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Emx1 and *Emx2* functions in development of dorsal telencephalon

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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 roof 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 (Boncinelli 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 *Apa*I site (A) of the *Emx1* gene and the *Msc*I 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 *Not*I and introduced into TT2 ES cells derived from an F_1 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′-CTGACAGCTCCCTAGACACTCTTGG-3′ and 5′-GTTGCTATACTCTGCCTACAAACGTAACTG-3′ as the 3′ side primers (antisense strand oligonucleotides) in the *Emx1* and *Emx2* genes, respectively, and 5′-GCCTGCTTGCCGAATATCATGGTGGAAAAT-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 *Xba*I-*Eco*RI-digested 700 bp fragment of *Emx1*, *Sac*I-*Spe*I-digested 500 bp fragment of *Emx2* and *Pst*I-digested 600 bp fragment of the *neo* gene (indicated in Fig. 1A) were used as probes of those analyses. Two mutant mouse strains were generated from two independent homologous recombinant ES clones in each disruption. The genotype of each mutant mouse or embryo was routinely determined on tail or yolk sac by PCR with the primers described above. To detect normal allele primers 5′-AGCGACGTTCCCCAGGACGGGCTGC-3′ and 5′-CCGAGAGTTTCCTTTTGCACAACGC-3′ were used as the 5′ side primers (sense strand oligonucleotide) in the *Emx1* and *Emx2* genes, respectively. The genotype was confirmed by Southern blot analyses when necessary. The mice were housed in environmentally controlled rooms of the Laboratory Animal Research Center of Kumamoto University School of Medicine under the guidelines of Kumamoto University for animal and recombinant DNA experiments.

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′-CCGAGAGTTTCCTTTTGCACAACGC-3′,
5′-GCCTGCTTGGTAGCAATTCTCCACC-3′,
5′-AGCGACGTTCCCCAGGACGGGCTGC-3′ and
5′-TGCGTCTCGGAGAGGCTGAGGCTGC-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 *Pvu*II digestion and the 300 bp cDNA fragment obtained by PCR that covers the stop codon and the *Hin*dIII 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 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 ($Emx1^{HD/HD}$) 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 $Emx1^{HD/HD}$ mice, defects were subtle and restricted to the

