Emx1 and Emx2 functions in development of dorsal telencephalon

Michio Yoshida¹, Yoko Suda¹², Isao Matsuo¹, Norimasa Miyamoto¹³, Naoki Takeda¹, Shigeru Kuratani¹ and Shinichi Aizawa¹,*

¹Department of Morphogenesis, Institute of Molecular Embryology and Genetics (IMEG), Kumamoto University School of Medicine, 2-2-1 Honjo, Kumamoto, Kumamoto 860
²Japan Immunoresearch Laboratories Co. Ltd, 351-1 Nishiyokote-cho, Takasaki, Gunma 370
³Department of Drug Discovery, Tsukuba Research Laboratories, Eisai Co. Ltd, 5-1-3 Tokodai, Tsukuba, Ibaraki 300-26, Japan

*Author for correspondence (e-mail: saizawa@gpo.kumamoto-u.ac.jp)

SUMMARY

The genes Emx1 and Emx2 are mouse cognates of a Drosophila head gap gene, empty spiracles, and their expression patterns have suggested their involvement in regional patterning of the forebrain. To define their functions we introduced mutations into these loci. The newborn Emx2 mutants displayed defects in archipallium structures that are believed to play essential roles in learning, memory and behavior: the dentate gyrus was missing, and the hippocampus and medial limbic cortex were greatly reduced in size. In contrast, defects were subtle in adult Emx1 mutant brain. In the early developing Emx2 mutant forebrain, the evagination of cerebral hemispheres was reduced and the root between the hemispheres was expanded, suggesting the lateral shift of its boundary.

Defects were not apparent, however, in the region where Emx1 expression overlaps that of Emx2, nor was any defect found in the early embryonic forebrain caused by mutation of the Emx1 gene, of which expression principally occurs within the Emx2-positive region. Emx2 most likely delineates the palliochoroidal boundary in the absence of Emx1 expression during early dorsal forebrain patterning. In the more lateral region of telencephalon, Emx2-deficiency may be compensated for by Emx1 and vice versa. Phenotypes of newborn brains also suggest that these genes function in neurogenesis corresponding to their later expressions.

Key words: Emx1, Emx2, homeobox, dorsal telencephalon, regionalization, limbic system, mutant mice

INTRODUCTION

Hippocampus and dentate gyrus are major archipallium structures that play essential roles in learning, memory and behavior (Jarrard, 1993). During embryogenesis, they originate from the telencephalic connection to the non-evaginated roof; this region develops into a series of structures collectively called the limbic system. An increasing number of studies are focusing on the molecular basis of the higher brain functions (Abeliovich et al., 1993a,b; Grant et al., 1992; Sakimura et al., 1995), but no clue has yet been obtained as to the molecular plan behind the development of these structures.

It is generally believed that the vertebrate neural tube is segmented along the neuraxis into developmental compartments called neuromeres. Each neuromere is likely to undergo its specific developmental pathway under the control of coordinated genetic codes: the Hox code is thought to play an essential role in specification of the hindbrain and trunk regions. In the hindbrain, nested expressions of Hox genes are limited at the levels of the rhombomere boundaries, thus providing compartment-based molecular cascades for each segment (reviewed by McGinnis and Krumlauf, 1992; Marshall et al., 1992). The more rostral portion of the brain is also believed to be composed of developmental compartments, though not showing simple constrictions; there are compartments divided by cell lineage restriction where no cells migrate into neighboring segments once the boundary has been established (Figdor and Stern, 1993). Unlike the rhombomeres in the hindbrain, the forebrain neuromeres tend to develop not only as developmental units but also as anatomical and functional subdomains of the brain; the nerve tracts and commissures developing at the boundaries are maintained in adulthood as is the boundary between the dorsal and ventral thalamus. Details of how the forebrain is segmented or compartmented, however, is still an issue of dispute. Figdor and Stern (1993) assumed that the telencephalon is the most anteriorly located neuromere, while Rubenstein and his colleagues (Bulfone, 1993; Puelles and Rubenstein, 1993) have proposed that the telencephalon is the lateral outgrowth of the alar plate of the most rostral neuromere called the secondary prosencephalon (SP); SP and diencephalon are subdivided from the primary prosencephalon. The controversy is inherently associated with the anterior end of the neuraxis. Expression patterns of several regulatory genes seem to favor the latter view.

