EMX2 Regulates Sizes and Positioning of the Primary Sensory and Motor Areas in Neocortex by Direct Specification of Cortical Progenitors

Tadashi Hamasaki,1 Axel Leingärtner,1 Thomas Ringstedt,2 and Dennis D.M. O’Leary*
Molecular Neurobiology Laboratory
The Salk Institute
10010 North Torrey Pines Road
La Jolla, California 92037

Summary

Genetic studies of neocortical area patterning are limited, because mice deficient for candidate regulatory genes die before areas emerge and have other complicating issues. To define roles for the homeodomain transcription factor EMX2, we engineered transgenic mice that overexpress EMX2 in cortical progenitors coincident with expression of endogenous Emx2 and survive postnatally. Cortical size, laminarization, thalamus, and thalamocortical pathfinding are normal in homozygous nestin-Emx2 mice. However, primary sensory and motor areas are disproportionately altered in size and shift rostrally. Heterozygous transgenics have similar but smaller changes. Opposite changes are found in homozygous Emx2 knockout mice. Fgf8 expression in the commissural plate of nestin-Emx2 mice is indistinguishable from wild-type, but Pax6 expression is downregulated in rostral cortical progenitors, suggesting that EMX2 repression of PAX6 specification of rostral identities contributes to reduced rostral areas. We conclude that EMX2 levels in cortical progenitors disproportionately specify sizes and positions of primary cortical areas.

Introduction

The mammalian neocortex, the largest region of the cerebral cortex, is tangentially organized into subdivisions, called areas, that serve unique functions such as sensory perception or motor control. Areas are also distinguished by distinct architecture, distributions of neuron types, and axon projections. The development of areas is proposed to be controlled by transcription factors (TFs) that specify positional or area identities of cortical neurons and thalamocortical axons (TCAs) that relay visual, auditory, and somatic input to the neocortex (Rakic, 1988; O’Leary, 1989; O’Leary and Nakagawa, 2002). TFs high in the genetic hierarchy controlling arealization should confer positional, or areal, identity to progenitors in the neocortical ventricular zone (VZ) that is imparted to their progeny and also specify the expression of guidance molecules that control area-specific targeting of TCAs. The related homeodomain TFs Emx1 and Emx2 are expressed by cortical progenitors in the VZ of dorsal telencephalon (dTel) in a low rostral-lateral to high caudal-medial gradient (Simeone et al., 1992a, 1992b) and are proposed to control arealization (O’Leary et al., 1994). Changes in patterns of gene markers and area-specific TCA projections in embryonic Emx2 mutant mice provide evidence consistent with a role for EMX2 in arealization (Bishop et al., 2000, 2002; Mallamaci et al., 2000a). In contrast, analyses of Emx1 mutants and Emx1/Emx2 double mutants suggest that EMX1 does not regulate arealization (Bishop et al., 2002).

Studies of genetic regulation of areal patterning have been indirect, because mice deficient for Emx2 or other TFs that are proposed to be primary regulators, for example, PAX6, die perinatally or earlier, before areas emerge. In addition, they have reduced cortical size, which is suggested to be due to region-specific loss of cortical tissue, introducing caveats into interpretations of marker shifts (Muzio et al., 2002b). Further, TCAs either do not reach cortex, as in Pax6 mutants (Jones et al., 2002), or only a proportion do, as in Emx2 null mice (Lopez-Bendito et al., 2002), which can alter areal distributions of TCAs (Garel et al., 2002). Thus, evidence for genetic regulation of arealization is controversial.

The goal of this study was to determine whether EMX2 has a primary role in patterning of the neocortex into defined areas. Therefore, we made mice with an Emx2 transgene driven by promoter elements of the nestin gene (ne), resulting in its expression being limited to the same progenitors and the same time frame as endogenous Emx2 in wild-type (wt) cortex. We complement these gain-of-function studies with analyses of heterozygous Emx2 knockout mice. Our analyses are focused on primary cortical areas because they can be clearly delineated, and markers for higher-order areas are lacking. We also determined the influence of the Emx2 transgene on expression of the morphogen Fgf8 in the commissural plate, a domain at the rostral midline of the nascent dTel, because a recent report concluded that EMX2 does not directly control arealization but instead acts solely by repressing Fgf8 (Fukuchi-Shimogori and Grove, 2003). Our findings lead us to conclude that EMX2 expressed in cortical progenitors disproportionately controls in a direct, concentration-dependent manner the sizes and positioning of primary cortical areas.

Results

ne-Emx2 Transgenic Mice and Emx2 Transgene Expression

Figure 1A schematizes Emx2 expression in wt and ne-Emx2 mice and our findings of overexpressing Emx2 on patterning of the neocortex into primary areas. ne-Emx2 transgenic mice were generated using regulatory sequences of the nestin gene (ne) to drive cDNA of the Emx2 coding region (Figure 1B) specifically in nestin-positive cells in the nervous system, which are predominantly progenitors in neuroepithelia (Zimmerman et al., 1994). ne-Emx2 mice are identified by PCR (Figure 1C); transgene expression is detected by qualitative RT-PCR in all brain regions analyzed (Figure 1D and data not shown). Northern blot analysis on forebrain at E11.5 and...
E13.5 and telencephalon at E15.5, ages that encompass most of cortical neurogenesis, reveals in wt and ne-Emx2 mice a 1.7 kb band corresponding to endogenous Emx2 mRNA; an additional 1 kb band corresponding to transgene Emx2 mRNA is observed only in ne-Emx2 mice (Figure 1E). Densitometry of Northern blots indicates that transgene expression in homozygous ne-Emx2 mice is about half that of endogenous Emx2, and in heterozygous ne-Emx2 mice the level is about a quarter.

The ne vector drives expression across the neocortical VZ in the same progenitors that normally express Emx2 over a similar period of development (Emx2, Simone et al., 1992a, 1992b; nestin, Dahlstrand et al., 1995; nestin transgene, Ringstedt et al., 1998; Magdaleno et al., 2002). To localize expression, in situ hybridization was performed on sections through forebrain of wt and ne-Emx2 mice using an Emx2 riboprobe at successive days from E10.5 to E15.5 (Figure 1F and data not shown; also Figures 7A and 7B). Figure 1F illustrates expression at E14.5, a peak of cortical neurogenesis, of endogenous Emx2 in wt and combined expression of endogenous and transgene Emx2 in ne-Emx2 mice. In wt, Emx2 transcripts are distributed in a high caudomedial to low rostral-lateral gradient in the cortical VZ, and a low expression is detected in the ganglionic eminence. In ne-Emx2 mice, a high caudomedial to low rostral-lateral gradient of Emx2 transcripts is evident in the cortical VZ, and expression is higher than that in wt (Figure 1F). Enhanced Emx2 expression is also evident in the
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ganglionic eminence of ne-Emx2 mice, and moderate expression is detected in other nestin-positive proliferative zones, such as in dTh, where expression is not detected in wt (Figure 1F).

