

Dentate gyrus formation requires *Emx2*

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SUMMARY

Emx 1 and *2* are the murine homologues of the *Drosophila empty spiracles* gene and based on their expression pattern may be involved in the regional specification of the mammalian forebrain. During early embryogenesis, *Emx2* is expressed in the presumptive cerebral cortex and olfactory bulbs and later, in the hippocampus proper and dentate gyrus. The latter are involved in memory processes. To understand the role of *Emx2* in vivo, we have mutated the gene in mice. Homozygous embryos die postnatally because of severe urogenital alterations. These mice present cerebral hemispheres with a reduced size and exhibit specific morphological alterations in allocortical

structures of the medial wall of the brain. The dentate gyrus is missing and the hippocampus proper is reduced. The medial limbic cortex is also severely shortened. The development of the dentate gyrus is affected at the onset of its formation with defects in the neuroepithelium from which it originates. These findings demonstrate that *Emx2* is required for the development of several forebrain structures.

Key words: *Emx2*, dentate gyrus, medial limbic cortex, cerebral hemispheres, cerebral cortex, telencephalon

INTRODUCTION

The mechanisms underlying the development of the forebrain with its complex organization is a central problem in neurobiology. The processes controlling the development of the rostral brain are mostly unknown. Different hypotheses have been formulated (Shatz, 1992) to explain the region-specific morphogenesis of the brain and in particular the laminar organization of the cerebral cortex and its segregation from the basal ganglia.

Recently, several families of genes with a restricted pattern of expression including the telencephalon have been isolated. Many of these genes code for transcription factors and may be involved in patterning the telencephalon into its two major subdivisions, the cerebral cortex and the basal ganglia. Among these genes are *Emx* (Simeone et al., 1992a), *Otx* (Simeone et al., 1993), *Pax* (Chalepakidis et al., 1993; Stoykova and Gruss, 1994; Stoykova et al., 1996), *Dlx* (Price et al., 1991; Porteus et al., 1991), *BF-1* (Tao and Lai, 1992), *Nkx-2.2* (Price et al., 1992), *Shh* (Echelard et al., 1993) and some POU-domain genes (Alvarez-Bolado et al., 1995).

Besides expression data, new information about the mechanisms controlling the forebrain patterning is coming from gene targeting experiments. Knock out experiments of the winged helix transcription factor *BF-1* have shown that this gene is essential for the development of the cerebral hemispheres, most probably by enhancing the growth of the telencephalon (Xuan et al., 1995). In *BF-1* homozygous mutant mice, the development of the ventral telencephalon was more severely affected than that of the dorsal part.

The nested expression patterns of four homeobox genes in the rostral brain, *Emx1*, *Emx2*, *Otx1* and *Otx2* (Simeone et al., 1992b), have raised the possibility that they might exert a control in forebrain development comparable to the role of Hox genes in the regionalization of hindbrain and spinal cord.

Emx2 is expressed in dorsal telencephalon fated to give rise to the cerebral cortex (Simeone et al., 1992a). Based on the expression pattern, *Emx2* could be involved in patterning this structure. Furthermore, recent data have localised *Emx2* expression restricted to cells of the germinal ventricular zone of the cerebral cortex (Gulisano et al., 1996), which favours the hypothesis that *Emx2* might be a factor regulating neuronal proliferation in the dorsal telencephalon.

In order to analyse its function in vivo, we have mutated the *Emx2* gene by homologous recombination and produced mutant mice carrying a null allele.

MATERIALS AND METHODS

Construction of the targeting vector and screening strategy

A classical replacement vector was designed (Fig. 1B; Capecchi, 1989). After deleting a *PvuII-PvuII* 250 bp fragment in the 5' part of the homeobox, the neomycin resistance (NEO) gene, driven by the PGK promoter and ending with the polyA, was inserted. The PGKNeo plasmid was kindly provided by P. Soriano (Soriano et al., 1991). The thymidine kinase (TK) gene was inserted in one extremity of the construct. 25 µg of linearized targeting construct were added to 10⁷ R1-ES cells in a volume of 800 µl of PBS and electroporated with a

