Absence of Cajal-Retzius cells and subplate neurons associated with defects of tangential cell migration from ganglionic eminence in Emx1/2 double mutant cerebral cortex

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SUMMARY

Emx1 and Emx2, mouse orthologs of the Drosophila head gap gene, ems, are expressed during corticogenesis. Emx2 null mutants exhibit mild defects in cortical lamination. Segregation of differentiating neurons from proliferative cells is normal for the most part, however, reelin-positive Cajal-Retzius cells are lost by the late embryonic period. Additionally, late-born cortical plate neurons display abnormal position. These types of lamination defects are subtle in the Emx1 mutant cortex. In the present study we show that Emx1 and Emx2 double mutant neocortex is much more severely affected. Thickness of the cerebral wall was diminished with the decrease in cell number. Bromodeoxyuridine uptake in the germinal zone was nearly normal; moreover, no apparent increase in cell death or tetraploid cell number was observed. However, tangential migration of cells from the ganglionic eminence into the neocortex was greatly inhibited. The wild-type ganglionic eminence cells transplanted into Emx1/2-double mutant telencephalon did not move to the cortex. MAP2-positive neuronal bodies and RC2-positive radial glial cells emerged normally, but the laminar structure subsequently formed was completely abnormal. Furthermore, both corticofugal and corticopetal fibers were predominantly absent in the cortex. Most importantly, neither Cajal-Retzius cells nor subplate neurons were found throughout E11.5-E18.5. Thus, this investigation suggests that laminar organization in the cortex or the production of Cajal-Retzius cells and subplate neurons is interrelated to the tangential movement of cells from the ganglionic eminence under the control of Emx1 and Emx2.

Key words: Neocortex, Cortical lamination, Radial cell migration, Tangential cell migration, Emx1, Emx2, Cajal-Retzius cells, Subplate neurons, Pioneer neurons, Interneurons, Ganglionic eminence, Mouse

INTRODUCTION

The mammalian cortex is the most complex structure in animate nature. Neuronal differentiation begins around E11.5 in the cortex, with the major period occurring between E12-E18 (Marin-Padilla, 1998). First, the preplate is formed; Cajal-Retzius (CR) cells and subplate neurons are pioneer neurons that differentiate at this earliest stage. By E13.5, the preplate has split into the marginal zone and the subplate. Subsequently, the cortical plate is formed between these regions by migration of differentiated neurons through the subplate. Projection neurons that are born in the ventricular zone migrate radially and accumulate in the cortical plate. At this juncture, neurons born later pass those born earlier, and settle in progressively more superficial levels in the cortical plate. Thus, deep cortical layers develop earlier; in contrast, progressively younger neurons form upper cortical layers. As a result, five layers are generated in the cortical plate. This ‘inside-out corticogenetic gradient’ is a general feature of the mammalian cortex (Angevine and Sidman, 1961; Caviness and Rakic, 1978).

CR cells localize in the marginal zone and play an essential role in cortical lamination. Reelin, which encodes an extracellular matrix protein, is expressed in the CR cells and is a key molecule in the orchestration of the radial migration of cortical plate neurons (D’Arcangelo et al., 1995; Ogawa et al., 1995; Alcántara et al., 1998; Curran and D’Arcangelo, 1998; Rice and Curran, 1999). A spontaneous mouse mutant reeler exhibits a mutation in the reelin gene. The CR cells develop, but reelin is not produced in the mutant. In its cortex the preplate does not split into the marginal zone and subplate.
Consequently, this structure constitutes a “superplate” in the most superficial region of the cortex. Cortical plate neurons accumulate beneath the superplate; young neurons cannot migrate outward by passing across pre-existing cell layers (Caviness, 1982).

Radial glial cells are also among the earliest cell types to emerge in the developing mammalian forebrain (Rakic, 1972; Misson et al., 1988). Their fibers extend radially across the entire cerebral wall from the ventricular surface to the pial surface, resulting in the formation of a dense scaffold. The scaffold is thought to provide a cellular substratum that supports and directs the migration of young cortical neurons. Radial glial cells persist throughout corticogenesis. However, they proliferate throughout cortical development and may be multipotent progenitor cells that give rise to various cell types, including neurons (Chans-Sacre et al., 2000; Malatesta et al., 2000; Hartfuss et al., 2001; Miyata et al., 2001; Noctor et al., 2001). The glial cells transdifferentiate into astrocytes during the perinatal period following the completion of neuronal migration. Both radial glial and CR cells are transient pioneer cells, appearing and disappearing at similar stages.

As pioneer neurons, subplate neurons play a role in the guidance of cortical afferents such as thalamocortical afferents (Allenendorfer and Shatz, 1994; Molnar and Blakemore, 1995; Nothias et al., 1998; Mackarehtschian et al., 1999; Zhou et al., 1999). In lower mammals, a significant portion of thalamic fibers enters the neocortex via the marginal zone; in contrast, entry is predominantly via the subplate in higher mammals (O’Leary, 1989; Super et al., 1998). During evolution, the subplate appears to have increased in thickness while the marginal zone has become relatively thinner. These early growing subplate axons have been suggested to control, in turn, neocortical development, area specification and lamination (Rakic et al., 1991; Nothias et al., 1998; Mackarehtschian et al., 1999; Zhou et al., 1999). Corticogenesis of the layer IV neurons occurs at the identical developmental stage as the onset of thalamic innervation into the subplate (Kennedy and Dehay, 1993; Zhou et al., 1999). However, little is known regarding the molecular basis of subplate neuron development.

In addition to these glutamatergic projection neurons, interneurons constitute another major cell population within the cortex. GABAergic interneurons are born in the ganglionic eminence and migrate tangentially into the cortex (de Carlos, 1993; Zhou et al., 1999). However, the number of other sites of origin of cortical cells and the extent of cell influx from each of these sites remain unknown.