No Hox genes are expressed in rostral brain. In Drosophila, HOM-C genes are not expressed in the head either, and the Drosophila head is thought to develop under gap genes unique to this region: orthodenticle (otd), empty spiracles (ems) and buttonhead (btd) (Finkelstein et al., 1990; Dalton et al., 1989; Walldorf and Gehring, 1992; Cohen and Jürgens, 1990). Vertebrate cognates of Drosophila otd and ems have been isolated such as Otx1, Otx2, Emx1, Emx2, and they show a nested
pattern of expressions in the forebrain and midbrain at the stage when patterning is established in these regions, the pharyngula stage corresponding to 8.0-11.5 dpc (days post coitus) in mouse embryos (Simeone et al., 1992a,b, 1993). Defects resulting from an Otx2 heterozygous mutation were found in most anterior and most posterior regions of Otx2 expression where Otx1 is not expressed, demonstrating its essential role in patterning of the forebrain and midbrain (Matsuo et al., 1995). Expressions of Emx genes at the pharyngula stage are suggestive of their roles in delineation of the telencephalic evagination (Bonaccielli et al., 1993; Morita et al., 1995). Later Emx expressions suggest their parts in neurogenesis of cerebral cortex and the hippocampal region. There is a shift in Emx1 and Emx2 expressions, the latter being expressed earlier and declining earlier. For example, Emx1 expression is more pronounced in hippocampus at 16.5 dpc. Emx2 is also expressed in the olfactory system; its Drosophila cognate, ems, regulates development of olfactory sense organs. To define their functions, mutations were introduced into the Emx1 and Emx2 genes by homologous recombination in embryonic stem (ES) cells. In this study we focused on Emx functions at the pharyngula stage in forebrain regional patterning. The results have provided clues to the development of the limbic system and the regionalization of the dorsal telencephalon.

MATERIALS AND METHODS

The generation of Emx1 and Emx2 mutant mice
In constructing the targeting vectors, the neo cassette was inserted into the Apral site (A) of the Emx1 gene and the Msc1 site (M) of the Emx2 gene in the second exons. Lengths of the homologous regions were 6.7 kb and 0.9 kb in the Emx1 targeting vector and 9.0 kb and 0.7 kb in the Emx2 targeting vector at the 5’ and 3’ sides of the neo cassette, respectively. These targeting vectors were linearized with NotI and introduced into TT2 ES cells derived from an F1 embryo between C57BL/6 and CBA mice (Charles River Inc. Japan) by electroporation as previously described (Yagi et al., 1993a). Homologous recombinant ES clones were initially identified by PCR and finally confirmed by Southern blot analyses. Polymerase chain reaction (PCR) primers used were 5’-CTGACAGCTCCCTAGACACTTT-GG-3’ and 5’-GGTGGTATATCCTGCTCTAAACGTAATC-3’ as the 3’ side primers (antisense strand oligonucleotides) in the Emx1 and Emx2 genes, respectively, and 5’-GCCGGATGGCAATATCATG-GGGAAAT-3’ as the 5’ side primer (sense strand oligonucleotide) in the neo gene. Genomic DNAs extracted from ES cells were digested with several restriction enzymes, and Southern blot analyses were performed as described (Yagi et al., 1993b; Matsuo et al., 1995).

The XbaI-EcoRI-digested 700 bp fragment of Emx1, SacI-3’-digested 500 bp fragment of Emx2 and PstI-digested 600 bp fragment of the neo gene (indicated in Fig. 1A) were used as probes of those Southern blot analyses. They were then injected into ICR 8 cell-stage embryos to generate chimeric mice, and male chimeras were mated with C57BL/6 females to generate heterozygous mutant mice. The mice were normally obtained from two independent mutant ES clones in each disruption and intercrossed to generate homozygous mutants; no difference was found between mouse strains derived from the two clones in each disruption, and no specification is made as to which strain was used in each experiment. The homozygous mutants developed beyond the pharyngula stage when the expression of these genes was established (Simeone et al., 1992a) (Fig. 1B,C), and RT-PCR analyses indicated the absence of normal transcripts beyond the pharyngula stage when the expression of these genes was established (Simeone et al., 1992a) (Fig. 1B,C), and RT-PCR analyses indicated the absence of normal transcripts in each mutation (Fig. 1D). The disruption, however, might be ruled out by the present study.