Bio-Rad Gene Pulser. R1-ES cells were kindly provided by A. Nagy (Nagy et al., 1993a). Drug selection (G418: 300 µg/ml + gancyclovir 2 µM) was started 24 hours later, and after 8 days resistant colonies were picked and grown for DNA analysis and for storage of the different clones. ES cells were cultured as described by Robertson (1987). DNA from 66 independent resistant clones were digested with *EcoRI* and screened by genomic Southern blot, using the external probe PR20 (Fig. 1D). The size of the bands for the expected homologous recombination event are 10.1 kb for the wild-type allele and 6.4 kb for the mutated allele. Five recombinant clones showed the expected bands and clone number 19 was used to generate chimeras.

Generation of mutant mice

Clone number 19 was used to generate chimeras by morulae aggregation and germ line transmission of the mutated allele (Nagy and Rossant, 1993b). Chimeras were mated with C57BL/6 mice to obtain germline transmission. Probe PR20 was used to genotype tail DNA from F₁ and F₂ generations after digestion with *EcoRI* (Fig. 1E).

In situ hybridization and histology

Brains or heads from 14.5 and 18.5 days p.c. embryos were dissected in PBS, and photographed or fixed in Bouin's solution for 24 hours. After dehydration and inclusion in paraffin, 8 to 12 µm horizontal and frontal head sections were obtained with a rotary microtome. The sections were stained with cresyl violet or silver-stained using a modification of the Bodian's method (Rager et al., 1979). In situ hybridization on sections was performed as previously described (Stoykova

and Gruss, 1994). Sections were hybridized using the ³⁵S-labelled *Emx2* probe previously reported (Simeone et al., 1992a).

RESULTS

Emx2^{-/-} mutant mice die postnatally

While heterozygous mice were healthy and fertile, the homozygous mice died within a few hours after birth. Anatomical inspection of *Emx2*^{-/-} newborns revealed that the kidneys and other parts of the urogenital system were missing while the suprarenal glands appeared unaffected (data not shown). These observations concur with the previously reported expression of *Emx2* in the primordia of the urogenital system during embryogenesis (Simeone et al., 1992a) and indicate a requirement of *Emx2* in the development of the urogenital system.

Analysis of the brains in 18.5 days p.c. *Emx2*^{-/-} embryos

Macroscopical inspection of 18.5 days p.c. *Emx2*^{-/-} brains revealed a reduction in the size of the cerebral hemispheres and the olfactory bulbs (Fig. 2). Light microscopy of cresyl violet-stained sections from these brains shows major alterations at the level of allocortical structures of the medial wall of the brain (Figs 3, 4). These cortical areas are termed allocortex. In contrast to the six layer lamination of the isocortex the allocortex has a mostly 3-layered structure. The hippocampal formation shows the most interesting morphological alterations. This complex structure is formed by the hippocampus proper or Ammon's horn, the dentate gyrus and the subiculum. In *Emx2*^{-/-} mutant mice, the dentate gyrus is completely missing, while Ammon's horn is reduced in size (Fig. 3B,D). The fimbria and the fornix, the main efferent systems of the hippocampus, are severely reduced (Fig. 3B,D). The commissural component of the fornix (hippocampal commissure) is absent or greatly reduced and a grey matter formation, which probably corresponds to the nuclei of the posterior division of septal region, directly contacts corpus callosum (Figs 3B, 4D). An abnormally large communication between the two lateral ventricles and the third ventricle along with a dilatation of the choroid plexus of the third ventricle is also evident at this level (Fig. 3B).