Thalamic Patterning, TCA Pathfinding, Cortical Size, and Lamination Are Normal in ne-Emx2 Mice

To address whether potential changes in principal dTh nuclei and TCA input influence cortical patterning, we show using gene markers and histological stains that patterning and size of dTh in ne-Emx2 mice at E15.5 and P0 (Figures 2A–2C and data not shown) are indistinguishable from wt (see Nakagawa and O’Leary, 2001). Dii labeling from dTh confirms that TCA pathfinding is normal in ne-Emx2 mice (Figure 2D and data not shown; for wt see Braisted et al., 1999). Emx2 null mice have lamination defects (Mallamaci et al., 2000b), and cortical area is reduced by 30% at P0 (Bishop et al., 2003). However, cortical area, laminar thickness and differentiation, and TCA terminations are normal in ne-Emx2 mice, at early and late postnatal ages (see Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/43/3/359/DC1).

S1 Is Reduced in Size and Shifts Rostrally and Laterally in ne-Emx2 Cortex

We predicted that Emx2 overexpression would result in a reduced size of S1 and a rostral and lateral shift in its location (Figure 1A). To delineate S1, we performed cytochrome oxidase (CO) histochemistry on tangential sections through flattened cortices of P7 wt and ne-Emx2 mice to reveal the body representation in S1 that parallels its functional organization (Figure 3) (Wong-Riley and Welt, 1980). S1 is significantly reduced in size and shifted rostrally in ne-Emx2 mice compared to wt (Figure 3A; Table 1). To assess S1 size, we measured the area of the postero medial barrel subfield (PMBSF), the representation in S1 of large facial whiskers, and related it to the area of the entire neocortex. PMBSF is 25% smaller in ne-Emx2 mice compared to wt (Figure 3B; Table 1). The CO-negative cortical field rostral to S1, where motor areas are located, is significantly reduced in ne-Emx2 mice (Figure 3C; Table 1), whereas the cortical field caudal to S1, where visual areas are located, is significantly increased (Figure 3D; Table 1).

To quantify the rostral shift of S1 in ne-Emx2 mice, we plotted the position of the C3 barrel, located near the center of PMBSF, C3 is positioned more rostrally and laterally in ne-Emx2 mice than in wt (Figure 3E; Table 1). Distributions of C3 barrel positions in ne-Emx2 and wt mice do not overlap along the rostral-caudal cortical axis, indicating that the shifts in areal patterning are present in each ne-Emx2 mouse that was analyzed.

Primary Sensory Areas Show Disproportionate Changes in Sizes and Position Shifts in ne-Emx2 Cortex

To analyze the effect of Emx2 overexpression on each primary sensory area, we used serotonin immunostaining on tangential sections through flattened cortices of P7 wt and ne-Emx2 mice to mark TCA terminations of the ventroposterior nucleus (VP), dorsal lateral geniculate nucleus (dLG), and ventral division of the medial geniculate nucleus (MGv) in layer 4 (Fujimya et al., 1986), revealing the size and position of the primary sensory areas to which they project, the primary somatosensory area (S1), primary visual area (V1), and primary auditory area (A1), respectively (Figure 4A). V1 is significantly larger in homozygous ne-Emx2 mice compared to wt (Figures 4A–4C); V1 length is 38% greater than in wt (Figure 4D), and its area is increased 52% relative to overall cortical area (Figure 4E). The rostral border of V1 shifts rostrally, whereas its caudal border remains fixed near the caudal flexure of the hemisphere. A1 is also significantly shifted rostrally and laterally in homozygous ne-Emx2 mice compared to wt (Figure 4F). Positions of A1 in the two populations do not overlap along either cortical axis. These data show that the primary sensory areas exhibit disproportionate changes in their sizes in ne-Emx2 mice compared to wt, with S1 decreasing and V1 increasing, and the primary sensory areas, including A1, shift rostrally and laterally (Table 1).

Disproportionate Changes in Motor and Visual cad8 Domains in ne-Emx2 Cortex

To address whether cortical plate neurons exhibit changes in positional, or areal, identities, we analyzed the expression pattern of cadherin8 (cad8), a cell adhesion protein expressed by cortical plate neurons in patterns that mark V1 and the motor area in postnatal mice (Suzuki et al., 1997). Because the cad8 expression do-
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Figure 3. Reduced Size and Rostrolateral Shift of S1 in ne-Emx2 Mice

(A) CO histochemistry on tangential sections of flattened cortices of P7 wild-type (wt) and ne-Emx2 mice. CO is a mitochondrial enzyme enriched in TCA terminations and presynaptic dendrites in layer 4, and by P7, CO staining reveals S1 that includes the barrel pattern of facial whiskers in posteromedial barrel subfield (PMBSF). In ne-Emx2 mice, S1 is reduced in size and shifted rostrally compared to wt (dashed line is at the same rostral-caudal level in both). F(M), frontal cortex (motor areas); O(V), occipital (visual areas); P(S), parietal (somatosensory areas). Scale bar, 1 mm.

(B) PMBSF ratio, defined as the ratio of BA (the area of PMBSF) to TA (the area of the entire cortex).

(C) Frontal ratio, defined as the ratio of F (the length from the frontal pole to the rostral edge of PMBSF) to T (the length from the frontal pole to the occipital pole).

(D) Occipital ratio, defined as the ratio of O (the length from the occipital pole to the caudal edge of the PMBSF) to T.

(E) Scatter plot of C3 barrel position, located near the center of PMBSF. Coordinate axes were drawn on sections as shown: the caudal-rostral (C-R) axis extends from the occipital pole to the frontal pole, and the medial-lateral (M-L) axis is at the widest aspect of the section. In ne-Emx2 mice (black dots), C3 is rostral and lateral to C3 in wt (open circles). The difference between the C-R and M-L location of C3 in wt and ne-Emx2 is significant (C-R, p < 0.001; M-L, p < 0.05). In (B)–(D), ** indicates significance of p < 0.01 in unpaired Student’s t test. See Table 1 for summary.

mains develop independent of TCA input (Nakagawa et al., 1999) and expand or contract in Emx2 and Pax6 mutants, respectively, as predicted, cad8 is considered a bona fide positional marker in neocortex (Bishop et al., 2000, 2002).