Emx1 and Emx2, mouse orthologs of the Drosophila head gap gene, ems, are expressed during corticogenesis (Simeone et al., 1992; Gulisano et al., 1996; Mallamaci et al., 1998). Emx2 expression begins around the 3 somite stage and covers the cortical region, a portion of the lateral ganglionic eminence and the diencephalon anterior to the zona limitans by E10.5. Emx1 expression occurs around E9.5 in the cortical region. Our previous studies involving Emx1 and Emx2 single mutants suggested that these genes play essential complementary roles in the development of archipallium structures (Yoshida et al., 1997). The investigations also suggested that these genes may function in cortical lamination at a later stage. Indeed, Mallamaci et al. (Mallamaci et al., 2000) recently demonstrated that reelin-positive CR cells are initially formed at E12 in Emx2 single mutants, but they are subsequently lost in the neocortical region. Concomitantly, neurons constituting the cortical plate display an abnormal migration pattern in the single mutants. Defects in neocorticogenesis are subtle in Emx1 single mutants (Yoshida et al., 1997).

The present investigation examined neocorticogenesis defects in Emx1 and Emx2 double mutants. The analyses suggest that Emx1 and Emx2 cooperate to promote cell influx from the ganglionic eminence and operate in tandem in the development of CR cells and subplate neurons.

MATERIALS AND METHODS

Mouse mutants

Emx1+/– single homozygous and Emx2+/– single heterozygous mice were obtained as previously described (Yoshida et al., 1997). They were mated to generate double mutants as described in Results. Mice were housed in an environmentally controlled room of the Laboratory Animal Research Center of Kumamoto University under University guidelines for animal and recombinant DNA experiments. Genotypes of newborn mice and embryos were routinely determined by PCR analyses as described (Yoshida et al., 1997). Confirmation, when necessary, was effected by Southern blot analyses. Genomic DNAs utilized in the analyses were prepared from tails or yolk sacs. Noon of the day on which the vaginal plug was detected was designated as E0.5.

Histological analysis

Brains were fixed with Carnoy’s fixative solution at room temperature for 18-24 hours. Specimens were dehydrated and embedded in paraffin (Paraplast; Oxford). Serial sections (thickness of 10 μm) were prepared and stained with 0.1% Cresyl Violet (Sigma).

RNA probes and in situ hybridization

Embryos were dissected in phosphate-buffered saline (PBS) and fixed overnight at 4°C in 4% paraformaldehyde (PFA) in PBS. Specimens were gradually dehydrated in ethanol/H2O up to 95% ethanol and stored at –20°C. The protocol for in situ hybridization of embryos was as described (Wilkinson, 1993). Single-stranded digoxigenin-UTP-labeled (Boehringer Mannheim) RNA probes were employed. The probes were as follows. BF1 (a KpnI/XhoI fragment of cDNA) (Tao and Lai, 1992), Dlx1 (Bulfone et al., 1993), Emx1 and Emx2 (Yoshida et al., 1997), Mxsl (a EcoRI/XbaI fragment isolated by RT-PCR) (Hill et al., 1989), NEX (Schwab et al., 1998), Ngn2 (Sommer and Anderson, 1996), Pax6 (Walther and Gruss, 1991), reelin (D’Arcangelo et al., 1995), SCIP (Monuki et al., 1989) and Lhx2 (Porter et al., 1997).

Immunohistochemistry

In the immunohistochemical staining with antibodies against calretinin, MAP2, CSPGs, GAP43, TAG1 and L1, brains were fixed in Carnoy’s fixative, embedded in paraffin and subsequently sectioned at 8 μm thickness. In the staining with monoclonal antibodies against calretinin, GABA, GFAP and glialtune, brains were fixed in PFA (4% paraformaldehyde in PBS), and in the staining with the RC2 antibody they were fixed in PLP (2% paraformaldehyde and 1.35% lysine in phosphate buffer). Fixed brains were soaked in 20% sucrose in PBS and embedded in OCT compound (TISSUE-TEK, USA). Cryostat sections of 10-30 μm thickness were prepared (MICROM HM500M; Carl Zeiss).
Paraffin sections were deparaffinized with xylene, rehydrated through a descending ethanol series and immersed in Tris-buffered saline supplemented with 1% Triton X-100 (TST). Specimens were subsequently treated with 0.3% hydrogen peroxide and 30% methanol in TST for 30 minutes. Sections were then incubated with primary antibodies overnight at 4°C. After rinsing, specimens were incubated with the corresponding secondary antibodies. Immunostaining was effected in 0.05 M Tris buffer containing 0.01% dianisidinobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxide. The following primary antibodies were utilized: polyclonal anti-calretinin (AB149; Chemicon), 1:500; monoclonal anti-chondroitin sulfate (clone CS-56; Sigma), 1:600; monoclonal anti-GAP43 (clone GAP-7B10; Sigma), 1:2000; polyclonal anti-L1 (Fukuda et al., 1997), 1:1000; monoclonal anti-MAP2 (clone HM-2; Sigma), 1:500; polyclonal anti-TAG1 (Fukuda et al., 1997), 1:1000.

Frozen sections were stained by the free-floating method, followed by incubation with primary antibodies in PBS overnight at 4°C. After rinsing, specimens were incubated with corresponding secondary antibodies. Immunostaining was effected in 0.05 M Tris buffer containing 0.01% dianisidinobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxide. Immunofluorescence was performed with fluorescein-conjugated secondary antibodies (Vector). The following primary antibodies were utilized: polyclonal anti-calretinin (AB149; Chemicon), 1:500; monoclonal anti-GFAP (clone GF12.24; Progen), 1:500; monoclonal anti-GABA (clone GB-69; Sigma), 1:2000; monoclonal anti-glutamate (clone GLU-4; Sigma), 1:10000; monoclonal anti-RC2 (tissue culture supernatant cell line, a generous gift from Dr. M. Yamamoto, Tsukuba University, Japan) (Mission et al., 1988).