RT-PCR analysis
RT-PCR analysis was performed with total RNAs isolated from 13.5 dpc mutant embryos and reverse-transcribed with oligo(dT)17 (Ilic et al., 1995). The sequences of the primers p1, p2 for Emx2 and p3, p4 for the Emx1 indicated in Fig. 1A were:

5’-CCGAGATTCTTGTGACACAAGC-3’, 5’-GCCGCTTGGTGACAAACCTGACC-3’, 5’-AGCGAGCGCTCCACAGGAGGCCTGC-3’ and 5’-TGCGTCTCAGAGAGCCGGCTGTC-3’, respectively. The primers for HPRT expressions were as described by Ilic et al. (1995).

Histological analysis
Embryos were fixed in Bouin’s or Carnoy fixative solutions, embedded in paraffin, sectioned at 8 μm thickness and stained with 0.1% cresyl violet (sigma) solution for Nissl staining or with Haematoxylin-Eosin staining solution.

In situ hybridization
In situ hybridization analyses were carried out as described by Wilkinson (1993). The probes used were as described for Dlx-1 (Bulfone et al., 1993), BF-1 (Tao and Lai, 1992), Wnt-3a (Roelink and Nusse, 1991), noggin (Shimamura et al., 1995) and Wnt1 (Shimamura et al., 1994). For Emx1 and Emx2, the 500 bp cDNA fragment obtained by PvuII digestion and the 300 bp cDNA fragment obtained by PCR that covers the stop codon and the HindIII site in the 3’ untranslated region (Simeone et al., 1992a) were used, respectively.

RESULTS

Generation of mutant mice
Targeting vectors were constructed by inserting the neomycin phosphotransferase (neo) gene that bears no polyadenylation signal in front of homeobox domains of the Emx1 and Emx2 genes, respectively, and using diphteria toxin A fragment (DT-A) for negative selection of homologous recombinants (Fig. 1A). The vectors were introduced into the TT2 embryonic stem cells, and six homologous recombinant clones were assessed among 1176 and 3035 G418 resistant clones for Emx1 and Emx2 genes, respectively, and using diphtheria toxin A fragment (DT-A) for negative selection of homologous recombinants (Fig. 1A). The vectors were introduced into the TT2 embryonic stem cells, and six homologous recombinant clones were assessed among 1176 and 3035 G418 resistant clones for Emx1 and Emx2, respectively, by PCR and confirmed by Southern blot analyses. They were then injected into ICR 8 cell-stage embryos to generate chimeric mice, and male chimeras were mated with C57BL/6 females to generate heterozygous mutant mice. The mice were normally obtained from two independent mutant ES clones in each disruption and intercrossed to generate homozygous mutants; no difference was found between mouse strains derived from the two clones in each disruption, and no specification is made as to which strain was used in each experiment. The homozygous mutants developed beyond the pharyngula stage when the expression of these genes was established (Simeone et al., 1992a) (Fig. 1B,C), and RT-PCR analyses indicated the absence of normal transcripts in each mutation (Fig. 1D). The disruption, however, might have yielded the truncated products 5’ upstream of the neo integration site, and their effects on mutant phenotypes cannot be ruled out by the present study.

Forebrain phenotypes in newborn Emx mutants
Emx1 homozygous mutant (Emx1HHDH) mice were normally born in a Mendelian ratio and could grow to adults although about half died neonatally for an unknown reason. In brains of Emx1HHDH mice, defects were subtle and restricted to the
forebrain. Indusium griseum and taenia tecta were always missing (Fig. 2C,D). Disorganized fasciculation in the corpus callosum and anterior commissure were coincidentally evident in a significant portion of the Emx1HD/HD mutants (Fig. 2A-F). In the severest case, the callosal commissure axons were stacked and failed to cross the midline into the opposite hemisphere (Fig. 2A,B). The cerebral cortical layer was often poorly differentiated; the cortical plate and white matter were thin, and the cortical subplate was hardly visible (Fig. 2I,J). Hippocampus was sometimes smaller (Fig. 2G,H), but was always present. No defects were found in the olfactory bulb or in the hippocampal commissure. These defects are most likely to correspond to Emx1 expressions at stages later than pharyngula as discussed below. For example, Emx1, but not Emx2, is expressed at 16.5 dpc in mesocortical subplate and a portion of cells that form the glial sling (Fig. 2K,L) (Hankin and Silver, 1988). They are considered essential for the guidance of callosal fibers.