We performed whole-mount in situ hybridizations for cad8 on P7 brains (Figure 5A) and measured the sizes of the motor and V1 cad8 domains (Figure 5B). The motor cad8 domain is 36% smaller in ne-Emx2 mice compared to wt (Figure 5B; Table 1), whereas the V1 cad8 domain is 24% larger (Figure 5C; Table 1). These findings show that Emx2 overexpression in cortical progenitors changes the positional identities of their progeny, including cortical plate neurons. These disproportionate changes in cad8 domains in ne-Emx2 mice are consistent with our CO (Figure 3) and serotonin (Figure 4) analyses that show that the frontal (motor) cortical field rostral to S1 is decreased in ne-Emx2 mice and that the occipital (visual) cortical field caudal to S1, as well as V1 itself, are increased (Table 1).

Caudal Areas Exhibit Enhanced Sensitivity to Emx2 Overexpression

P7 heterozygous ne-Emx2 mice have a subset of phenotypes described above for homozygous ne-Emx2 mice. Analyses of CO- and serotonin-stained tangential sections from P7 flattened cortices do not reveal significant differences in S1 size or shifts in its location in heterozygous ne-Emx2 mice (data not shown). However, analyses using serotonin immunostaining show that V1 in P7 heterozygous ne-Emx2 mice (Figure 4B) is intermediate in size to V1 in wt (Figure 4A) and homozygous ne-Emx2 mice (Figure 4C) and significantly different from either (Figures 4D and 4E; Table 1). In heterozygous ne-Emx2 mice, V1 length is 16% greater than in wt (Figure 4D), and V1 area is increased 25% (Figure 4E). In addition, A1 is significantly shifted rostrally and laterally in heterozygous ne-Emx2 mice (Figures 4B and 4F) compared to wt (Figures 4A and 4F) (Table 1). These findings indicate that caudal neocortex is particularly sensitive to Emx2 overexpression and support the suggestion that EMX2 preferentially imparts caudal area identities.

Fgf8 Expression in the Commissural Plate Is Normal in ne-Emx2 Mice

Emx2 expression in dTel begins at E8.5 (Simeone et al., 1992a, 1992b), similar to Fgf8 expression in the commissural plate (Shimamura and Rubenstein, 1997). Fukuchi-Shimogori and Grove (2003) report that ectopic Emx2 expression in the Fgf8 domain in the commissural plate that is achieved by electroporation results in a dramatic downregulation of Fgf8 and a substantial reduction in

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Table 1. Summary of Statistical Analyses of Neocortical Areas in ne-Emx2 Transgenic Mice

<table>
<thead>
<tr>
<th>Neocortical Area (frontal cortex)</th>
<th>Parameter</th>
<th>Marker/Experiment</th>
<th>wt ± SEM</th>
<th>n</th>
<th>ne-Emx2 Mice</th>
<th>hetero ± SEM</th>
<th>inc/dec/sh</th>
<th>n</th>
<th>homo ± SEM</th>
<th>inc/dec/sh</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motor area</td>
<td>Motor area frontal length (mm)</td>
<td>CO, flat cortex</td>
<td>3.94 ± 0.10</td>
<td>14</td>
<td>3.38 ± 0.14**</td>
<td>14% dec</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Motor area frontal area (mm²)</td>
<td>cad8, WMISH</td>
<td>3.34 ± 0.11</td>
<td>16</td>
<td>1.98 ± 0.13***</td>
<td>41% dec</td>
<td>8</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Motor area frontal area ratio</td>
<td>cad8, WMISH</td>
<td>0.501 ± 0.009</td>
<td>14</td>
<td>0.433 ± 0.006***</td>
<td>14% dec</td>
<td>6</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Motor area occipital area ratio</td>
<td>CO, flat cortex</td>
<td>1.58 ± 0.03</td>
<td>14</td>
<td>1.17 ± 0.09**</td>
<td>26% dec</td>
<td>6</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Somatosensory area (parietal cortex)</td>
<td>PMBSF area (mm²)</td>
<td>CO, flat cortex</td>
<td>0.044 ± 0.001</td>
<td>14</td>
<td>0.034 ± 0.002**</td>
<td>25% dec</td>
<td>6</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>PMBSF area ratio</td>
<td>CO, flat cortex</td>
<td>0.044 ± 0.001</td>
<td>14</td>
<td>0.034 ± 0.002**</td>
<td>25% dec</td>
<td>6</td>
<td></td>
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<tr>
<td></td>
<td>C3 C-R location</td>
<td>CO, flat cortex</td>
<td>−2.05 ± 0.12</td>
<td>12</td>
<td>−0.77 ± 0.09***</td>
<td>6% R/sh</td>
<td>6</td>
<td></td>
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<tr>
<td></td>
<td>C3 M-L location</td>
<td>CO, flat cortex</td>
<td>−3.30 ± 0.15</td>
<td>12</td>
<td>−0.68 ± 0.21*</td>
<td>3% L/sh</td>
<td>6</td>
<td></td>
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<tr>
<td>Auditory area (temporal cortex)</td>
<td>A1 C-R location</td>
<td>SHT, flat cortex</td>
<td>−5.27 ± 0.19</td>
<td>10</td>
<td>−4.31 ± 0.12**</td>
<td>5% R/sh</td>
<td>7</td>
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<tr>
<td></td>
<td>A1 M-L location</td>
<td>SHT, flat cortex</td>
<td>1.83 ± 0.19</td>
<td>10</td>
<td>3.12 ± 0.19**</td>
<td>6% L/sh</td>
<td>7</td>
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<tr>
<td>Visual area (occipital cortex)</td>
<td>occipital length (mm)</td>
<td>CO, flat cortex</td>
<td>2.49 ± 0.07</td>
<td>14</td>
<td>2.99 ± 0.12**</td>
<td>20% inc</td>
<td>6</td>
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<tr>
<td></td>
<td>occipital area (mm²)</td>
<td>cad8, WMISH</td>
<td>3.21 ± 0.06</td>
<td>16</td>
<td>3.75 ± 0.14**</td>
<td>17% inc</td>
<td>7</td>
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<tr>
<td></td>
<td>V1 length (mm)</td>
<td>SHT, flat cortex</td>
<td>1.92 ± 0.05</td>
<td>15</td>
<td>2.65 ± 0.16***</td>
<td>38% inc</td>
<td>4</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>V1 area (mm²)</td>
<td>SHT, flat cortex</td>
<td>2.19 ± 0.07</td>
<td>15</td>
<td>3.00 ± 0.23***</td>
<td>37% inc</td>
<td>4</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>V1 occipital length ratio</td>
<td>CO, flat cortex</td>
<td>0.317 ± 0.007</td>
<td>14</td>
<td>0.384 ± 0.010***</td>
<td>21% inc</td>
<td>6</td>
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<tr>
<td></td>
<td>V1 occipital area ratio</td>
<td>cad8, WMISH</td>
<td>0.165 ± 0.004</td>
<td>16</td>
<td>0.205 ± 0.005**</td>
<td>24% inc</td>
<td>7</td>
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<tr>
<td></td>
<td>V1 occipital length ratio</td>
<td>SHT, flat cortex</td>
<td>0.235 ± 0.008</td>
<td>15</td>
<td>0.272 ± 0.005**</td>
<td>16% inc</td>
<td>7</td>
<td></td>
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<tr>
<td></td>
<td>V1 occipital area ratio</td>
<td>SHT, flat cortex</td>
<td>0.059 ± 0.002</td>
<td>15</td>
<td>0.073 ± 0.001**</td>
<td>25% inc</td>
<td>7</td>
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</table>