**Cell proliferation and birthdate studies**

For birthdate studies, timed-pregnant female mice were injected intraperitoneally at several stages of pregnancy with a single pulse (50 mg/kg body weight) of 5-bromo-2'-deoxyuridine (BrdU) (5 mg/ml dissolved in sterile PBS; Nacalai Tesque, Japan). Subsequently, distribution of BrdU-positive cells was determined at E18.5 or E19.5. In order to investigate cell proliferation, BrdU was delivered to pregnant mice 2 hours before sacrifice. Brains were fixed with Carnoy's fixative and embedded in paraplast. Sections were subsequently prepared through a descending ethanol series and immersed in Tris-buffered 10% formaldehyde at E11.5-16.5 were subjected to TUNEL assay (7-amino-actinomycin D).

**Flow cytometric analysis of DNA content**

Pregnant female mice at E12.5 were injected intraperitoneally with a single pulse (100 μl) of BrdU (10 mg/ml dissolved in sterile PBS; BrdU Flow Kit; Pharmingen). Two days later, the neocortical tissue was excised from each embryo; three embryos of each genotype were subjected to the analysis. The tissue was dispersed by pipetting and cells were collected at a concentration of 1x10⁶ cells/tube. DNA content analysis was conducted by flow cytometry (FACS Calibur; Becton Dickinson) based on the manufacturer's protocol following staining with FITC-labeled monoclonal anti-BrdU and 7-AAD (7-aminocytidine D).

**TUNEL assay**

Serial paraffin sections (8 μm) derived from embryos fixed in 10% formaldehyde at E11.5-16.5 were subjected to TUNEL assay according to the manufacturer's protocol (In Situ Cell Death Detection Kit, AP; Boehringer Mannheim).

**RESULTS**

**Emx1/2 cortex is thin with the decrease in cell number**

Emx1/2– mutants are viable and fertile (Qiu et al., 1996; Yoshida et al., 1997), however, Emx2– mutants die soon after birth as a result of urogenital defects (Pellegrini et al., 1996; Miyamoto et al., 1997; Yoshida et al., 1997). Emx1+/–/Emx2+/– double heterozygotes were obtained via crosses between Emx1+/– single homozygotes and Emx2+/– single heterozygotes. These mutants were obtained in Mendelian ratio and were fertile; moreover, their crosses with Emx1+/–...
homozygotes yielded fertile $\text{Emx1}^{-/-}\text{Emx2}^{+/+}$ double mutants in approximately a quarter of the offspring. These double mutants were propagated and maintained via intercrosses or by crosses with $\text{Emx1}^{-/-}$ single homozygotes as the source to generate $\text{Emx1}^{-/-}\text{Emx2}^{-/-}$ ($\text{Emx1/2}$) double mutants. The $\text{Emx1/2}$ double homozygous mutants died soon after birth, as did the $\text{Emx2}^{-/-}$ ($\text{Emx2}$ single) mutants; however, the double mutants occurred in Mendelian ratio prior to delivery.

In E18.5 $\text{Emx1/2}$ double mutants, the medial pallium was lost, and the cortex was greatly reduced not only in area but also in thickness (Fig. 1). At E11.5, the point at which cell differentiation begins in the cortex, the loss of the medial and medial regions of the ventricular zone in the cortex exhibited nearly the same level of BrdU labeling, with the exception of the fimbria region (Reznikov and Kooy, 1995). No difference was apparent in the frequency of BrdU-labeled cells of the double mutant cortex relative to that of the wild-type or $\text{Emx2}$ single mutant cortex at either E11.5 or E16.5 (Fig. 2C). Consistent with this finding, the thickness of the ventricular zone was nearly normal in the $\text{Emx1/2}$ double mutants (Fig. 6D-F). Total cell number decreases with gestational age, consequently, this observation indicates that the frequency of labeled cells per total cells, or the labeling index, increases in the double mutant cortex (Fig. 2D).

Fig. 2. BrdU incorporation. (A) Boxed areas indicate 100 μm wide sampling areas. (B,C) Numbers of total cells (B) and BrdU-positive cells (C) were counted in nine sections obtained from three embryos of each genotype. (D) The labeling index, or BrdU-positive cells per total cells. Scale bars, 100 μm.

Fig. 3. Frequency of tetraploid cells in the neocortex. The frequency was determined for BrdU-incorporating (R1) and non-incorporating (R2) populations by 7-AAD staining. The averages of R1 and R2 in three independent analyses were 7.3 and 6.6% in wild type (A), 8.2 and 7.3% in $\text{Emx2}$ single mutants (B) and 7.9 and 5.8% in $\text{Emx1/2}$ double mutants (C), respectively.
The decrease in cell number with the normal pool of BrdU-incorporating cells could occur as a result of defects in cell division following final DNA duplication. This possibility was assessed by DNA content analysis on the second day following BrdU labeling at E12.5 (Fig. 3). The analysis suggested no difference in the frequency of tetraploid cells either in BrdU-positive or negative populations among wild-type, Emx2 single mutant and Emx1/2 double mutant cortex. Furthermore, cell death could also account for the decline in cell number in the Emx1/2 double mutant cortex. Using the TUNEL assay, prominent cell death was detected exclusively in the roof area during E11.5-16.5 (Gavrieli et al., 1992). Additionally, increased cell death was not observed in the Emx1/2 double mutant cortex (data not shown).