Homozygous Emx2 mutant mice (Emx2HD/HD) were also born in Mendelian ratio, but they died within a few hours having no kidneys or reproductive organs, the details of which will be reported elsewhere. External morphology of the newborn Emx2HD/HD brain was obviously abnormal; the cerebral hemisphere was significantly reduced in size, and the olfactory bulb was small (Fig. 3A,B). Histologically, anomalies in the cerebral hemisphere were restricted to its dorsal structures (Fig. 3C-J), and the medial limbic cortex and hippocampal region were particularly affected. Dentate gyrus was always completely absent (Fig. 3F). The hippocampus was usually greatly reduced in size and infrequently even missing. When it developed, the S-shaped configuration of the pyramidal layer was apparently normal. Development of fascicles of the fimbria and fornix, the major efferent fibers from the hippocampus, was poor (Fig. 3D), and the hippocampal commissure was barely visible (data not shown). It is of note that the junctional region between the cerebral hemisphere and thalamus was malformed. Choroid plexus in the third ventricle was always hyperplastic, although that in the lateral ventricle was frequently reduced. In the diencephalon, the dorsal roof of the third ventricle was expanded (Fig. 3I),

**Fig. 1.** The generation of Emx1 and Emx2 mutant mice. (A) Targeting vectors for Emx1 and Emx2 mutations. Exons are indicated by rectangles; filled boxes indicate homeodomain. The predicted sizes of normal and targeted alleles and the probes used in Southern analyses (B,C) are shown. Arrowheads indicate the positions of primers in RT-PCR analyses, respectively. (B,C) Examples of Southern blot analyses by EcoRI digestion of 13.5 dpc. Emx1 and Emx2 mutant embryos, respectively. The analyses were also performed with several restriction enzymes and probes, and the results were all consistent with the homologous nature of the recombination in the absence of any random integrations. (D) RT-PCR analyses of Emx1 and Emx2 disruptions in 13.5 dpc embryos. The 183 bp and 247 bp bands represent Emx1 and Emx2 transcripts, respectively. Primers used are indicated in A, and amplifications of HPRT (hypoxanthine phosphoribosyl transferase) gene expression were used as control. +/- heterozygous and HD/HD homozygous mutants. A, ApaI; B, BamHI; E, EcoRI; M, MscI; N, NotI; S, SalI.
and the epiphysis was somewhat atypical, but defects were not apparent in other diencephalic structures such as epithalmus or habenula.

In the cortex, the medial portion was not formed (Fig. 3I), and lateral cortical layers were poor; both the cortical plate and white matter were thin, and the subplate was hardly visible (Fig. 3J). Anteriorly, the posterior part of the anterior commissure was absent (data not shown). The corpus callosum had a decreased number of fibers but there were always fibers crossing to the other hemisphere. These commissure defects were apparently different from those in the Emx1 mutant; in the latter the number of fibers was unchanged but their guidance was aberrant. No obvious defect was found in the ventrolateral structures of the telencephalon such as corpus striatum (Fig. 3I). In the olfactory system, the olfactory nerve was found to be normal and the lateral olfactory tract was present in the Emx2 mutant. However, there was no connection between the nerve and bulb, suggesting that most of the olfactory axons failed to project to the olfactory bulb (Fig. 3K,L). Within the bulb, the mitral cell layer was disorganized.