Table presents mean data and standard error of the mean (SEM) for the number of cases (n) indicated. For each neocortical area data set, the first set of data are absolute measurements, whereas the second set of data (indicated as "ratio") are proportional relative to overall area or length. Proportional data are presented in the text. Some cases were only suitable for one or the other type of measurements. wt, wild type; inc/dec, increase or decrease rate compared to wild type; CO, cytochrome oxydase; cad8, cadherin8; WMISH, whole-mount in situ hybridization; PMBSF, posteromedial barrel subfield; C3, barrel C3; C-R, caudal to rostral; sh, shift toward rostral pole in percent of caudal to rostral cortical axis; M-L, medial to lateral; R/sh, shift toward lateral margin in percent of medial to lateral cortical axis; A1, primary auditory area; 5HT, 5-hydroxytryptamine (serotonin); V1, primary visual area. *p < 0.05; **p < 0.01; ***p < 0.001, compared to wt by unpaired Student's t test. †p < 0.05; ††p < 0.01; †††p < 0.001, compared to wt by ANOVA/Bonferroni's multiple comparison.
its expression domain and at later ages in a rostral shift of S1. They conclude that EMX2 does not directly control arealization but acts solely by repressing Fgf8 expression in the commissural plate. Therefore, we examined in wt and ne-Emx2 mice the relationships between expression of Fgf8, nestin, and Emx2 in transverse forebrain sections on consecutive days from E9.5 to E12.5 and Fgf8 expression in the commissural plate in whole mounts at E9.5 and E10.5 (Figure 6).

At E9.5, a low to moderate level of nestin immunostaining is observed throughout most of the telencephalic wall, but a much lower level of nestin staining is observed in the Fgf8 domain in the commissural plate (Figures 6A–6C). By E10.5, nestin staining is stronger in the telencephalon and includes the Fgf8 domain (Figures 6D–6F). This overlap in nestin immunostaining and the Fgf8 domain persists at E11.5 (data not shown) and E12.5 (Figures 6G–6I). In wt mice of these ages, Emx2 expression is not detected in the Fgf8 domain, although we find strong Emx2 expression dorsal to it (data not shown).

In contrast, in ne-Emx2 mice, the relationship of Emx2 expression to the Fgf8 domain in the commissural plate parallels that of nestin immunostaining, since nestin promoter elements drive expression of the Emx2 transgene. As a result, in ne-Emx2 mice at E9.5, Emx2 expression is very low or nondetectable in the midline Fgf8 domain, but moderate levels of Emx2 expression are observed in it at E10.5 (data not shown) and E12.5 in ne-Emx2 mice (Figures 6J–6L). In ne-Emx2 mice at E9.5 through E12.5, much stronger Emx2 expression is seen in the cortical VZ dorsal to the Fgf8 domain (Figures 6J–6L and data not shown), due to the combined expression of endogenous and transgene Emx2.

The Fgf8 expression domain in the commissural plate has the same appearance, both in size and level of expression, in transverse sections through the telencephalon in wt and ne-Emx2 mice at E10.5 (data not shown) and E12.5 (Figures 6H, 6I, 6K, and 6L) and in

Figure 4. Disproportionate Changes in Sizes of Primary Sensory Areas and Shifts in Their Locations in an Emx2 Concentration-Dependent Manner in ne-Emx2 Mice
(A–C) Serotonin immunostaining on tangential sections of flattened cortex of P7 wild-type (wt; [A]), heterozygous ne-Emx2 (het; [B]), and homozygous ne-Emx2 (homo; [C]) mice. In ne-Emx2 homo (C), V1 expands compared to wt, S1 is reduced in size and shifts rostrally, A1 shifts rostrolaterally, and the domain remaining for motor areas (M) is reduced compared to wt. In ne-Emx2 het (B), these changes are intermediate. Scale bar in (C) is 1 mm for (A–C).
(D) Histogram for V1 length ratio, defined as ratio of V1 length (Vl) relative to the overall cortical length (Tl).
(E) Histogram for V1 area ratio, defined as ratio of V1 area (VA) relative to the overall cortical area (TA).
(F) Scatter plot for position of A1. The center of A1 was plotted in coordinate axes similar to the C3 barrel in Figure 3E. The difference of the C-R and M-L locations of A1 between wt, ne-Emx2 het, and ne-Emx2 homo is statistically significant. In (D) and (E), * indicates a significance of p < 0.05, and ** indicates a significance of p < 0.01. See Table 1 for summary.