The medial limbic cortex is normally represented by the

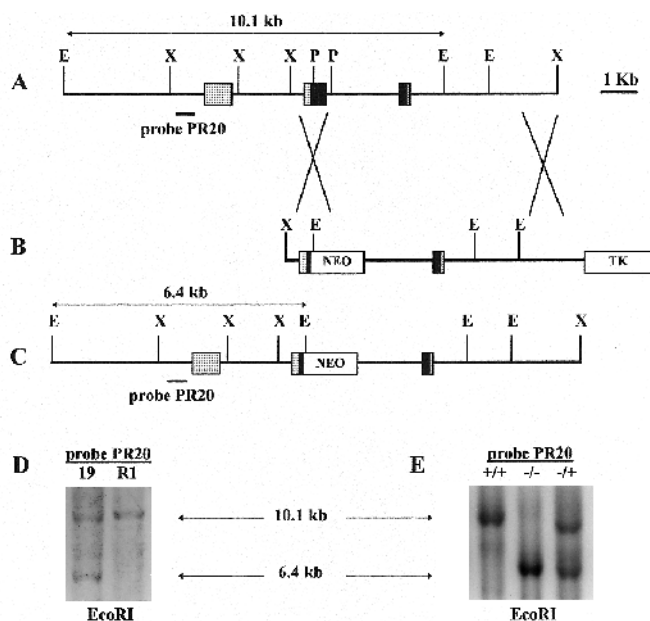


Fig. 1. Production of *Emx2* mutant mice. (A) *Emx2* genomic map. Exons are represented by light boxes while the 5' and the 3' components of the homeobox are represented by black boxes. (B) The targeting construct is a replacement vector with the neomycin resistance gene (NEO) replacing the second and part of the third helix of the homeobox. The thymidine kinase gene (TK) is inserted at one extremity of the construct. (C) The mutated allele generated after the expected recombination event. E, *EcoRI*; P, *PvuII* (more *PvuII* sites are not shown); X, *XhoI*. (D,E) Southern blot analysis of R1-ES clone DNA (D), and of F₂ litter DNA (E), genotyped with the external probe PR20 after *EcoRI* digestion. In D the wild-type band (size: 10.1 kb) in the parental R1-ES cell line and the wild-type with the mutated allele (size: 6.4 kb) bands in clone number 19 are shown. (E) The results obtained with an F₂ litter.

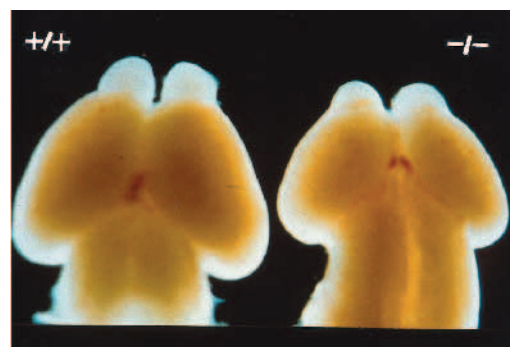


Fig. 2. Cerebral hemispheres reduction in *Emx2*^{-/-} embryos. A case of severe reduction of the size of cerebral hemispheres and olfactory bulbs at 18.5 days p.c. *Emx2*^{-/-} mutant, compared with a control embryo (+/+).