**Early embryonic defects in Emx mutant forebrains**

To define the functions of Emx genes in regional patterning of forebrain, the mutant phenotype was traced back to the pharyngeal stage. The Emx1HD/HD embryonic brains were indistinguishable from wild-type brain at this stage (Fig. 4G-I). In the Emx2HD/HD brains, however, the mutant phenotype was apparent morphologically by 11.5 dpc, i.e., the anlagen of the cerebral hemispheres were significantly smaller than normal, and the non-evaginated roof between the two hemispheres (hereafter referred to simply as the roof) was exposed in the dorsal view (Fig. 4A,D). In the sagittal sections, anomalies were seen in the dorsal portion of the forebrain, while the ventral structures such as ganglionic eminence and mammillary region did not show any defects (Fig. 4B,E). The defects in the dorsal structures were most apparent in the region corresponding to the pallio-choroidal boundary. In the caudal and medial portion of the telencephalon at this stage, normally there is a protrusion called Ammon’s horn which develops later into the hippocampus. The neuroepithelium between the diencephalon and the protrusion is characteristically thin compared...
with the rest of the brain and develops the choroid plexus into the lateral ventricle (Fig. 4B). In the Emx2 mutant brain, Ammon’s horn was present but its caudal and medial portion was strikingly shortened (Fig. 4E). In contrast, the roof was expanded and the neuroepithelium was thickened and irregular (Fig. 4F). Later, at 13.5 dpc, the shortening of the medial region of the pallium juxtaposed to the choroid plexus was more obvious, and development of the protrusion of Ammon’s horn into the lateral ventricle was poor (Fig. 4J,K). The choroid plexus did not extend into the lateral ventricle but was stacked at the dorsal midline (data not shown). Earlier, anomalies in dorsal forebrain were already found at 9.0 dpc like the reduction of the telencephalic evagination (Fig. 4L,M).

In these stages morphological landmarks are poor, and we resorted to in situ hybridization analyses with several molecular markers to define the affected region in the Emx2 forebrain. As expected from the morphological observations, the region that expresses Dlx-1, a mouse homologue of Drosophila distal-less homeobox gene (Bulfone et al., 1993), was normally present in the mutant ventral telencephalon (Fig. 5A,B). In the wild-type brain, BF-1, a fork-head family gene (Tao and Lai, 1992) was expressed in almost the entire region of the telencephalon except the medial regions adjacent to the roof and diencephalon (Fig. 5C,E). In the Emx2 mutant brain, the BF-1-positive region was normally present, but the negative region was greatly reduced (Fig. 5D,F); the border of the expression was close to the morphological sulci that delineated the pallium from the roof in the mutant (Fig. 5D). The Emx1 expression in the normal brain extended beyond the BF-1-positive margin to a part of the medial region of the telencephalon, but did not extend to the sulcus or the roof (Fig. 5D). The Emx1 expression in the normal brain extended beyond the BF-1-positive margin to a part of the medial region of the telencephalon, but did not extend to the sulcus or the roof (Fig. 5D). The Emx1 expression in the normal brain extended beyond the BF-1-positive margin to a part of the medial region of the telencephalon, but did not extend to the sulcus or the roof (Fig. 5D). The Emx1 expression in the normal brain extended beyond the BF-1-positive margin to a part of the medial region of the telencephalon, but did not extend to the sulcus or the roof (Fig. 5D). The Emx1 expression in the normal brain extended beyond the BF-1-positive margin to a part of the medial region of the telencephalon, but did not extend to the sulcus or the roof (Fig. 5D). The Emx1 expression in the normal brain extended beyond the BF-1-positive margin to a part of the medial region of the telencephalon, but did not extend to the sulcus or the roof (Fig. 5D).
these molecular markers, the most medial region of the pallium appeared to be transformed into the non-evaginated roof in the
Emx2 mutant brain as suggested morphologically. Unexpected, however, was that Wnt-1 which is normally not expressed in
the roof was expressed, though faintly, in the Emx2 mutant roof (Fig. 5M,N).

Expressions of Emx genes in the dorsomedial part of early forebrain
As reported by Simeone and his colleagues (Simeone et al., 1992a,b), Emx1 and Emx2 are expressed in normal embryonic
forebrain in very similar but not completely overlapping domains. Emx2 expression takes place at 8.5 dpc, whereas
Emx1 expression occurs at 9.0 dpc. Of note is the fact that Emx2 and Emx1 expressions are absent from their beginning
in the dorsal midline of forebrain that corresponds to the future
roof (Fig. 6A,B), and this region expands as development proceeds. As is apparent in 10.5 and 11.5 dpc forebrains (Fig.
6C,E), the Emx2 expression extended to the most medial region of the telencephalic evagination and caudally ended at the telen-diencephalic boundary; it was not expressed in choroid plexus anlage of either the lateral or third ventricle or in the rostral diencephalic roof. The boundary of Emx1 expression was largely overlapping with Emx2 expression in the pallial
region, but it ended more laterally than the Emx2-positive domain as was especially evident in the posterior hippocampal region (Fig. 6C-F). Thus, there exists the Emx2-positive but Emx1-negative region in the caudal and medial portion of the early pallium that corresponds to the future hippocampus and dentate gyrus.