Figure 5. Disproportionate Changes in Size of Motor Area and V1 Domains of cad8 Expression in ne-Emx2 Mice
(A) In situ hybridization for cad8 using digoxigenin-labeled riboprobes on whole mounts of P7 wild-type (wt) and ne-Emx2 brains to mark the motor area (M) and V1. ne-Emx2 brain has smaller M and larger V1 cad8 expression domains. Scale bar, 1 mm. (B and C) Histograms for ratio of the area of M cad8 domain (B) and V1 cad8 domain (C) relative to the dorsal neocortical surface area. ** indicates significance of p < 0.01. See Table 1 for summary.
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Figure 6. Fgf8 Expression in the Commissural Plate of ne-Emx2 Mice Is Indistinguishable from Wild-Type

(A–C) Coronal sections through the midline Fgf8 expression domain in commissural plate (cp) of dTel in E9.5 wt. (A and C) Nestin immunostaining. (B and C) Nestin immunostaining (brown) and Fgf8 in situ hybridization (blue). High-power views of Fgf8 domain (between arrowheads in [A] and [B]) are shown in (C) and (C'). Scale bar in (B) is 200 µm for (A) and (B). Scale bar in (C) is 100 µm for (C) and (C'). E9.5 analyzed, n = 7.

(D–F) Coronal sections through Fgf8 domain in the cp of E10.5 wt. (D) Nestin immunostaining. (E and F) Nestin immunostaining (brown) with Fgf8 in situ hybridization (blue). High-power view of Fgf8 domain (between arrowheads in [E]) is shown in (F). Scale bar in (E) is 200 µm for (D) and (E). Scale bar in (F) is 200 µm. E10.5 analyzed, n = 5; E11.5 analyzed, n = 2 (data not shown).

(G–I) Adjacent coronal sections through Fgf8 domain in the cp of E12.5 wt. Nestin immunostaining (G), Fgf8 in situ hybridization (H), and nestin immunostaining (brown) with Fgf8 in situ hybridization (blue) (arrowheads mark same points in [G]). E12.5 analyzed, n = 7.

(J–L) Adjacent coronal sections through Fgf8 domain in the cp of E12.5 ne-Emx2 mice. Emx2 in situ (J), Fgf8 in situ (K), and nestin immunostaining (brown) with Fgf8 in situ (blue) (L) (arrowheads mark same points in [J], [K], and [L]). Scale bar in (L) is 100 µm for (G), (J), and (L). ne-Emx2 mice analyzed: E9.5, n = 2; E10.5, n = 7; E12.5, n = 2.

(M and N) Dorsal view of embryo heads of E9.5 wt (M) and ne-Emx2 homozygous (N) mice. Arrows indicate the Fgf8 domain in the cp at the anterior dTel midline. Anterior is to the top. Scale bar in (N) is 250 µm for (M) and (N). (M' and N') Midline views of E9.5 wt (M') and ne-Emx2 homozygous (N') brains bisected at the sagittal midline. Arrowheads mark the Fgf8 expression domain in the cp. Anterior is to the left; dorsal is to the top. The lengths of the Fgf8 domain in the cp in wt (n = 5 hemispheres, 3 brains) and ne-Emx2 homozygous (n = 6 hemispheres, 3 brains) are 279.2 ± 4.6 SEM and 274.6 ± 6.3 µm, respectively; the difference is not significant (p = 0.286; unpaired Student’s t test). is, isthmus. Scale bar in (N') is 500 µm for (M') and (N')..

(O and P) Dorsal views of embryo heads of E10.5 wt (O) and ne-Emx2 homozygous (P). np, nasal pit; mx, maxillary component of the first pharyngeal arch. Scale bar in (P) is 500 µm for (O) and (P). (O' and P') Midline views of E10.5 wt (O') and ne-Emx2 homozygous (P') brains. The lengths of the Fgf8 domain in the cp in wt (n = 4 hemispheres, 2 brains) and ne-Emx2 homo (n = 6 hemispheres, 3 brains) are 541.0 ± 4.6 and 529.9 ± 9.0 µm, respectively; the difference is not significant (p = 0.155; unpaired Student’s t test). rp, roof plate. Scale bar in (P') is 500 µm for (O') and (P').

Expression of Candidate Arealization Genes in ne-Emx2 Cortex

PAX6 (Bishop et al., 2000, 2002) and COUP-TF1 (Zhou et al., 2001) are implicated in cortical patterning, and...
LHX2 is hypothesized to influence it (Nakagawa et al., 1999; Monuki et al., 2001). Therefore, we examined their expression using in situ hybridization with 35S-labeled riboprobes on sagittal sections of wt and ne-Emx2 brains at E10.5 (Figure 7), when the first neocortical neurons are generated, and at E12.5 and E15.5 (data not shown). In wt and ne-Emx2 mice, Emx2 has a high caudal to low rostral graded expression in the cortical VZ (Figures 7A and 7B). In contrast, the low caudal to high rostral graded expression of Pax6 in the cortical VZ of wt (Figure 7C) is flattened in ne-Emx2 mice, due to diminished expression rostrally, where Pax6 expression is normally strongest (Figure 7D; data not shown). We detect no difference in expression of Lhx2 (Figures 7E and 7F) or COUP-TF1 (data not shown) in ne-Emx2 cortex.

To quantify relative gradients of Pax6 and Emx2 expression in the VZ of wt and ne-Emx2 neocortex, we counted silver grains at equivalent positions along the rostral-caudal axis of sagittal sections through E10.5 neocortex. A robust high rostral to low caudal gradient of Pax6 and an opposing high caudal to low rostral gradient of Emx2 are evident in wt (Figure 7G). In ne-Emx2 mice, Emx2 retains a robust high caudal to low rostral graded expression, whereas the slope of Pax6 expression is flattened (Figure 7H). This apparent repression of Pax6 by Emx2 overexpression in ne-Emx2 mice could contribute to the reduced sizes and rostral shifts of rostral areas, such as motor and S1.

Heterozygous Emx2 Knockout Mice Have Opposite Changes in Primary Areas to Those in ne-Emx2 Mice

To test whether a decrease in Emx2 expression influences primary cortical areas in an opposing manner to increasing Emx2 levels (Figure 8A), we performed on heterozygous Emx2+/− knockout mice and wt littermates analyses similar to those that were performed on ne-Emx2 mice. Northern blot analysis of telencephalon at E12.5, E13.5, and E15.5 reveals in wt and Emx2+/− mice a 1.7 kb band that corresponds to endogenous Emx2 mRNA; densitometry indicates that Emx2 expression is diminished by a third in Emx2+/− mice compared to wt (Figure 8B).