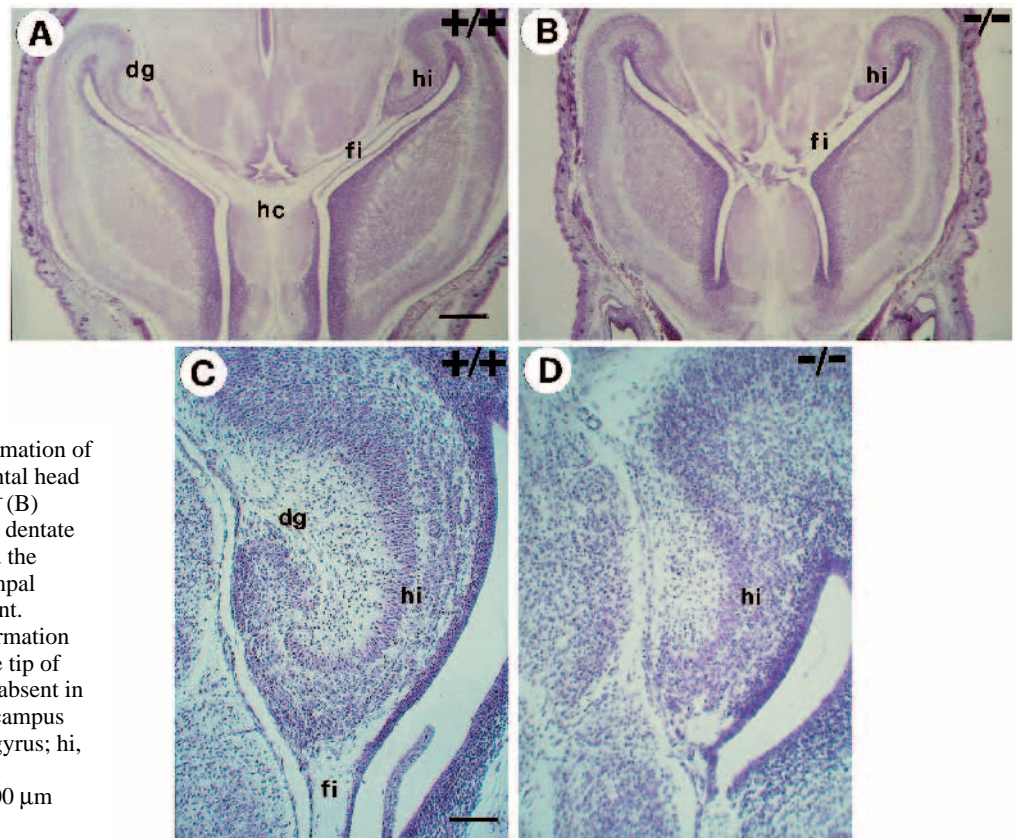


Fig. 3. Defects in the hippocampal formation of 18.5 days p.c. *Emx2*^{-/-} brains. Horizontal head sections from control (A) and *Emx2*^{-/-} (B) embryos. Note in B the absence of the dentate gyrus, the reduction of the fimbria and the alterations at the level of the hippocampal commissure, which is reduced or absent. (C) Details of normal hippocampal formation with the dentate gyrus surrounding the tip of Ammon's Horn. (D) Dentate gyrus is absent in *Emx2*^{-/-} embryos; note that the hippocampus proper is reduced in size. dg, dentate gyrus; hi, hippocampus proper; hc, hippocampal commissure; fi, fimbria. Scale bars, 500 μ m (A,B); 95 μ m (C,D).

cingulate, infralimbic and retrosplenial areas (Bayer and Altman, 1991). In *Emx2*^{-/-} mutant mice the medial limbic cortex is particularly reduced, as is the corresponding wall of the lateral ventricle which is clearly shortened (Fig. 4B). The smaller cerebral hemispheres are connected by a reduced corpus callosum and by a thinner and distorted anterior commissure.

***Emx2* is required for normal dentate gyrus development**

In order to better understand the role of the *Emx2* gene in

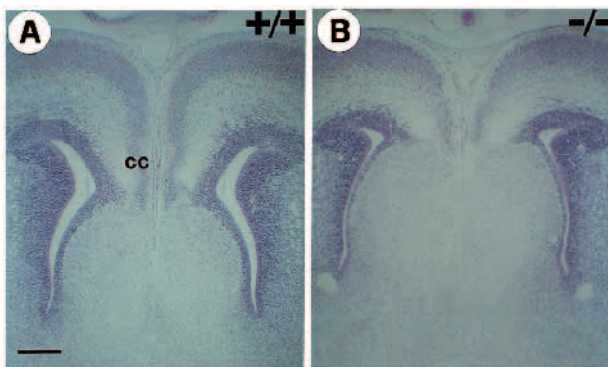


Fig. 4. Medial limbic cortex defects. Frontal head sections at the level of the cingulate cortex (A,B) from 18.5 days p.c. embryos. B shows a reduction of the cingulate cortex as of the corresponding wall of the lateral ventricle in *Emx2*^{-/-} mutants, compared to its normal size in control mice (A). cc, cingulate cortex. Scale bar, 270 μ m.

dentate gyrus development, we have analysed silver-stained sections from the hippocampal region and performed in situ hybridization at this level in 14.5 days p.c. embryos, a period in which the anlage of the dentate gyrus should be evident (Stanfield and Cowan, 1988). At 14.5 days p.c., in control embryos, a strong hybridization signal in the primary neuroepithelium and in the dentate gyrus anlage is evident (Fig. 5A). The strong hybridization signal persists in the dentate gyrus at birth (Fig. 5D). In *Emx2*^{-/-} embryos at 14.5 days p.c., the ventricular zone corresponding to the site of origin of the hippocampus and dentate gyrus is particularly reduced while the secondary proliferative zone or dentate gyrus anlage is absent as shown by the histological analysis (Fig. 5C).