**DISCUSSION**

The results obtained in the present study provide the first molecular information on the development of dentate gyrus and hippocampus, which are currently sites of broad attention as centers for spatial learning, memory and emotional behavior (Grant et al., 1992; Abeliovich et al., 1993a; Jarrard, 1993; Sakimura et al., 1995), demonstrating that Emx2 specifies the limbic region of the telencephalon. Three EMX2 mutations were recently reported in human schizencephaly characterized by a full-thickness cleft within the cerebral hemisphere (Brunelli et al., 1996). The phenotypes of the mutant mice in this study, however, show no apparent evidence to confirm that the mutations are responsible for the disease. Our aim was to explore how the limbic region becomes specified in the early neuroepithelial patterning. Subdivisions in brain are believed to occur sequentially from 8 dpc to 9.4 dpc (Sakai, 1987; Puelles et al., 1987). Early Emx2 and Emx1 expressions correspond to this stage, but their significance has remained a matter for morphogenetic analysis.

Emx2 may define the pallio-choroidal boundary

The non-evaginated roof between two cerebral hemispheres corresponds to the telencephalon impar according to Kuhlenbeck (1973) and the roof of the secondary prosencephalon according to Puelles and Rubenstein (1993): the choroidal or SP roof. The region of dorsal telencephalon juxtaposed to this roof is the pallium, and the medial part of the pallium develops...
Fig. 6. Emx1 and Emx2 expressions in dorsal forebrain at 9.5-11.5 dpc. (A,C,E) Emx2 expression; (B,D,F) Emx1 expression. Arrows indicate the medial boundary of Emx2 expression, and arrowheads that of Emx1 expression. (A,B) Cross sections at 9.5 dpc. Both genes are expressed in the telencephalic evagination from the secondary prosencephalon, however, they are not expressed in the dorsal-median region, the future non-evaginated roof between hemispheres. In addition, Emx2 expression extends into the more medial region beyond the Emx1 expression. This pattern is the same in the more anterior part of the forebrain. (C,D) Cross sections at 11.5 dpc. In the caudal region of the telencephalon, the expression of Emx2 extends into the most medial region of telencephalon, but the Emx1 expression ends more laterally; the presence of the Emx2-positive and Emx1-negative region (indicated by arrows and arrowheads) is evident. (E,F) Sagittal sections at 10.5 dpc. As in C and D, the Emx2-positive and Emx1-negative region is detected in the caudal portion of the telencephalon, in the telencephalic boundary. This region corresponds to that affected in Emx2 mutants (cf. Fig. 4B,F). os, optic stalk; also see Fig. 4 legend.

into a series of structures collectively called the limbic system. In early brain development, Emx2 expression largely overlaps with Emx1 expression, but there is an Emx2-positive and Emx1-negative region in this pallio-choroidal transition. In addition, there is a delay in Emx1 expression as compared to Emx2 expression that starts around 8.5 dpc (Simeone et al., 1992a,b). Unfortunately, those molecular markers that are expressed uniquely in the Emx2-positive but the Emx1-negative pallium are unavailable at present. Nevertheless, the present analyses suggested that the most medial pallium was reduced and the roof was expanded by the Emx2 mutation. We speculate that in the absence of Emx1 expression, Emx2 normally defines the pallio-choroidal boundary, and its disruption has shifted the boundary laterally to the site of Emx1 expression (Fig. 7); Emx1 expression now terminates at the new sulcus between the pallium and expanded roof of the mutant (Fig. 5H). Dentate gyrus, choroid plexus in lateral ventricles, hippocampus and medial limbic cortex may be completely or partially lost by this shift of the boundary; they originate from the medial pallium that was transformed into the choroidal roof in the Emx2 mutant. It should be kept in mind, however, that the expanded roof delineated by the sulci was morphologically atypical. In addition, noggin expression was somewhat lower and Wnt-1 was faintly expressed in the expanded roof of the mutant forebrain. Wnt-1 is expressed in the diencephalic roof, but is never expressed in the more rostral roof (Parr et al., 1993). noggin expression in normal diencephalic roof appears lower than that in the roof between two cerebral hemispheres (our unpublished data). These findings might suggest the diencephalization of the roof between two cerebral hemispheres, but the normal noggin and Wnt-1 expressions change temporally being finely regulated. The significance of the observations is left to future studies.