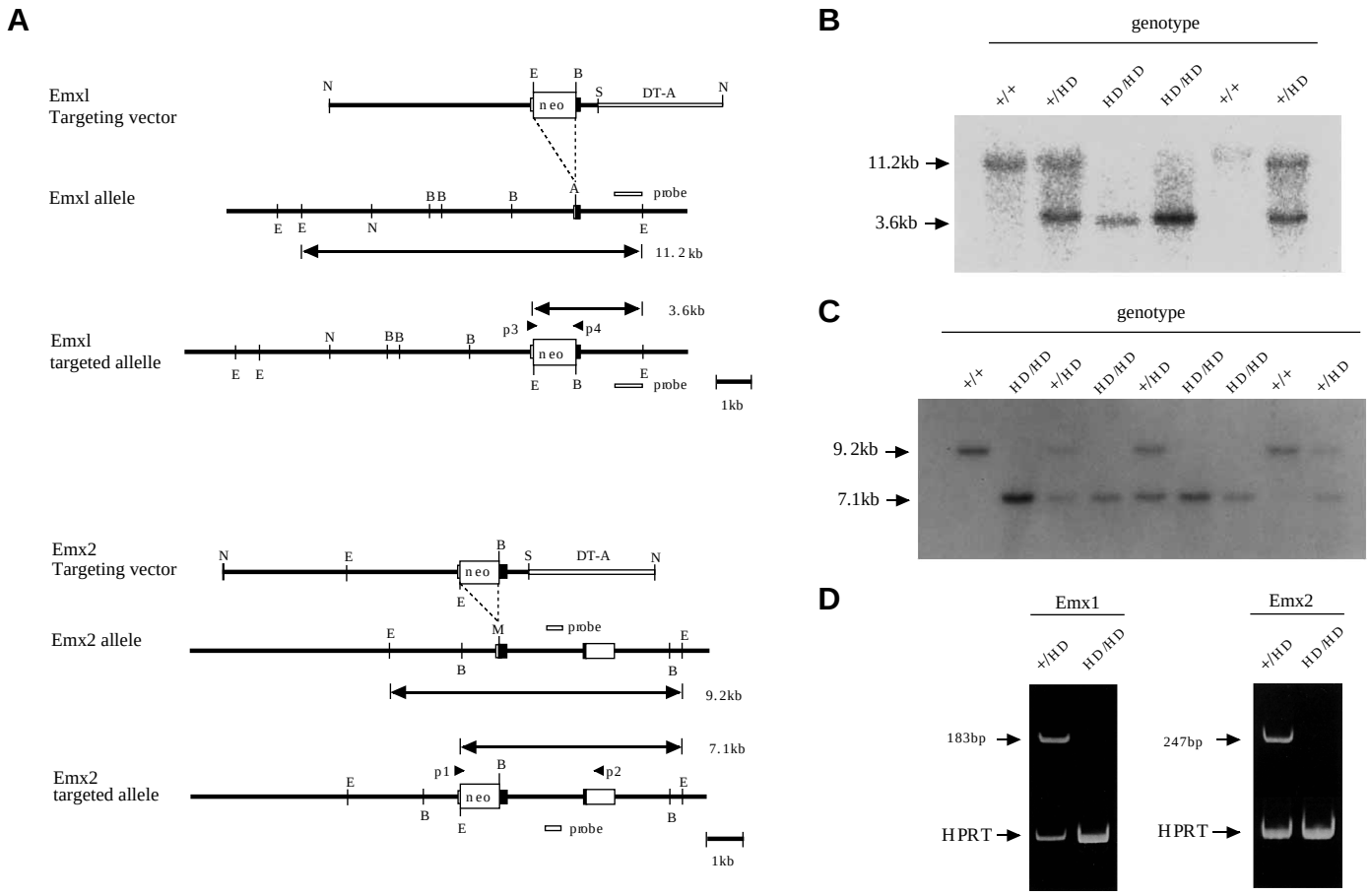

Fig. 1. The generation of *Emx1* and *Emx2* mutant mice. (A) Targeting vectors for *Emx1* and *Emx2* mutations. Exons are indicated by rectangles; filled boxes indicate homeodomain. The predicted sizes of normal and targeted alleles and the probes used in Southern analyses (B,C) are shown. Arrowheads indicate the positions of primers in RT-PCR analyses, respectively. (B,C) Examples of Southern blot analyses by *Eco*RI 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. +/HD heterozygous and HD/HD homozygous mutants. A, *Apa*I; B, *Bam*HI; E, *Eco*RI; M, *Msc*I; N, *Not*I; S, *Sal*I.

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 *Emx1*$^{HD/HD}$ 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 (*Emx2*$^{HD/HD}$) 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 *Emx2*$^{HD/HD}$ 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),