DISCUSSION

The dentate gyrus belongs to the hippocampal formation and is functionally a part of the limbic system. The hippocampus and the dentate gyrus are required for declarative memory in humans, and patients with bilateral lesions at this level are not able to consolidate the representation of facts and events into long term memory (Bliss and Lomo, 1973; Victor and Agmanolis, 1990; Squire et al., 1991). Rodents with specific bilateral lesions in the dentate gyrus and/or hippocampus show impairment in spatial (McNaughton et al., 1989; Emerich and Walsh, 1990; Vaher et al., 1994;) and non-spatial (Bunsey and Eichenbaum, 1996) memory tasks. Moreover loss of dentate hilar neurons have been associated with the pathogenesis of temporal lobe epilepsy as a result of a reduced inhibitory effect on dentate granule neurons (Lowenstein et al., 1992; Sloviter,

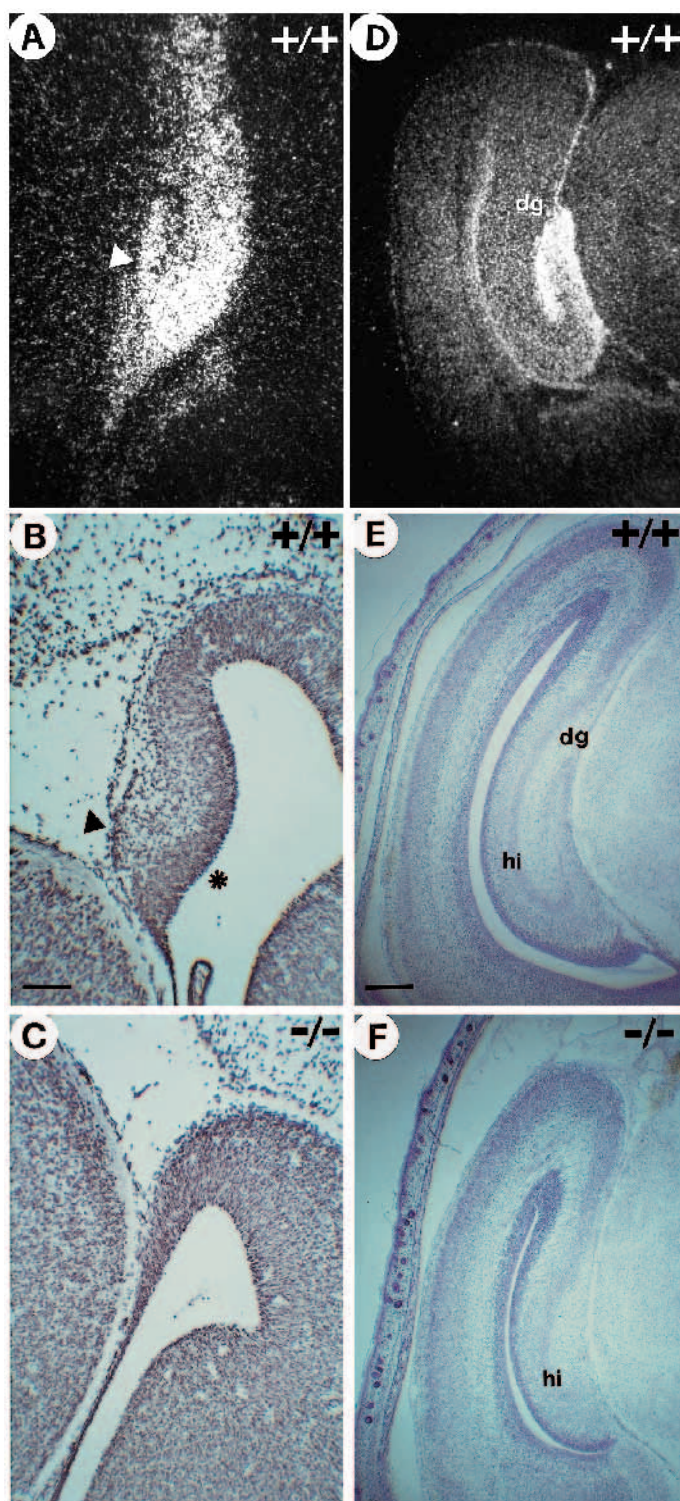


Fig. 5. Dentate gyrus developmental alterations. (A) In situ hybridization analysis of *Emx2*^{+/+} brains from 14.5 days p.c. embryos. Section at the level of the dentate gyrus. A strong hybridization signal is evident in the dentate gyrus primary proliferative neuroepithelium and at the position of the secondary proliferative zone or anlage of the dentate gyrus, marked by the arrowhead. (B,C) Silver-stained horizontal head sections from *Emx2*^{+/+} (B) and *Emx2*^{-/-} (C) 14.5 days p.c. embryos. (B) Arrowhead marks the row of neuroblasts which migrate and form the dentate gyrus anlage. Asterisk marks the area of the primary neuroepithelium of the hippocampus proper and dentate gyrus. (C) In *Emx2*^{-/-} embryos the primary neuroepithelium from which dentate gyrus and hippocampus originate is particularly reduced while the cluster of neuroblasts forming the dentate gyrus anlage is absent. (D) In situ hybridization of frontal head sections from 18.5 days p.c. control embryos showing a strong hybridization signal at the level of the dentate gyrus. In E and F, the corresponding cresyl violet-stained frontal head sections show the normal morphology of the dentate gyrus in *Emx2*^{+/+} (E) and its absence in *Emx2*^{-/-} embryos (F). dg, dentate gyrus; hi, hippocampus proper. Scale bars: 95 μ m (A-C); 270 μ m (D-F).