**Defects in cell migration into cortex from ganglionic eminence**

Projection neurons are born in the cortical ventricular zone and migrate radially, whereas interneurons in the cortex are born in the ganglionic eminence and migrate tangentially into the cortex. The decrease in cell number can be explained by the decrease in this cell influx. Influx was examined in slice cultures by DiI-labeling of the medial ganglionic eminence (Powell et al., 2001). In slices prepared from E13.5 brain, 4.0-4.5% of the cells were DiI-positive in the neocortical area of the wild type, as determined by counting of dissociated cells 8 hours after DiI-positive cells first reached the cortical/subcortical boundary. In contrast, no positive cells were found in the double mutant neocortex. In Emx2 single mutants, migration was initially retarded (Fig. 4E), however, the cells were able to migrate into the neocortex after 2 days in culture (Fig. 4H,K). In contrast, in Emx1/2 double mutants, DiI-positive cells could not cross the cortical/subcortical border even after 2 days in culture (Fig. 4I,L).

Of note was the obvious increase in volume of the subcortical area during culture. This increase was probably the result of the lack of cell migration into the cortical area. Indeed, the ganglionic eminence was normal in size at E11.5, but became hypertrophic at subsequent stages (Fig. 1).

Emx1 is not expressed in the subcortex (Tole et al., 2000b) and the migration defects characteristic of double mutants are most probably due to cortical defects resulting from the double mutation rather than to ganglionic eminence cells per se. To confirm this, the migration was analyzed in double mutant or wild-type slices transplanted with the wild-type or double mutant ganglionic eminence cells, respectively. Three types of assays were conducted as shown in Fig. 5. In all three the double mutant cells were able to move into the cortex when transplanted into the medial ganglionic eminence of wild-type slices. In contrast, wild-type cells could not migrate into the cortex when transplanted into Emx1/2 double mutant slices.

**Pan-neural differentiation is normal in the double mutant**

Neuronal differentiation begins around E12.5. The possible disturbance in neuronal differentiation was examined in the mutants through the expression of pan-neuronal markers. Normally a layer of MAP2-positive neurons emerges at E12.5 beneath the pial surface. These neurons were normally observed in Emx1/2 double mutants (Fig. 11G-I). At E15.5 in Emx1/2 double mutant cortex, differentiated neuronal cells were separated normally from the undifferentiated ventricular zone as determined with NEX, a bHLH gene unique to differentiated neurons (Fig. 6A-C) (Bartholomae and Nave, 1994; Shimizu et al., 1995), and Lhx2, a marker for proliferative undifferentiated cells (Fig. 6D-F) (Porter et al., 1997).

Radial glial cells are among the earliest cells to emerge and persist throughout cortical development, constituting a major cell type of the developing brain (Super et al., 1998; Chans-Sacre et al., 2000). The glial cells transdifferentiate into astrocytes upon completion of neural migration at an early postnatal stage. RC2 and GFAP mark these radial glial cells and astrocytes, respectively (Misson et al., 1988; Sancho-Tello et al., 1995). RC2-positive radial glial cells were present in Emx1/2 double mutants (Fig. 6G-I), although the radial

![Fig. 4. Cell influx from the ganglionic eminence. DiI was placed in the E13.5 medial ganglionic eminence (A-C). Subsequently, slices were cultured for 1 (D-F) and 2 days (G-I). (J-L) Enlarged views of the boxed areas in G-I, respectively. (A,D,G,J) wild type, (B,E,H,K) Emx2 single and (C,F,I,L) Emx1/2 double mutants. Yellow dotted lines show the cortical/subcortical borders. Scale bars: (A-I) 100 μm; (J-L) 400 μm.]
alignment of their fibers was greatly disturbed. The disruption was more severe than in Emx2 single mutants. However, as in wild type, no GFAP-positive astrocytes were evident in the E18.5 double mutant cortex, suggesting the absence of accelerated differentiation into astrocytes (Fig. 6J-L).

Cortical architecture is greatly impaired in Emx1/2 double mutants

The E18.5 neocortex is laminated into the marginal zone, cortical plate, subplate, intermediate zone and subventricular/ventricular zone (Fig. 7A). Each zone was clearly discernible in Emx1 single mutants as in wild-type neocortex (Fig. 7B,F) (Yoshida et al., 1997). The laminar organization, while established, was somewhat perturbed in Emx2 single mutants (Fig. 7C) (Yoshida et al., 1997; Mallamaci et al., 2000). White matter was thin and the subplate was less distinct. The marginal zone, which is normally cell body poor, displayed an increased number of cell bodies (Fig. 7G); moreover, the cortical plate border on the marginal zone was wavy in the lateral portion of the Emx2 single mutant neocortex. In contrast, laminar organization was severely impaired in Emx1/2 double mutants (Fig. 7D). Heavy accumulation of cell bodies was evident in the most superficial region of the neocortex beneath the pial surface where, normally, the molecular layer should exist (Fig. 7H). The cortical plate was obscured and the subplate could not be identified. Relatively cell body-rich and -poor zones existed. In the former, however, neurons were distributed in a diffuse and disorganized fashion. The white matter was sparse, particularly at the medial portion, and abnormal axon bundles occasionally invaded the cell body-rich zone.

The defects in the cortical architecture were subsequently assessed using immunohistochemical analysis of MAP2, which specifically identifies dendrite extensions of post-mitotic cortical neurons. In the wild-type cortex, MAP2-positive neuronal processes are arranged radially and form a tight, palisade-like pattern in the cortical plate (Fig. 7I). MAP2 also stains the horizontal processes of subplate neurons (Luskin and Shatz, 1985; Wood et al., 1992) but not those in the marginal and intermediate zones (Ringstedt et al., 1998). This MAP2 distribution was normal in Emx1 single homozygotes (Fig. 7J).

