing neocortex and confer positional identities to cortical cells. In addition, we show that EMX1 has no apparent role in regulating arealization. Figure 1 summarizes our predictions and interpretations. Because *Emx2* and *Pax6* (*Sey*/*Sey*) mutant mice die on the day of birth before areas become anatomically and functionally distinct, we have used genetic markers to assess changes in positional, or area, identity of cortical cells and presumably associated changes in arealization of the neocortex. We are confident in basing our interpretations on these data because each of the seven marker genes analyzed exhibits opposing expansions or contractions, and border shifts, in its expression domain in *Emx2* and *Pax6* mutants, as predicted by the opposing gradients of *Emx2* and *Pax6* expression (Fig. 1). The changes in marker expression indicate that arealization of the neocortex is disproportionately altered in *Emx2* and *Pax6* mutant mice in opposing manners: in *Emx2* mutants, rostrolateral areas are expanded compared with wild type, whereas caudomedial areas are reduced, and in *Pax6* mutants the opposite changes occur. We conclude that EMX2 and PAX6 act in opposing manners to regulate arealization and to confer positional, or area, identity to neocortical cells. EMX2 appears to preferentially impart caudomedial area identities, and PAX6 preferentially imparts rostrolateral area identities.

We expected to observe similar changes in neocortical arealization in *Emx1* mutants as in *Emx2* mutants, because in the ventricular zone, *Emx1* has a graded expression similar to that of *Emx2* (Fig. 1). However, we find that each of the seven marker genes exhibits a normal expression pattern in the neocortex of *Emx1* mutants. In addition, the area-specific organization of TCA projections is normal in *Emx1* mutants. These findings suggest that unlike EMX2, EMX1 is not involved in arealization. An alternative is that Emx function is redundant in arealization and that EMX2 in particular can more fully compensate for the loss of EMX1 than vice versa. This is suggested by the coincident expression of the Emx genes in the cortical ventricular zone, their high sequence homology, and that in the dorsal telencephalon *Emx2* expression begins at E8.0–8.5, whereas *Emx1* expression begins at E9.5 (Simeone et al., 1992). However, our marker analysis of *Emx1; Emx2* double mutants reveals that the changes in patterns of gene expression are similar to those observed in