Analyses of CO- and serotonin-stained tangential sections through P7 flattened cortices of wt (Emx2+/+) (Figure 8C) and Emx2+/− littermates (Figure 8D) revealed opposite changes in arealization in Emx2+/− than those observed in ne-Emx2 mice. Overall cortical area in wt and Emx2+/− mice is not significantly different (Figure 8E). However, V1 is significantly reduced in size in Emx2+/− mice compared to wt: V1 length is 25% less than in wt (Figure 8F; Table 2), and V1 area is reduced by 23% (Figure 8G; Table 2). Consistent with these changes, the rostral-caudal length of occipital cortex compared to overall cortical length is significantly decreased in Emx2+/− mice by 13% (Figure 8H; Table 2). In contrast, frontal cortex rostral to S1, where motor areas are located, is significantly increased by 8% in length in Emx2+/− mice (Figure 8I; Table 2). PMBSF is 12% larger, and the C3 barrel is positioned more caudally and medially in Emx2+/− mice than in wt, but these changes do not achieve statistical significance (Figure 8J; Table 2).
These findings show that V1 is reduced in size in Emx2+/− mice compared to wt and that S1 and motor areas are increased in size and shifted caudally. Thus, decreasing Emx2 expression in cortical progenitors has the opposite effect on areal patterning of the neocortex as increasing Emx2 expression.

Discussion

EMX2 Levels in Cortical Progenitors Disproportionately Control Sizes and Positioning of Primary Cortical Areas

Emx2 is expressed highest in progenitors that generate caudal-medial areas of neocortex, such as V1, and lowest in progenitors that generate rostral and lateral areas, such as S1 and motor (Simeone et al., 1992a, 1992b; Leingartner et al., 2003). If EMX2 controls arealization, it should preferentially impart caudal-medial area identities. Recent studies present evidence consistent with this hypothesis (Bishop et al., 2000, 2002; Mallamaci et al., 2000a; Leingartner et al., 2003; Muzio and Mallamaci, 2003). However, the findings are controversial, and reinterpretation has been suggested because of defects in TCA pathfinding (Lopez-Bendito et al., 2002) and a potential region-specific loss of cortical tissue (Muzio et al., 2002b) in Emx2 null mice, and a report concluding that EMX2 acts indirectly in arealization by repressing Fgf8 expression in the commissural plate (Fukuchi-Shiromori and Grove, 2003).

The present study does not suffer from these caveats, because cortical size and TCA pathfinding are normal in ne-Emx2 mice and Emx2+/− mice, and Fgf8 expression in ne-Emx2 mice is indistinguishable from wt. We show that in ne-Emx2 mice the primary sensory and motor cortical areas have disproportionate changes in their sizes and shifts in position compared to wt (Figure 1A). V1, a caudomedial area, is significantly increased in size, whereas rostral areas S1 and motor are significantly reduced in size, and all three areas shift rostrally; S1 and A1 shift laterally as well as rostrally. We also find significant changes in the size and positioning of V1 and A1 in heterozygous ne-Emx2 mice intermediate to wt and homozygous ne-Emx2 mice, whereas rostral areas S1 and motor do not exhibit significant changes in heterozygous ne-Emx2 mice.

Complementing the gain-of-function studies in ne-Emx2 mice, we show that in Emx2+/− mice, which have reduced Emx2 expression, V1 is significantly reduced in size, and its rostral border is shifted caudally. S1 and the rostral cortical field that contains motor areas are
modestly increased in size and shifted caudally, and S1 is shifted medially, but only the effects on motor area size and S1 position are significant. We conclude that EMX2 operates by a concentration-dependent mechanism in cortical progenitors to specify disproportionately the sizes and positioning of the primary cortical areas and that higher levels of EMX2 preferentially impart caudal-medial area identities, such as those associated with V1.

**EMX2 and Cortical Size**

*Emx2* null mice have a cortical hemisphere that is 70% of wt size at P0, a reduction that is evident as early as E12.5 (Bishop et al., 2003). However, *Emx2* mutants do not have decreased proliferation rates or increased progenitor cell death during cortical neurogenesis (Shinohara et al., 2002; Bishop et al., 2003), suggesting that the reduced size is due at least in part to a defect in an earlier patterning event that allocates early telencephalic progenitors to specific fates (Muzio et al., 2002a). *Emx2* overexpression in vitro increases cortical clone size (Heins et al., 2001). Although these findings suggest a relationship between EMX2 and cortical size and cell number, we find no difference in cortical surface area or laminar appearance and thickness between postnatal wt, *ne-Emx2* mice, and *Emx2*−/− mice.

**EMX2 Influences on TCA Projections Are Limited to Their Area-Specific Patterning**

The *Emx2* transgene is expressed in progenitors throughout the CNS, but its effect is limited. For example, dTh patterning is normal in *ne-Emx2* transgenics, even though dTh progenitors ectopically express *Emx2*. In *Emx2* nulls, TCAs exhibit subcortical pathfinding errors (Lopez-Bendito et al., 2002; Bishop et al., 2003), but TCA pathfinding is normal in *ne-Emx2* mice, further suggesting that dTh and subcortical structures that are involved in TCA guidance develop normally in *ne-Emx2* mice.

Our findings indicate that EMX2 not only confers area identity to cells that form the cortical plate but also specifies the expression of guidance molecules that control area-specific targeting of TCAs that are presumed to act in the subplate (Ghosh et al., 1990). Leingartner et al. (2003) have also shown that TCAs from dLG, which normally target V1, aberrantly invade S1 coincident with high levels of deep layer Emx2-Adv infection, indicating that higher levels of EMX2 specify cortical cells to have TCA guidance properties normally associated with V1. Shifts in area-specific TCA projections in *Emx2* mutants are consistent with this interpretation (Bishop et al., 2000; Mallamaci et al., 2000a) but are subject to the caveat that TCAs from dLG have aberrant subcortical pathfinding in *Emx2* nulls (but not *Emx2*−/− mice) (Lopez-Bendito et al., 2002). Inconsistent with our findings is the report that TCAs have a normal area projection to cortex of neonatal Fgf8 hypomorphic mice that have higher levels of graded *Emx2* (Garell et al., 2003). Large ablations of neocortex in the marsupial Monodelphis domestica, before TCAs arrive, result in a compression of area-specific TCA projections (Huffman et al., 1999), but whether genetic respecification is involved is not known.