In Emx2 single mutants, the MAP2-negative superficial layer was thin; however, the palisade-like neuronal processes and the subplate neurons were observed (Fig. 7K). In contrast, this
pattern was severely disordered in *Emx1/2* double homozygotes. MAP2-positive dendrites were scattered throughout the neocortical wall except for the ventricular zone; in addition, the palisade-like pattern and the subplate neurons had disappeared (Fig. 7L). The MAP2-negative superficial layer was absent, and a line of strong signals was present just beneath the pial surface.

At E16.5, both GABA- and glutamate-immunoreactivities are prominent in the marginal zone, subplate and intermediate-subventricular border (Fig. 7M,P) (Del Rio et al., 1992; Del Rio et al., 1995). These GABA- and glutamate-positive layers were present in *Emx2* single mutants, albeit diffusely. In *Emx1/2* double mutants, however, GABA-positive neurons were scattered and did not exhibit layer-like distribution (Fig. 7O) and glutamate-positive neurons were scarce (Fig. 7R). SCIP is expressed in cortical plate neurons at E18.5 (Frantz et al., 1994). SCIP expression was present but diffuse in *Emx2* single mutants (Tole et al., 2000a) but was not detected at all in the *Emx1/2* double mutant cortex (Fig. 7S-U). Thus, the cortical architecture was severely affected in the double mutants.

**Impairment of ‘inside-out’ lamination pattern**

To examine possible defects associated with the “inside-out” corticogenesis, neuronal birthdate analysis was then performed in E13.5 and E15.5 embryos. Pregnant mice were injected with a single dose of BrdU. The embryos were sacrificed at E19.5, and the nuclei of those neurons that had incorporated BrdU while undergoing their final cell division at the time of injection were identified. In wild-type and *Emx1* homozygotes, neurons born at E13.5 and E15.5 assumed their proper positions in the deeper layers (Fig. 8A,B) and in the most superficial layer of the cortical plate (Fig. 8E,F), respectively, at E19.5. The cell migration pattern was slightly affected in *Emx2* single mutants (Mallamaci et al., 2000) in that, neurons born both at E13.5 and E15.5 were somewhat scattered in the cortex, however, many E15.5 neurons had migrated to the superficial aspect of the cortical plate (Fig. 8C,G). In contrast, *Emx1/2* double mutant brain demonstrated a severely impaired neuronal migration pattern. The E13.5 neurons were scattered throughout the cortex (Fig. 8D) and the E15.5 neurons failed to migrate to the superficial aspect of the cortical plate and remained in the periventricular zone (Fig. 8H). Thus, the corticogenetic gradient was disturbed in *Emx1/2* double mutants.

**Lack of Cajal-Retzius cells and subplate neurons**

Reelin signaling plays a critical role in radial migration. In wild-type embryos at E13.5 and E15.5, reelin mRNA is present in CR cells in the marginal zone throughout the entire cerebral cortex (Fig. 9A,D,G) (Alcántara et al., 1998). At E13.5, though reduced in number, reelin-positive cells were observed in *Emx2* single mutants (Mallamaci et al., 2000) (Fig. 9B). These cells were predominantly absent in neocortex and paleocortex regions at E15.5 (Fig. 9E,H) but present in the most medial region of the cortex, the presumptive cingulate cortex and hippocampal anlage. In contrast, reelin expression was completely absent in *Emx1/2* double mutants in the superficial portion of the entire cerebral cortex both at E13.5 and E15.5 (Fig. 9C,F,I).

In mouse embryonic cerebral cortex, reelin is expressed exclusively in CR cells (Alcántara et al., 1998). Therefore, the absence of reelin expression suggests the lack of CR cells in *Emx1/2* double mutants. This possibility was assessed using several molecular markers. GAP43 is a neuron-specific phosphoprotein (Benowitz et al., 1988). At E16.5, the antibody against this protein stains the horizontal axons of CR cells, nerve terminals of apical dendrites that synapse with the CR horizontal axons in the marginal zone and subplate neurons (Fig. 10A). GAP43 was present in the subplate of *Emx2* single mutants but could not be detected in the marginal zone (Fig. 10B). In contrast, GAP43 was not evident throughout the

![Fig. 6. Marker analyses of neuronal differentiation. (A-C) NEX expression at E15.5; (D-F) Lhx2 expression at E15.5; (G-I) RC2 staining at E16.5; (J-L) GFAP staining at E18.5. (A,D,G) Wild-type, (B,E,H,K) *Emx2*–/– and (C,F,I,L) *Emx1/2*–/– cortices. Scale bars, 100 µm.](image-url)
cortex in Emx1/2 double mutants (Fig. 10C). In E16.5 wild-type cortex, chondroitin sulfate proteoglycans (CSPGs) also distribute in the marginal zone and subplate (Fig. 10D) (Sheppard and Pearlman, 1997), suggesting that they are produced mainly by CR cells and subplate neurons. In Emx2 single mutants, CSPGs were present in the subplate. They were also detected in the medial portion of the marginal zone, but were greatly reduced in the lateral portion (Fig. 10E). In Emx1/2 double mutants, CSPGs were scarcely present throughout the cortex (Fig. 10F).

Calretinin is another marker of CR cells and subplate neurons (Fig. 8G) (Del Rio et al., 1995; Alcántara et al., 1998). In the marginal zone of Emx2 single mutants, calretinin was present, although its expression was greatly diminished (Fig. 10H) and its expression was relatively normal in the subplate (Mallamaci et al., 2000). In contrast, expression was not observed throughout the cortex of Emx1/2 double mutants (Fig. 10I).