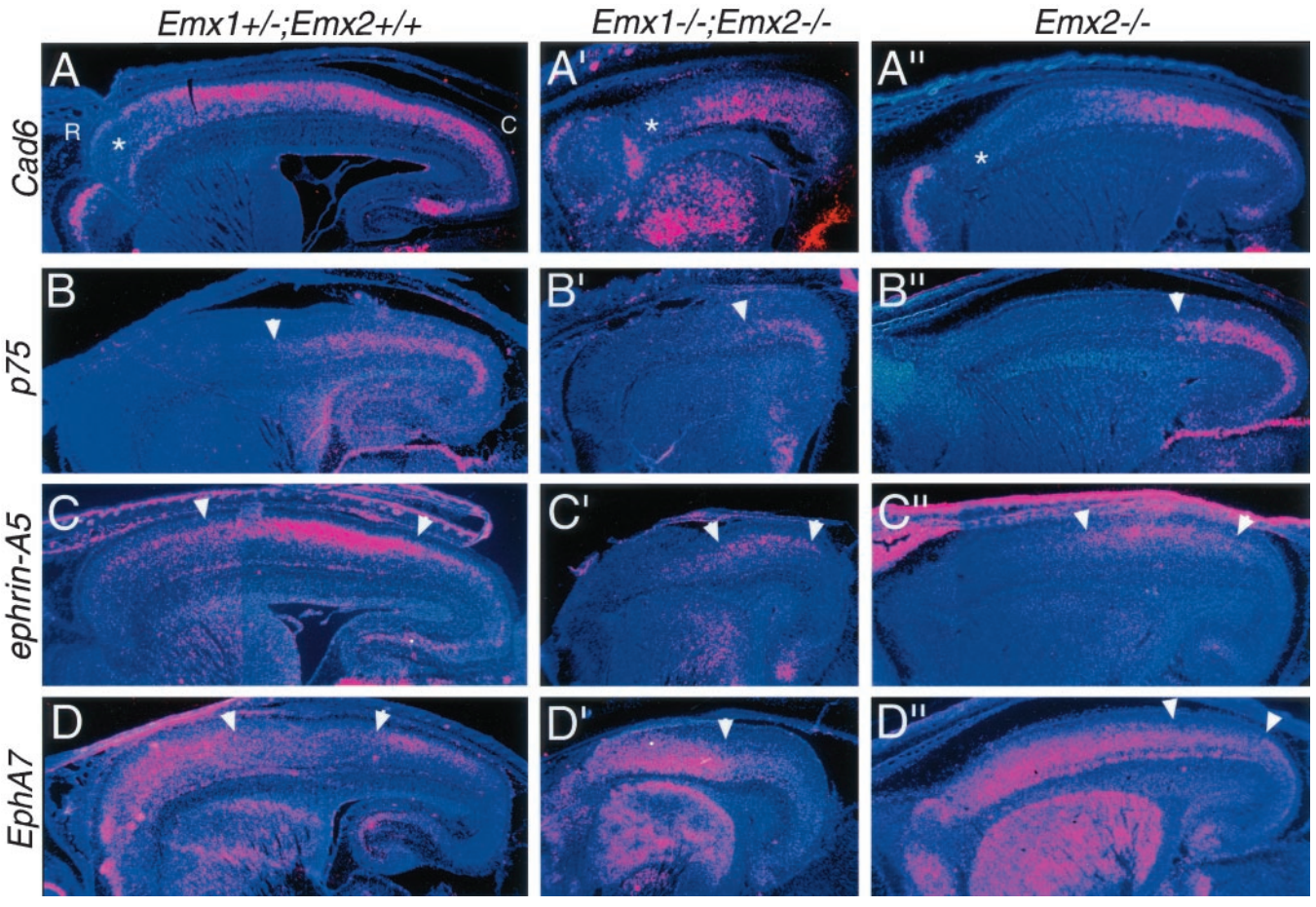

Figure 9. The relative positioning of expression domains of gene markers in *Emx1; Emx2* double mutant neocortex resembles that in *Emx2* single mutant neocortex. Shown are *in situ* hybridizations on sagittal sections through the forebrain of E18.5 (*A–D*) wild-type (i.e., *Emx1*+/−; *Emx2* +/+) (*A′–D′*) Emx double mutant (*Emx1*−/−; *Emx2*−/−), and (*A′′–D′′*) *Emx2* single mutant (*Emx2*−/−) mice using S^{35}-labeled riboprobes for *Cad6*, *p75*, *ephrin-A5*, or *EphA7* and later counterstained with bisbenzimide. Note that the neocortex of the Emx double mutant is reduced to approximately half of the wild-type area. Sections are taken from similar medial–lateral positions. Each *panel* is a montage of single-exposure photos using dark-field illumination and a red filter to view the silver grains and UV fluorescence to view the counterstain. The *asterisks* in *A–A′′* mark a domain of low *Cad6* expression normally characteristic of far rostral neocortex. This domain of low expression expands and shifts caudally in Emx double mutants and *Emx2* mutants. *p75* is expressed in the deep layers in roughly the caudal half of the wild-type neocortex (*B*); this expression domain constricts caudally in Emx double mutants and *Emx2* mutants (*B′*, *B′′*). The *arrowheads* mark the rostral limit of expression. In wild-type mice, *ephrin-A5* has high expression centered on the somatosensory area (*A*, *B*), whereas *EphA7* has low expression centered on the somatosensory area (*C*, *D*). These domains shift caudally in Emx double mutants and *Emx2* mutants (*C′*, *D′*, *C′′*, *D′′*). *Arrowheads* mark the domains of high *ephrin-A5* or low *EphA7* expression. See Results for details. *C*, Caudal; *R*, rostral.

Emx2 single mutants. Thus, unlike EMX2, EMX1 does not appear to play a role in regulating arealization of the neocortex.

Emx genes may function in the patterning of neocortical areas in the same way that their *Drosophila* ortholog, the *empty spiracle* (*ems*) gene, specifies in the developing head early populations of neuroblasts that at later stages form specific brain structures (Younossi-Hartenstein et al., 1997; Hartmann et al., 2000). If the Emx genes act in this manner, we would predict that a deletion of both would result in the complete loss of caudomedial neocortical tissue, along with areas that differentiate within it, such as visual areas. However, our findings are inconsistent with this scenario, because rather than a complete loss of tissue in which caudal areas would form, our marker analyses indicate that rostral neocortical areas expand and shift caudally into domains that would normally develop caudal area identities and that caudal areas are present but contracted. It seems more likely that EMX2 confers positional identity to cortical neurons around the time they are generated. Further support for this interpretation is our finding that the patterned expression of the marker genes and the positioning of their expression domains retain the same relative relationships with each other, and with the neocortex as a whole, between *Emx2* single mutants and *Emx1; Emx2* double mutants, although the double mutant cortex is approximately half the normal size.