The defects seen in newborn mice, however, appear to extend beyond the Emx2-positive and Emx1-negative region at 9.5-11.5 dpc. One plausible explanation would be that determination of the boundary marginally precedes the onset of the Emx1 expression, and the compensation for Emx2-deficiency by Emx1 for the boundary determination occurs more laterally than the normal boundary of Emx1 expression. The reduced evagination of telencephalon at 9.0 dpc may suggest that demarcation of the pallio-choroidal boundary has already been accomplished by this stage. Alternatively, the defects in the region beyond the Emx2-positive and Emx1-negative region may be secondary to the loss of the most medial structures that was brought about by the shift of the pallio-choroidal boundary. The role of Emx2 expression in the hippocampal region at a later stage might also partly contribute to this discrepancy. On the other hand, defects in early embryonic Emx2/HOXD forebrain were not apparent in the more lateral regions of its expression where it overlaps Emx1 expression; nor were any apparent defects found in early embryonic forebrain of Emx1 mutants. Thus, in these regions Emx2 deficiency may be compensated for by Emx1 and vice versa; the Emx genes themselves may function in delineating the whole telencephalic evagination. Indeed, our preliminary analysis of Emx2/Emx1 double mutant forebrain is consistent with this assumption. This is reminiscent of En1 and En2 genes in cerebellar development (Joyner, 1996), and the detailed analyses of the Emx2/Emx1 double mutant forebrain are eagerly awaited.

The molecular mechanisms governing how Emx genes define the boundary remain for future studies, but it is speculated that the Emx transcriptional factors probably interact counteractively with a gene(s) responsible for development of the roof and dorsal diencephalon. Wnt family genes would be such a candidate(s); there are several cognates uniquely expressed in the dorsal forebrain (Parr et al., 1993). It is worthwhile noting that Emx protein binding sites have been identified in the regulatory region of Wnt-1, which is expressed in the roof of the diencephalon but not in the more rostral roof (Iler et al., 1995). Furthermore, transgenic analyses have indicated that the lack of this enhancer leads to ectopic expression of Wnt1 in the region which was affected in the Emx2 mutant, suggesting that Emx2 might function as a negative regulator of Wnt-1 expression; this might relate to the expression of Wnt-1 in the expanded roof. In Drosophila engrailed, which is functionally closely related to wingless, a
Drosophila homologue of Wnt family genes has been suggested to interact with ems in segmentation of the brain neuromere (Cohen and Jürgens, 1990; Hirth et al., 1995). Expressions of noggin in the normal roof where Emx2 is not expressed and in the expanded roof of the Emx2 mutant are also suggestive of the interaction between noggin and Emx for demarcation of the pallio-choroidal boundary.

**Forebrain defects due to later Emx expressions**

Emx1 and Emx2 expression patterns change with forebrain development, and the newborn brain phenotypes provide some clues about the function of these genes in later neurogenesis. The Emx1 mutation displayed no apparent defects at the pharyngula stage, and most, if not all, of the subtle defects in the adult may be attributable to its later expression. Defects such as those in indusium griseum and taenia tecta were never found in Emx2 mutants and may correspond to later expressions unique to Emx1. The defects in corpus callosum in Emx1 mutants may also be associated with the gene’s expression in the mesocortical subplate and a portion of the cells that form the glial sling at 16.5 dpc. According to Harkin et al. (1988), this structure mediates the axonal growth of callosal fibers. The hippocampus was sometimes small as a result of the Emx1 mutation, but this may also correspond to Emx1 expression at the later stage (Simeone et al., 1992a; Boncinelli et al., 1993). At 16.5 dpc Emx2 expression is weak and restricted to a small part of the hippocampus region, while Emx1 expression is intensive and covers most of the region, even extending further into the intermediate zone of hippocampal and pallial regions which never express Emx2. Defects in the cortical layer of Emx1 mutants, though morphologically similar to those of Emx2 mutants, may also reside in its later expression in the intermediate zone of the cortex.