organization, the dentate gyrus and the hippocampus proper are two brain formations particularly well studied (Stanfield and Cowan, 1979a; Cowan et al., 1980; Stanfield and Cowan, 1988; Reznikov, 1991). The dentate gyrus is formed by a simple trilaminar cortex. The principal cellular layer is the granular layer, which consists of the bodies of the predominant class of neurons, the dentate granule cells. The dendrites of these neurons ascend into the outermost synaptic layer, or molecular layer where the afferent fibers make their synaptic contacts with granule cells. The innermost stratum is the polymorphic layer which forms part of the dentate hilar region. At 18.5 days p.c. the dentate gyrus of wild-type embryos is easily detectable in horizontal brain sections even though it does not as yet present the typical U shape surrounding the tip of Ammon's horn (or CA3) (Fig. 3A,C). The infrapyramidal blade (the medial limb of the U) becomes visible as a separate entity from CA3 region only some days later. In corresponding *Emx2*^{-/-} embryos the dentate gyrus is absent and a reduced Ammon's horn is visible (Fig. 3B,D). Moreover the fimbria and the fornix, which constitute the main efferent systems of the hippocampus, are severely reduced and the hippocampal commissure, or commissural component of the fornix, is absent or greatly reduced (Figs 3, 4). This may account for the abnormal conformation observed at this level where a grey matter formation (probably corresponding to the nuclei of the septal region) is dislocated and directly contacts corpus callosum (Fig. 4D). This results in an enlargement of the septal region in the mutant as compared with the wild-type brain (compare Fig. 3A,B and Fig. 4A,B).

Dentate gyrus development (Cowan et al., 1980; Stanfield and Cowan, 1979b; Stanfield and Cowan, 1988; Altman and Bayer, 1990a,b) is affected at the onset of its formation in *Emx2*^{-/-} embryos. Dentate gyrus precursor cells originate in a restricted area of the ventricular zone of the medial wall of the cerebral vesicles, next to the site of origin of the hippocampus proper (Fig. 5B, asterisk). These neuroblasts leave the primary dentate neuroepithelium and form a second proliferative area or dentate gyrus anlage, under the pia (Fig. 5B, arrowhead) (Stanfield and Cowan, 1988; Altman and Bayer, 1990a). Finally, granule cell precursors migrate radially and aggregate

1994). Unfortunately, *Emx2*^{-/-} mutant mice die a few hours after birth, most likely due to the absence of the urogenital system. It is for this reason that the expected loss of declarative memory could not be determined yet. However, experiments are under way to partially rescue the *Emx-2* kidney phenotype which then will enable us to study this aspect in detail.