Because surface area of the cortex is reduced by ~25% in *Emx2* mutants and *Pax6* mutants (Bishop et al., 2000), the disproportionate areal expansions and contractions in these mutants may be caused in part by a decreased growth of caudomedial cortex in *Emx2* mutants and rostrolateral cortex in *Pax6* mutants (Muzio et al., 2002). However, several aspects of our findings cannot be accounted for by a regional decrease in cortical growth and therefore strongly argue that positional identity and arealization of the neocortex are altered in *Emx2* and *Pax6* mutants (Bishop et al., 2000; present study). As discussed above, these include the similarities in changes in expression domains of areal markers between *Emx2* single and *Emx1; Emx2* double mutants,

although cortical size in the double mutant is substantially decreased compared with the single mutant. Other aspects of our findings supporting this argument include that the expression domains of areal markers exhibit the following: (1) increases or decreases in their proportional size relative to that of the neocortex which significantly exceed that predicted by a region-specific decrease in growth, and for some markers, even increases in absolute size of domains of positive or negative expression, and (2) changes in relative positioning and coverage of the rostrocaudal axis of the neocortex that cannot be accounted for by a region-specific decrease in growth.

Potential mechanisms for EMX2 and PAX6 regulation of arealization

The details of how PAX6 and EMX2 regulate arealization are unknown. The loss of one or the other may result in a complete change or switch in area identity. PAX6, for example, has been implicated more generally in regulating the identity of ventrolateral regions of the cerebral cortex. In *Pax6* mutants, progenitor cells in the ventrolateral regions lose expression of cortical regulatory genes and acquire expression of subcortical regulatory genes. As a result, ventral cortical regions (e.g., olfactory cortex) are hypoplastic, whereas adjacent subcortical areas expand (Stoykova et al., 2000; Toresson et al., 2000; Kim et al., 2001; Yun et al., 2001; Muzio et al., 2002). Evidence for cross-repression between EMX2 and PAX6 in the neocortex (Muzio et al., 2002) suggests that they may cooperate with each other, and possibly with other transcription factors (e.g., LHX2) (Nakagawa et al., 1999; Monuki et al., 2001), to confer positional or area identity to neocortical neurons. For example, EMX2 and PAX6 may participate in a combinatorial code of regulatory proteins that specifies the area identity of cortical neurons, as suggested for the specification of subtypes of motor neurons and interneurons in the spinal cord (Jessell, 2000). If so, the loss of either EMX2 or PAX6 may perturb the area identity of cortical neurons, resulting in an aberrant or chimera area identity, rather than a complete change from one area identity to another.

One protein that may collaborate with EMX2 and PAX6 in regulating arealization is COUP-TFI, an orphan nuclear receptor that has a high caudal to low rostral graded expression across the neocortex within the ventricular zone, subplate, and cortical plate (Liu et al., 2000). An analysis in *CoupTfI* mutants of the expression of the marker genes, *Cad8*, *Id2*, and *RORβ* (*RZRβ*), shows that they all lose their normally restricted, areal expression patterns and instead are broadly expressed across the neocortex (Zhou et al., 2001). This finding differs from that in *Emx2* and *Pax6* mutants, in which the marker genes retain specific patterns of expression, but the patterned expression is either expanded or contracted in a manner that opposes the graded expression of *Emx2* or *Pax6*. In contrast, in *CoupTfI* mutants, *Emx2* and *Pax6* show their normal graded patterns of expression. Thus, *CoupTfI* mutants have an apparent loss of areal specificity in patterns of marker expression, with the exception of *Emx2* and *Pax6,* suggesting that COUP-TFI does not directly regulate arealization but is required for the proper action of EMX2 and PAX6 in this process.

Establishment of graded expression of arealization genes across the neocortex

Evidence is emerging for a role for secreted signaling proteins with known patterning functions in establishing and maintaining the graded expression of regulatory genes, in particular *Emx2*, across the neocortical ventricular zone. One such signaling protein is FGF8, which is expressed in proximity to rostral parts of the cortical anlage at neural plate stages, in the anterior neural ridge, and later the rostrodorsal midline of the telencephalon (Crossley and Martin, 1995; Shimamura and Rubenstein, 1997; Crossley et al., 2001). Electroporation in E11.5 mouse cortex of vectors to overexpress FGF8 or a soluble FGF8 receptor body to diminish endogenous FGF8 results in shifts in areal markers (Fukuchi-Shimogori and Grove, 2001) similar to those expected if the graded expression of *Emx2* was decreased or increased. That these effects are likely caused by FGF8 regulation of *Emx2* is implied by the finding that FGF8-soaked beads implanted into dorsal telencephalon of embryonic chicks locally repress *Emx2* expression (Crossley et al., 2001).