**Roles for EMX2 and FGF8 in Cortical Patterning**

The graded expression of *Emx2* and likely other TFs in the cortical VZ is established by patterning centers that secrete signaling molecules, including FGFs expressed in the commissural plate at the rostral midline of the nascent neocortex (Shimamura and Rubenstein, 1997) (Figure 9A) that repress *Emx2* (Crossley et al., 2001) (Figure 9B) and Bmps and WNTs expressed in the cortical hem along the caudodorsal midline (Furuta et al., 1997; Grove et al., 1998) that positively regulate *Emx2* expression (Ohkubo et al., 2002; Theil et al., 2002). Electroporation in mice to overexpress Fgf8 or diminish endogenous Fgf8 results in repressed or enhanced expression of *Emx2* in the rostral VZ and S1 shifting caudally or rostrally, respectively (Fukuchi-Shimogori and Grove, 2001, 2003). Similarly, Fgf8 hypomorphic mice have a higher level of graded *Emx2* expression in the cortical VZ (Garell et al., 2003). Thus, the S1 shifts...
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Figure 9. EMX2 Regulation of Patterning of Neocortex into Primary Areas

(A) The low rostral-lateral (R-L) to high caudal-medial (C-M) graded expression of Emx2 in progenitors across the cortical ventricular zone (VZ) of dTel is established by signaling molecules, such as FGF8 secreted from the commissural plate, a midline domain positioned at the rostral margin of dTel. (B) FGF8 helps establish the graded expression of Emx2 through repression before cortical neurogenesis begins. EMX2 appears to repress Fgf8 expression and restrict its expression domain. Our findings show that the graded distribution of EMX2 in progenitors in the VZ directly participates in the specification of their positional, or area, identity, inherited by their neuronal progeny, which form the subplate and cortical plate. This EMX2 specification regulates axon guidance information in the subplate to establish area-specific TCA projections and determines the sizes and positioning of the primary cortical areas (also see Figures 1A and 5A). (C) EMX2 likely cooperates with other TFs, such as PAX6, to specify area identity. EMX2 represses Pax6 expression, diminishing the influence of Pax6 in imparting rostral area identities and thereby contributing to the reduced sizes and rostral shifts of the rostrally located areas motor and S1 that are observed in ne-Emx2 mice. See the Discussion for details. L, lateral; M, motor areas; R, rostral; S1, primary somatosensory area; V1, primary visual area.

are consistent with a model in which FGF8 regulates the level of graded Emx2 expression in cortical progenitors, and EMX2 specifies the shift of S1 directly and/or by repressing Pax6.

However, Fukuchi-Shimogori and Grove (2003) conclude that EMX2 does not directly control arealization but solely acts indirectly by repressing Fgf8 expression in the commissural plate. They report that ectopic expression of Emx2 in the Fgf8 domain in the commissural plate by electroporation in E10.5 cortical explants results in a dramatic downregulation of Fgf8 and a later rostral shift in S1 in mice similarly electroporated in utero at E10.5. The shifts that we observe in S1 in homozygous ne-Emx2 mice match the description of cases with rostral electroporation of Emx2 in wt mice that result in a virtually complete loss of Fgf8 expression in the commissural plate. Therefore, for the arealization changes in ne-Emx2 mice to be solely mediated by the Emx2 transgene repressing Fgf8 expression would require that the Fgf8 expression domain in the commissural plate be substantially reduced. However, this Fgf8 domain in ne-Emx2 mice is indistinguishable from wt at embryonic ages during and after FGF8 exerts its patterning influence.

Therefore, our data demonstrate that changes in arealization in ne-Emx2 mice are not due to repression of Fgf8 expression. Instead, the disproportionate changes in sizes of primary cortical areas and shifts in their positions in ne-Emx2 mice must be due to the direct and combined effect of endogenous Emx2 and the Emx2 transgene in progenitors in the cortical VZ. This conclusion is supported by our findings that caudal primary areas V1 and A1 exhibit size changes and shifts in position in heterozygous ne-Emx2 mice intermediate to wt and homozygous ne-Emx2 mice, whereas rostral areas, such as S1, do not have significant changes; this selective effect on caudal areas is difficult to reconcile with a mechanism by which EMX2 acts solely by repressing Fgf8 in the rostrally located commissural plate.

Complementary Actions of EMX2, PAX6, and Other Regulatory Proteins in Arealization

Diminishing endogenous FGF levels “rescues” shifts of gene markers in embryonic Emx2 null mice (Fukuchi-Shimogori and Grove, 2003)—a finding that indicates the upregulation of other TFs that normally cooperate with EMX2 in arealization and compensate for its loss. For example, Coup-TF1, which is implicated in arealization (Zhou et al., 2001), is upregulated when FGF8 is diminished (Fukuchi-Shimogori and Grove, 2003; Garel et al., 2003).

Coup-TF1 (Liu et al., 2000) and Lhx2 (Nakagawa et al., 1999) have caudal to rostral graded expression that parallels Emx2 in the cortical VZ. We detect no change in their graded expression in embryonic ne-Emx2 mice but find a flattening of the normally strongly graded, high rostral to low caudal expression of Pax6, consistent with an analysis of Emx2 null mice suggesting that EMX2 represses Pax6 (Muzio et al., 2002b). Marker analyses of embryonic Pax6 mutant mice implicate Pax6 in areal patterning, particularly in imparting rostral area identities (Bishop et al., 2000, 2002). Thus, repression of Pax6 in rostral VZ of ne-Emx2 mice would diminish its influence in imparting rostral area identities and thereby contribute to the reduced sizes and shifts of rostral areas motor and S1 in ne-Emx2 mice (Figure 9C).

Model of Areal Patterning of the Neocortex

The “combinatorial code model” of neuronal specification defined in the developing ventral spinal cord has become the dominant mechanism for specification of
neuronal identities (Jessell, 2000). In the spinal cord VZ, sonic hedgehog that is secreted by notocord and floorplate represses or induces expression of different TFs in graded patterns that constrict into sharply bordered patterns through mutual repression. This mechanism results in genetically distinct domains of progenitors, which generate different types of interneurons and motor neurons that are definable by their expression of unique subsets of TFs and other proteins.

We suggest that the mechanism that operates in cortex to specify area identities has similarities to but also important differences from the combinatorial code model. As in spinal cord, in cortex the initial graded expression of TFs, for example, Emx2 and Pax6, in progenitors across the cortical VZ is established by signaling molecules secreted from patterning centers, and the graded expression of TFs across the cortical VZ is refined by crossexpression (present study; Muzio et al., 2002a). Later in the cortical plate, the expression of many genes, including ROR<jΡ/txn> (Nakagawa and O’Leary, 2003), Coup-TF1 (Liu et al., 2000), and Id2 (Rubenstein et al., 1999), develop disjunctive patterns that align with area borders. This patterned gene expression may develop by a progressive translation of the graded expression of Emx2 through concentration-dependent differences in binding efficacy to promoter and repressor elements or its participation in the combinatorial action of multiple activators and repressors of transcription (O’Leary and Nakagawa, 2002). However, at no time during corticogenesis are sharply bordered patterns of TFs evident in the neocortical VZ, and all retain graded expression across the entire neocortical VZ.