Calretinin (Del Rio et al., 1995) and GAP43 (Benowitz et al., 1988) are also expressed in thalamic and cortical axons. The absence of their expression indicates a lack of these projections in Emx1/2 double mutant cortex. Subplate neurons have been suggested to play important roles in guiding cortical afferents (Molnar and Blakemore, 1995; Super et al., 1998; Zhou et al., 1999). Expression of CSPGs in subplate has been reported to be associated with thalamic innervation (Bicknese et al., 1994); expression of CSPGs was scarce in Emx1/2 double mutants. Defects in the thalamic projection were then assessed with L1. L1-positive axons leave the diencephalon for the internal capsules and subsequently invade the cortex from the ventrolateral region along the subplate in E17.5 wild type (Fig. 10J) (Fukuda et al., 1997). In Emx2 single mutants, the axons projected into the internal capsule and then into the cortex. However, projections to each portion of the cortex were immature and fewer fibers accumulated below the cortical plate (Fig. 10K). In contrast, thalamocortical axons scarcely innervated the cortex in Emx1/2 double mutants with many turning basilaterally away from the cortex into the external capsule and amygdala (Fig. 10L).

TAG1 stains corticofugal axons (Fig. 10M) (Fukuda et al., 1997). TAG1 immunoreactivity resides in neurons of the cortical plate and thick fiber bundles in the cortex; these fibers are restricted to the intermediate zone and are not present in the subplate. They descend into the internal capsule and mingle with the thalamic axons. At E17.5 TAG1 immunoreactivity was reduced in the Emx2 single mutant cortex (Fig. 10N) and no TAG1 immunoreactivity was detected beyond the cortical/subcortical junction (data not shown). TAG1 immunoreactivity was scarce in Emx1/2 double mutant cortex (Fig. 10O).

**Emx1/2 in generation of CR cells and subplate neurons**

Emx2 expression occurs at E8.5, whereas Emx1 expression is initially observed at E9.5 in rostral brain (Gulisano et al., 1996; Briata et al., 1996; Mallamaci et al., 1998). Subcortex expresses Emx2 but not Emx1 (Tole et al., 2000b). Both

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**Fig. 7.** Cortical lamination. (A-H) Nissl’s staining; (E-H) enlarged views of marginal zone. (I-L) MAP2 staining. (M-O) GABA staining. (P-R) Glutamate staining. (S-U) SCIP expression. (A,E,I,M,P,S) Wild-type. (B,F,J) Emx1−/−. (C,G,K,N,Q,T) Emx2−/− and (D,H,L,O,R,U) Emx1+/− Emx2−/− cortices at E16.5 (M-R) and E18.5 (A-L,S-U), respectively. CP, cortical plate; IZ, intermediate zone; SP, subplate; SV, subventricular zone; MZ, marginal zone; VZ, ventricular zone. Scale bars, (E-L) 50 μm; others 100 μm.
genes are expressed in undifferentiated neuroepithelial cells in the cortex, but Emx1 is also expressed in differentiated cells. Thus, at E11.5, Emx1 and Emx2 expression overlaps in the ventricular zone (Fig. 11A,D). As corticogenesis progresses, Emx2 expression remains restricted to this zone (Fig. 11E), while Emx1 expression is evident throughout the cortex from the ventricular zone to the cortical plate (Fig. 11B). It is noteworthy that Emx2 expression marks CR cells in the marginal zone (Fig. 11F), but Emx1 is never expressed here (Fig. 11C) (Briata et al., 1996; Mallamaci et al., 1998). Emx2 expression in the marginal zone was faint at E13.5. It was not visible in preplate at E11.5. These Emx1 and Emx2 expression profiles suggest that the Emx1/2 double mutant neocortical defects, which are much more severe than the Emx2 single mutant defects, are due to their complementary functions in the ventricular zone of the cortex. Neuronal differentiation begins around E11.5 in neocortex with the generation of the preplate; this structure is the location where CR cells and subplate neurons first appear. These pioneer neurons are marked by the expression of MAP2, reelin and calretinin (Fig. 11G,J,M). Emx2 single mutant preplate expressed these genes normally as in the wild-type (Fig. 11H,K,N). In contrast, in E11.5 Emx1/2 double mutant cortex, MAP2-positive cells were present on the outer surface of the neuroepithelium, which was similar to the wild type (Fig. 11I) but neither reelin nor calretinin was detected there (Fig. 11L,O).

Finally, the fate of the neurons born at E11.5 was examined by BrdU labeling. At E18.5 in Emx2 single mutants, these cells were primarily harbored in the marginal zone and subplate, which was the same in the wild-type cortex, although some neurons were distributed throughout the cortical plate (Fig. 11P,Q). In contrast, in Emx1/2 double mutants the
neurons born at E11.5 were scattered throughout the cortex (Fig. 11R). Thus, in the absence of inhibition of neuronal production, the differentiation of CR cells and subplate neurons was arrested at the onset of corticogenesis in Emx1/2 double mutants.

DISCUSSION

In Emx1/2 double mutants the neocortex initially developed normally, but its wall thickness was greatly reduced at E18.5. No such reduction is seen in Dlx1/2, Mash1 or Nkx2.1 mutants that display defects in the migration of interneurons (Anderson et al., 1997; Casarosa et al., 1999; Sussel et al., 1999), nor in mutants of the reelin-signaling pathway that have defects in cortical lamination (Ohshima et al., 1996; Chae et al., 1997; Howell et al., 1997; Sheldon et al., 1997; Gilmore et al., 1998; Rice et al., 1998; Trommsdorff et al., 1999; Rice and Curran, 1999). Cell number decreased to 60% of that in wild-type neocortex at E16.5, and this decrease was mainly due to the failure of cell migration from the ganglionic eminence. Concomitantly, the ganglionic eminence expanded in the double mutants. However, it is possible that a delay of migration, but not an absence of migration, leads to this phenomenon, as the double mutants die at birth, at which time normal migration may not be complete.