Other candidate regulators of *Emx2* expression include members of the bone morphogenetic protein family, which along with Wnt family members are expressed in the cortical hem, a caudal midline structure adjacent to the hippocampal anlage (Furuta et al., 1997, Grove et al., 1998). Ectopic expression of *Bmp4* in the dorsal telencephalon of embryonic chicks appears to enhance *Emx2* expression, either directly or through the repression of *Fgf8* (Ohkubo et al., 2002). The zinc finger transcription factor *Gli3*, which is expressed broadly in the dorsal telencephalon, may be upstream to both Emx genes, because the expression of *Emx1* is lost (Thiel et al., 1999; Tole et al., 2000), and *Emx2* is lost (Thiel et al., 1999) or reduced (Tole et al., 2000) in the cortex of the *extra-toes^J* (*Xt^J*) mutant mouse, a naturally occurring *Gli3* mutant (Franz, 1994). In contrast, *Pax6* expression is maintained in the *Xt^J* mutant (Thiel et al., 1999; Tole et al., 2000). However, it is unclear whether *Emx2* is normally regulated directly by GLI3, by signaling molecules produced in the cortical hem that are lost in the *Xt* mutant, or by other molecular deficiencies in *Xt^J* mutants (Grove et al., 1998; Thiel et al., 1999; Tole et al., 2000).

Both PAX6 and EMX2 are implicated in regulating members of the Wnt family, which in turn may influence corticogenesis. In *Pax6* mutants, expression of *Wnt7b* and *SFRP2* in ventrolateral cortical progenitors is reduced (Kim et al., 2001), whereas *Wnt3a* and *Wnt8b* expression in the dorsomedial cortex is expanded (Muzio et al., 2002). On the other hand, *Wnt3a* expression is reduced in dorsomedial cortex in *Emx2* mutants (Muzio et al., 2002).

Defining area identity at the level of patterned gene expression

Many genes, including markers used in this study, exhibit abrupt transitions in their expression patterns within the cortical plate, and the areas themselves usually have abrupt borders. Therefore, the graded expressions of *Emx2* and *Pax6* are likely translated to generate downstream gene expression in restricted patterns with abrupt borders. Studies in *Drosophila* embryos have defined distinct mechanisms through which graded regulatory proteins can generate sharply bordered patterns of downstream gene expression, for example through concentration-dependent differences in binding efficacy to promoter and repressor elements (Rusch and Levine, 1996) or the combinatorial action of multiple activators and repressors of transcription (Stanojevic et al., 1991; Small et al., 1996). In the developing spinal cord, sonic hedgehog secreted by the notocord and floorplate represses or induces in the ventricular zone the expression of different classes of transcription factors in graded patterns, which are progressively converted into sharply bordered patterns through mutual repression (Jessell, 2000). This mechanism results in genetically distinct domains of progenitors, which generate different subtypes of spinal interneu-

rons and motor neurons definable by their expression of unique subsets of transcription factors.

Mechanisms similar to those used in spinal cord are likely coopted to generate neocortical areas and area-specific identities of neurons, but some differences appear to exist. For example, at no time during corticogenesis are sharply bordered patterns of regulatory genes observed in the ventricular zone; all retain graded expression patterns. Initially, even genes differentially expressed in the cortical plate have graded patterns, although at later stages of development many acquire an expression pattern with abrupt changes that correlate with borders between areas (Rubenstein et al., 1999; Sestan et al., 2001).

Area specificity may differ among layers

Genes with an expression pattern restricted to one area have not been identified (Liu et al., 2000). The only genetic marker with an expression pattern restricted to one area is the H-2Z1 transgene, which marks the granular parts of postnatal mouse S1 (Cohen-Tannoudji et al., 1994). It seems reasonable to conclude then that a neocortical area and the area identity of the neurons that comprise it are defined by the expression of a unique subset of genes, each of which is also expressed in other areas. However, the actual scenario is more complex because each layer has a unique profile of gene expression: most genes reported to be differentially expressed in the neocortex and expressed in more than one layer, including markers that we use here, have different expression patterns in each layer. The expression of *Id2* and *RZRβ* are clear examples described in this study (see Results). Thus, although neurons in different layers are generated by the same progenitors (Monuki and Walsh, 2001), they appear to have distinct positional or area identities, at least in terms of their expression of genes used as markers of these identities. This feature has significant implications for the genetic regulation of areas and how area identity is encoded in the ventricular zone and imparted by progenitors to their progeny. An understanding of these mechanisms will require the definition of areas at the level of gene expression, and defining the relationship between the specification of layer-specific and area-specific properties.

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