Defects in newborn forebrain were more extensive as a result of Emx2 disruption with the presence of defects in early regional patterning, and it is difficult to discriminate defects corresponding to later Emx2 expressions. The loss of the dentate gyrus and choroid plexus in the lateral ventricle was associated only with the Emx2 disruption and is likely to be related to failure in the early patterning of the forebrain; these structures originate from the pallio-choroidal boundary region. As discussed above, defects in the adjacent structures of hippocampus and medial limbic cortex may also be primarily related to the earlier defect. At the same time, defects in the
corpus callosum and the posterior part of anterior commissure caused by the Emx2 mutation appear different from those of the Emx1 mutation. In Emx1 mutants, the number of fibers was apparently unchanged and their axonal pathway was aberrant as discussed above, while in Emx2 mutants the number greatly decreased; this may correspond to the Emx2 expression in the ventricular zone of the cortex. Defects in the cortical layer of Emx2 mutants may also be due to its later expression in the subventricular zone of the neocortex. In Drosophila, ems mutants lack primordia of antennal sense organs, the main olfactory sensory structures. Defects in the olfactory system were found only as a result of the Emx2 mutation. Emx2 is expressed not only in olfactory placodes, olfactory bulbs and olfactory epithelia of nasal chambers, but also in several cerebral locations related to olfaction (Simeone et al., 1992a). No connection between the nerve and bulb and the disorganization of the mitral cell layer of the bulb was apparent in Emx2 mutant. Details of defects in the Emx2-positive structures in the olfactory system as well as defects corresponding to later Emx expressions, however, belong to future studies.

Genes for forebrain development

Several mutations have recently been reported in forebrain. Extra-toes mutants have mutations in Gli-3 gene (Schimmmang et al., 1992), a homologue of Drosophila segment polarity gene cubitus interruptus with a zinc finger motif (Hui et al., 1994). The mutant developed neither an olfactory bulb nor a choroid plexus in the lateral ventricles, displayed poor development of the limbic system and did not exhibit lamination in the cerebral cortex (Franz, 1994). These defects in forebrain appear to relate closely to Emx mutant phenotypes, and the intimate relation between Gli-3 and Emx genes is anticipated to be necessary for development of the dorsal telencephalon.

A mutation of the BF-1 gene, a gene with a fork-head domain, caused dramatic reduction in the size of the cerebral hemisphere, being more severe in the ventral telencephalon (Xuan et al., 1995). The BF-1-positive domain overlaps with Emx1- and Emx2-positive domains in the dorsolateral region. The telencephalon was, however, specified, and no change of forebrain regionalization appeared to be associated with the BF-1 mutation. BF-1 is likely to regulate proliferation and differentiation of neuroepithelial cells in the specified telencephalon. In contrast to Emx genes, Nkx-2.1, an NK-class of homeobox gene, is expressed in the ventral forebrain, and its disruption resulted in loss of the ventral forebrain (Kimura et al., 1996). Dlx genes are also expressed in the ventral portions of the telencephalon primordium (Bulfone et al., 1993). Although Dlx-2 mutants displayed no evident defect in the forebrain (Qiu et al., 1995), it may be involved in the development of this region redundantly with other Dlx genes.

Among genes uniquely expressed in forebrain, Emx genes are so far the only ones in the telencephalon of which the homologue is known to play an essential role in the development of the insect brain. The fly head develops under gap genes unique to this region, otD, ems and btd. The fly brain consists of three neuromeres (b1,2,3), and ems deficiency causes loss of b2 and b3 neuromeres, and otD-deficiency of b1 neuromere (Hirth et al., 1995). The mammalian homologue of otD, Otx2, is also essential in rostral head development (Matsuo et al., 1995; Acampora et al., 1995; Ang et al., 1996). In the evolutionary lineages leading to insects and vertebrates, cephalization is believed to have occurred independently in each group, and no direct equivalency can be ascertained in any of the brain structures between insects and mammals. Nevertheless, the conservation of the functions of the Emx and Otx families of genes in rostral head development may provide a clue to the origin of the rostral head in the animal kingdom; the head does not express Hox or HOM-C genes and may universally develop under the control of the ems and otD families of genes and their downstream gene cascades which are different from those of gap genes in the trunk region.

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