The question arises as to whether the lack of cell influx can fully explain the decrease in cell number. GABA-positive interneurons are reported to constitute up to 25% of all neocortical neurons (Gonchar and Burkhalter, 1997). Moreover, virtually all cortical GABAergic interneurons are derived from cells born in the ganglionic eminence (Stühmer et al., 2002). The medial pallium is lost in Emx1/2 double mutants (unpublished data), and there may also be no influx of cells born in the dorsal midline (Monuki et al., 2001). An additional source of cortical cells born in non-cortical areas might exist, and the influx of these cells might also be inhibited in the double mutants. Emx1 and Emx2 are co-expressed in the ventricular zone throughout cortical development. This would suggest that Emx1 and Emx2 promote proliferation or multipotential fate in precursor cells. However, the neocortical defects were not apparent at E11.5 and no significant decrease in the frequency of cells incorporating BrdU was observed at either E11.5 or E16.5 in the double mutants. No sign of premature differentiation of precursor cells or cell death was apparent, nor was there an apparent increase in tetraploid cells. However, the present analysis cannot exclude the possibility that each of these factors, while minor as independent components, contributes significantly in sum to the decrease in cell number.

Neuronal differentiation was normal in Emx1/2 double mutants as assessed by MAP2, NEX, Lhx2, RC2 and GFAP expression. Radial glial cells were generated and no obvious accelerated differentiation into astrocytes was evident. However, cortical architecture and layer-associated cell differentiation was greatly impaired in the double mutants. In E11.5-12.5 Emx1/2 double mutant cortex, a preplate-like structure was formed. The cells born at this stage later scattered throughout the cortex in the double mutants. Expression of reelin, calretinin, CSPGs or GAP43 was absent in the double mutant cortex. It is likely that no postmitotic neurons were able to differentiate into CR cells or subplate neurons in the double mutants. Residual CSPGs expression was detected but it is probable that cells other than CR cells and subplate neurons express CSPGs. MAP2, glutamate and GABA staining and failure in thalamic projection are also supportive of the absence
of subplate neurons. However, the nature of cells born around E11.5/12.5 in the double mutants remains to be evaluated in future investigations. These cells might be the immature cells in the normal lineage from which calretinin- and/or reelin-positive pioneer neurons arise. Alternatively, these cells might have differentiated abnormally, but survive, because of their ectopic placement; alternatively, these cells may be born heterochronically. It is also possible that they are unidentified normal pioneer cells, in which neuronal differentiation deviated as a consequence of the double mutation.

Recently, a set of mutants exhibiting defects in cortical lamination has been reported. Most of these are mutants involving the genes in the reelin-signaling pathway; the phenotype is reeler-like (Ohshima et al., 1996; Howell et al., 1997; Sheldon et al., 1997; Chae et al., 1997; Gilmore et al., 1998; Rice et al., 1998; Rice and Curran, 1999; Trommsdorff et al., 1999). Defects in cortical lamination due to a BDNF mutation can also be explained by its regulation of reelin expression in CR cells (Ringstedt et al., 1998). Defects in Tbr mutants might also be related to the decrease in reelin expression (Hevner et al., 2001). Obviously, lamination defects in Emx1/2 double mutants are far beyond these aberrations in reeler-like mutants. The lamination defects are most likely due to the failure in generation of CR cells and subplate neurons themselves. Mutants that fail to develop these pioneer cells have not been described.

In Emx2 single mutants, the marginal zone and subplate are formed and defects in cortical lamination were subtle in comparison to those in the double mutants. Reelin-positive CR cells are born and initially locate normally in the marginal zone but they are subsequently lost in the lateral portion. In the developing cortex, Emxl is expressed through the ventricular zone to the cortical plate, with the exception of the marginal zone (Briata et al., 1996; Mallamaci et al., 1998). In contrast, Emx2 is expressed in the ventricular zone and CR cells (Simeone et al., 1992; Gulisano et al., 1996; Mallamaci et al., 1998). Emx2 expression is not apparent at E11.5-12.5 in the marginal zone. These expression profiles and mutant phenotypes may lead to the interpretation that Emx2 acts to maintain CR cells of the marginal zone, whereas Emxl and Emx2 cooperate in the generation of CR and subplate neurons from progenitor cells in the ventricular zone. An alternative explanation, however, is obviously necessary to explain the persistence of the reelin-positive cells in the most medial region of the Emx2 single mutant cortex; that is, these cells would be a unique population of CR cells. One possibility is that they originated from a cortical hem that was present in Emx2 single mutants, but not in Emx1/2 double mutants (Monuki et al., 2001).