Thus, in contrast to the spinal cord VZ, the neocortical VZ does not become parcellated into genetically distinct domains of progenitors. We propose that absolute levels of EMX2 in cortical progenitors specify the area identity of their progeny. In this model, increasing or reducing EMX2 in cortical progenitors results in a complete change performed as described (Nakagawa et al., 1999). Relative gradients of their progeny. In this model, increasing or reducing 35S, Liu et al., 2000). DIG whole-mount in situ hybridization was performed (Wong-Riley, 1979). Images of CO or serotonin-tional guidelines. Animal care was in accordance with institutional guidelines. RT-PCR Detection of Transgene Expression

To detect specifically ne-Emx2 transgene mRNA, RT-PCR was done on total RNA as described (Leingartner et al., 1994). Transverse transcription (RT) was primed with an Emx2-specific antisense oligo (5’TGATCTCTTCGAAGCCG-3’), and PCR was performed with the same oligo and a nestin-specific sense primer (5’TCAACCCCTTAAAGGC TCC-3’), and an Emx2-specific antisense primer (5’GGACGGAGA GAAGCGGT-3’), resulting in a transgene-specific band of 587 bp.

**Northern Blot**

Northern blot analysis was performed as described (Leingartner et al., 1994). Relative levels of Emx2 expression were quantified using densitometry of digitized blot images (NIH image). Mean density was determined for each band, from which background was subtracted. The values presented are means from three or more blots.

In Situ Hybridization

S1-labeled or digoxigenin (DIG)-labeled riboprobes that were used are as follows: Emx2 (Leingartner et al., 2003), Fgfl (The Salk Institute), cad8, Lhx2, Lhx9 (Nakagawa et al., 1999), Pax6 (mouse full-length clone; O’Leary lab), Gbx2 (mouse full-length clone; G. Chapman, University of Adelaide, Australia), and Coup-TF1 (mouse clone; M.-J. Tsai, Baylor). In situ hybridization on 20–40 μm cryostat sections was performed as described (Díg, Nakagawa et al., 1999; Hs, Liu et al., 2000). DIG whole sections in situ hybridization was performed as described (Nakagawa et al., 1999). Relative gradients of Emx2 and Pax6 expression were quantified from sagittal sections of E10.5 wt and ne-Emx2 mice (n = 4 for each mouse line and gene) (Figures 7G and 7H). Silver grains were counted in fields of 1.0 × 10^5 μm^2 at the rostral end, caudal end, and midpoint in the VZ of the d’Tel. Data are expressed as the ratio of counts in each field to the total count in three fields in the same section.

Tangential Sections, Immunostaining, CO Histchemistry, Statistical Analysis, and Dil Tracing

Mice were perfused with cold 4% buffered paraformaldehyde; the cortical hemisphere was dissected free and postfixed between slide glasses and then cryoprotected. Tangential sections were cut at 40 μm. For immunostaining, sections were blocked, incubated with anti-serotonin (1:50,000; Immunostar) or anti-nestin (1:1,000; BD Biosciences Pharmingen) antibodies, detected with biotinylated secondary antibodies using Elite ABC kit (Vector). CO histochemistry was performed (Wong-Riley, 1979). Images of CO or serotonin-stained tangential sections were taken with a digital camera. Areas, lengths, and defined points were determined using Scion Image (Scion Corp) or Photoshop (Adobe). Excel (Microsoft) was programmed to calculate the axial position of barrel C3 and A1. Statistical analysis was done in SPSS (SPSS Inc.). Dil tracing of TCAs was done as described (Braisted et al., 1999).

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**Experimental Procedures**

**Mice**

Morning of the vaginal plug is E0.5; embryos were also staged (Kauffman, 1994). The first 24 hr after birth is P0. Analyses were done blind to genotype. Animal care was in accordance with institutional guidelines. Emx2<sup>+/−</sup> and Emx2<sup>−/−</sup> littermates were generated from heterozygous breeding pairs maintained on a C57/BL6 background (obtained from the Japan Society for the Promotion of Science. We thank U. Lendahl for the nestin promoter construct; P. Gruss for Emx2

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S1-labeled or digoxigenin (DIG)-labeled riboprobes that were used are as follows: Emx2 (Leingartner et al., 2003), Fgfl (The Salk Institute), cad8, Lhx2, Lhx9 (Nakagawa et al., 1999), Pax6 (mouse full-length clone; O’Leary lab), Gbx2 (mouse full-length clone; G. Chapman, University of Adelaide, Australia), and Coup-TF1 (mouse clone; M.-J. Tsai, Baylor). In situ hybridization on 20–40 μm cryostat sections was performed as described (Díg, Nakagawa et al., 1999; Hs, Liu et al., 2000). DIG whole sections in situ hybridization was performed as described (Nakagawa et al., 1999). Relative gradients of Emx2 and Pax6 expression were quantified from sagittal sections of E10.5 wt and ne-Emx2 mice (n = 4 for each mouse line and gene) (Figures 7G and 7H). Silver grains were counted in fields of 1.0 × 10^5 μm^2 at the rostral end, caudal end, and midpoint in the VZ of the d’Tel. Data are expressed as the ratio of counts in each field to the total count in three fields in the same section.

**Tangential Sections, Immunostaining, CO Histchemistry, Statistical Analysis, and Dil Tracing**

Mice were perfused with cold 4% buffered paraformaldehyde; the cortical hemisphere was dissected free and postfixed between slide glasses and then cryoprotected. Tangential sections were cut at 40 μm. For immunostaining, sections were blocked, incubated with anti-serotonin (1:50,000; Immunostar) or anti-nestin (1:1,000; BD Biosciences Pharmingen) antibodies, detected with biotinylated secondary antibodies using Elite ABC kit (Vector). CO histochemistry was performed (Wong-Riley, 1979). Images of CO or serotonin-stained tangential sections were taken with a digital camera. Areas, lengths, and defined points were determined using Scion Image (Scion Corp) or Photoshop (Adobe). Excel (Microsoft) was programmed to calculate the axial position of barrel C3 and A1. Statistical analysis was done in SPSS (SPSS Inc.). Dil tracing of TCAs was done as described (Braisted et al., 1999).
EMX2 Directly Specifies Primary Cortical Areas

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