For its important functional role and for its relatively simple

to form the granular layer of the dentate gyrus while continuing to proliferate in the deep aspect of this layer, possibly for the entire life of rodents (Stanfield and Cowan, 1988). Most granule cells are formed postnatally in the first 1-3 weeks. The protracted span of neurogenesis may explain, at least in part, the extraordinary capacity for extensive postlesion synaptic reorganization of this structure (Stanfield and Cowan, 1988). The dynamics of granule cell population of the dentate gyrus and its regeneration properties stress the importance of possible factors which might regulate these processes. *Emx2* is expressed in these cells during all these developmental phases as documented by in situ hybridization studies (Fig. 5A,D). In control embryos, a strong hybridization signal in the primary neuroepithelium, in the dentate gyrus anlage and in the dentate gyrus at birth is evident. In *Emx2*^{-/-} embryos at 14.5 days p.c., the part of the ventricular zone which corresponds to the site of origin of the hippocampus and dentate gyrus is extremely reduced while the secondary proliferative zone or dentate gyrus anlage is absent (Fig. 5C). These data demonstrate the absolute requirement of *Emx2* for dentate gyrus development.

Recent studies demonstrated that *Pax6* is required for the establishment of several expression boundaries that specify longitudinal domains within the developing forebrain (Stoykova et al., 1996). In this study we demonstrated that during development there is a progressive spreading of *Dlx1*-positive cells from the lateral ganglionic eminence into the cortex of the *Pax6* mutant brain which may contribute to the establishment of the cortical phenotype in the *Pax6*-*Small eye* mutant. In this context it is noteworthy (Figs 3A,B, 4A,B) that the septal region was enlarged in *Emx2*^{-/-} mutant mice while the cingulate cortex showed a prominent reduction. In analogy to the *Pax6*^{-/-} mutant mice it is conceivable that due to the loss of *Emx2* function, cells from septal nuclei might expand into a heterotopic location. We will attempt to study this exciting possibility in greater detail with the help of molecular markers.

Besides these specific anatomical alterations, *Emx2* inactivation has a more general effect on the cerebral cortex which displays a reduced extension. The cerebral hemispheres are connected by a reduced corpus callosum and by a thinner anterior commissure. Recent data show that *Emx2* expression is restricted to the proliferating neuroblasts of the ventricular zone of the developing cerebral cortex and is down-regulated in postmitotic cortical neurons (Gulisano et al, 1996). Therefore, it is possible that *Emx2* plays a role in controlling some parameters of cortical neuron proliferation. In *Emx2*^{-/-} embryos a reduced population of founder cells (Rakic, 1995; Caviness et al, 1995) might be responsible for the reduced cortical growth without affecting cortical lamination, which itself does not present evident morphological alterations in these mutants. The medial limbic cortex is particularly reduced, as is the corresponding wall of the lateral ventricle which is manifestly shortened (Fig. 4B). These severe defects may be due to a disturbed neuroblasts proliferation or to a more specific effect exerted by *Emx2* in patterning the medial limbic cortex. Experiments using BrdU labelling will soon allow us to precisely determine the proliferative activities in mutant mice. Recently, point mutations in the *Emx2* locus have been found in some heterozygous patients suffering from schizencephaly (Brunelli et al., 1996), a rare congenital disorder characterized by the absence of portions of the cerebral hemispheres. However, the cortical anatomy of *Emx2*^{+/-} mice did

not differ from that of wild-type mice. This discrepancy may be explained in a number of ways. Firstly, the phenotype may be related to the genetic background and heterozygous abnormalities may become apparent in a different mouse strain. Secondly, it may be due to an intrinsic difference between humans and mice in the geometry and pattern of growth of the cerebral hemispheres. Finally, it cannot as yet be excluded that the mutations observed in humans may involve gain or change of function or indeed that local homozygosity in the affected regions of the patients studied has manifested itself as the phenotype.

In summary, the data presented here may suggest a dual function for the *Emx2* gene. A more general effect on the patterning of forebrain regions and possibly a more specific role in the proliferation and/or specification of dentate gyrus precursor cells in the archicortex.

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