The view generally accepted is that CR cells are born around E11.5 in the cortical ventricular zone, migrate radially and persist in the marginal zone during corticogenesis. The majority of CR cells are glutamatergic (Del Rio et al., 1995). In mouse, the CR cells, but not the GABAergic neurons of the marginal zone, specifically express reelin (Alcantara et al., 1998). Despite innumerable publications regarding CR cells, however, these cells remain elusive. Their numbers obviously increase with corticogenesis (Alcantara et al., 1998) but the extent of the variety of cell types present in the marginal zone during development is disputed, along with what the CR cells are and their potentially numerous origins. In rat, CR cells are proposed to originate from smaller subpial neurons in the retrobulbar area and arrive

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**Fig. 11.** Onset of Emx1/2 double mutant neocortical defects. (A-C) Emx1 and (D-F) Emx2 expression in wild-type cortex at E11.5 (A,D) and at E15.5 (B,C,E,F). (C,F) Enlarged views of B,E at the marginal zone. (G-I) MAP2, (J-L) reelin and (M-O) calretinin expression in E12.5 (G-I) and E11.5 preplate (J-O). (P-R) Birthdate analysis of E11.5 pioneer neurons. BrdU was injected at E11.5, subsequently, the distribution of BrdU-positive cells was examined at E18.5. (G,J,M,P) Wild-type. (H,K,N,Q) Emx2−/− and (I,L,O,R) Emx1−/− Emx2−/− cortices. Abbreviations as in Fig. 7. Scale bars, C and F 25 μm; others 100 μm.
at the marginal zone late at different stages of fetal life (Meyer et al., 1998). The Emx2- and Emx1-dependence of CR cells might differ depending on their origin (Mallamaci et al., 2000).

Subplate neurons have been reported to play important roles in guiding thalamocortical pathways (Allendoer and Shatz, 1994; Molnar and Blakemore, 1995; Notthias et al., 1998; Mackarehtschian et al., 1999; Zhou et al., 1999). Chondroitin sulfate expression in subplate, which was scarce in Emx1/2 double mutants, has been shown to be associated with thalamic innervation (Bicknese et al., 1994). Axons from the thalamus grow directly into the superficially located subplate and then downward into layer IV in reeler mice (Molnar and Blakemore, 1995). Thalamic pathways were impaired in Tbr mutants, in which subplate cells, while present, underwent altered differentiation (Hevner et al., 2001). L1 staining, in addition to calretinin and GAP43 staining, indicated that the thalamocortical pathway was also impaired in Emx1/2 double mutant cortex, however, details await future study. Radial glial cells are also among the earliest cells detected. In Emx1/2 double mutants, these cells appeared to be generated normally, although the radial alignment of their fibers was greatly disturbed. CR cells have been suggested to maintain radial glial phenotype (Hunter-Schaedle, 1997; Soriano et al., 1997; Super et al., 2000) and in reeler mutants, the radial glial scaffold is less radially aligned and disappears prematurely. Thus, defects in the alignment of the radial fibers in Emx1/2 double mutants could be secondary to the loss of CR cells.

Intriguing questions arise as to whether and how the defects in tangential cell migration into neocortex and in cortical lamination are related. Emx1 is never expressed in the ganglionic eminence or in GABAergic neurons (Iwasato et al., 2000). Thus, the migration defects must be due to Emx1 and Emx2 functions in the cortex. Indeed, transplantation studies confirmed that migration defects do not reside in ganglionic eminence cells themselves. There is a possibility that not only interneurons, but also all neocortical reelin-positive CR cells are born in non-neocortical areas and migrate into the neocortical preplate or marginal zone (Lavdas et al., 1999). Emx1 and Emx2 might be essential in the cortex to promote the cell influx and the double mutant defects in cortical lamination might be secondary to the loss of CR, subplate and/or other cells from non-cortical areas caused by the defects in the cell influx. The laminar organization appears largely normal in Dlx1/2, Mashl and Ncx2.1 mutants (Anderson et al., 1997; Casarosa et al., 1999; Sussel et al., 1999), but their cortex does not lose all interneurons and has CR cells and subplate neurons. Alternatively and more probably, a laminar organization in the cortex or the production of CR cells and subplate neurons by Emx1 and Emx2 might be essential to the tangential movement of the interneurons. This cannot be a response to reelin, since interneurons migrate into the cortex in reeler mutants (T. M. and M. O., unpublished observation). A close association of tangentially oriented cells in the cortex and bundles of the corticofugal and thalamocortical fiber system has been reported (Metin and Godement, 1996), suggesting that migrating interneurons may use this fiber system as a scaffold for their migration into the neocortex. Recently TAG-1 present on corticofugal fibers, but not L1 present on thalamocortical fibers, was suggested to mediate the migration (Denaxa et al., 2001); both TAG-1 and L1-positive fibers were scarce in the Emx1/2 double mutant neocortex. Several motogenic and guidance factors for tangential migration have been described (Marin and Rubenstein, 2001) and alterations in the expression of these factors will be the subject of future investigation.

In contrast to Emx1/2, Pax6 is proposed to limit the invasion of the cortex by cells originating in the ganglionic eminence (Chapouton et al., 1999). In Pax6 mutants, the lateral ganglionic eminence (LGE) is re-specified to a more ventral fate, resulting in an expansion of the medial ganglionic eminence (MGE) and, the ventral and lateral cortex reallocates into derivatives of the ganglionic eminence, accompanied by severe disruption of the cortical/subcortical boundary (Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001). In the cortex of these Sey mutants CR cells are found in the marginal zone and basic laminar organization is established. However, proliferation of progenitor cells is accelerated with enlarged ventricular/subventricular zones, but a delineated intermediate zone and subplate are lacking (Stoykova et al., 2000). The cortical plate is thin without radial alignment of the cells, whereas the marginal zone is wide and hypercellular. Differentiation of cortical radial glia is impaired with defects in cellular morphology (Götz et al., 1998) and radial migration of late-born cortical neurons into the developing cortical plate is disturbed (Caric et al., 1997). During cortical arealization, Emx2 has been implicated in the development of the mediocaudal aspect, whereas Pax6 may be involved in laterorostral region formation (Bishop et al., 2000; Muzio et al., 2001). Thus, Pax6 and Emx1/2 appear to function differently in each step of cortical development; however, signals regulated by Emx1/2 and Pax6 need to be coordinated for normal development of cortex.

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