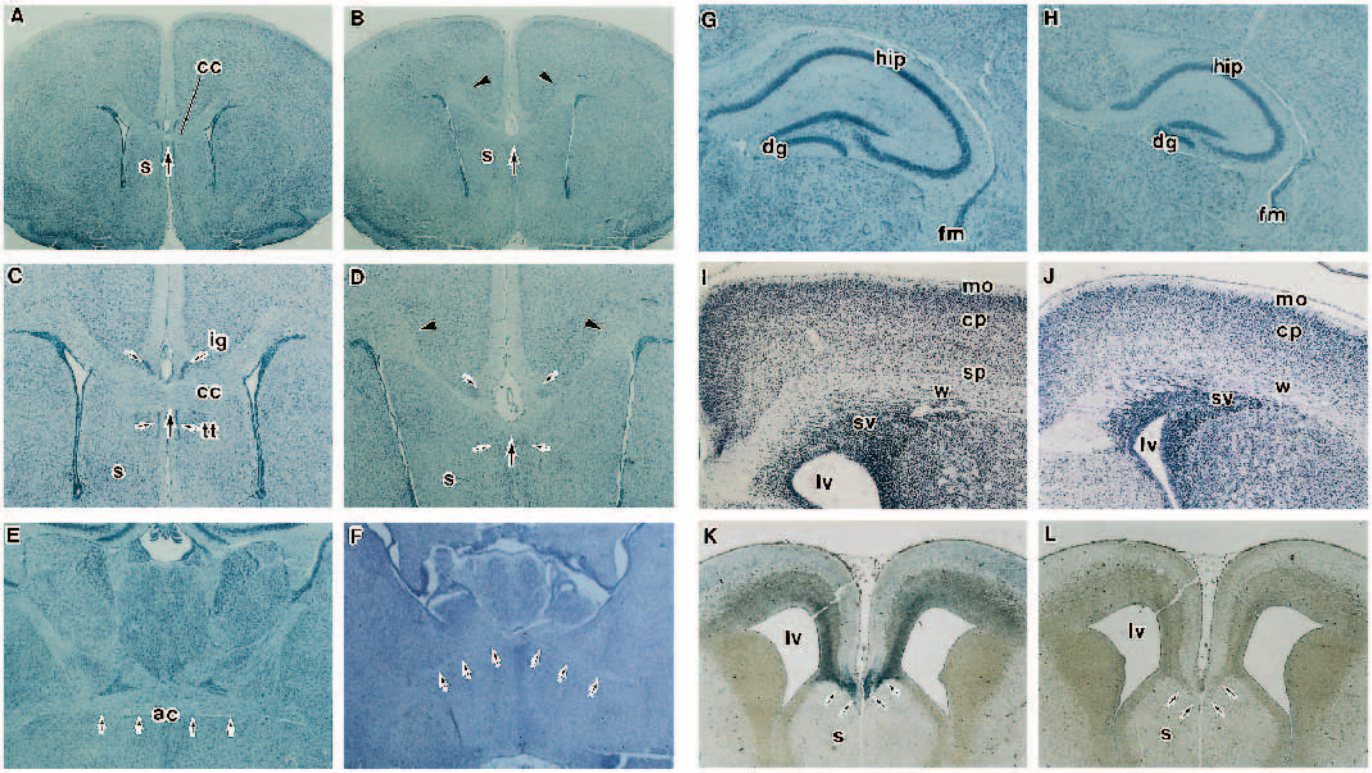

Fig. 2. Architecture of *Emx1*$^{HD/HD}$ brains. (A,C,E,G,I,K,L) Wild type; (B,D,F,H,J) *Emx1*$^{HD/HD}$. (A,B) Frontal sections of 3-week wild-type and one of the most severely affected *Emx1*$^{HD/HD}$ brains, respectively, and (C,D) highly magnified views of medial regions in A,B. In this mutant specimen (B,D), the commissural fibers of corpus callosum (cc) do not cross the midline into the opposite cerebral hemisphere (large arrow) and are tangled (arrowheads in B and D). Indusium griseum (ig) and taenia tecta (tt) are missing in all the *Emx1*$^{HD/HD}$ brains (small arrows in C and D). (E,F) Frontal sections at anterior commissure level. Disorganized fasciculation in anterior commissure (ac; arrows) is observed in a significant portion of the *Emx1*$^{HD/HD}$ mutants. (G,H) Frontal sections at the hippocampus level. In mutants the hippocampus (hip) is sometimes smaller, but is always present. (I,J) Frontal sections of dorsal part of the right cerebral hemisphere. The cortical layer is poorly differentiated and the subplate is hardly visible. (K,L) 16.5 dpc wild-type embryos showing the expressions of *Emx1* and *Emx2*, respectively, at the level of the corpus callosum. The high level *Emx1* expression is found in mesocortical subplate and a portion of cells that are migrating from the subventricular zone toward the dorsal fusion area (arrows in K); the cells form the glial sling that is essential for the guidance of callosal fibers (Hankin and Silver, 1988). No *Emx2* expression is found in them (L). ac, anterior commissure; cc, corpus callosum; cp, cortical plate; dg, dentate gyrus; fm, fimbria; hip, hippocampus; ig, indusium griseum; lv, lateral ventricle; mo, molecular layer; s, septum; sp, cortical subplate; sv, subventricular zone; tt, taenia tecta; w, white matter.

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 pharyngula stage. The *Emx1*$^{HD/HD}$ embryonic brains were indistinguishable from wild-type brain at this stage (Fig. 4G-I). In the *Emx2*$^{HD/HD}$ brains, however, the mutant phenotype was apparent morphologically by 11.5 dpc, i.e., the anlages 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

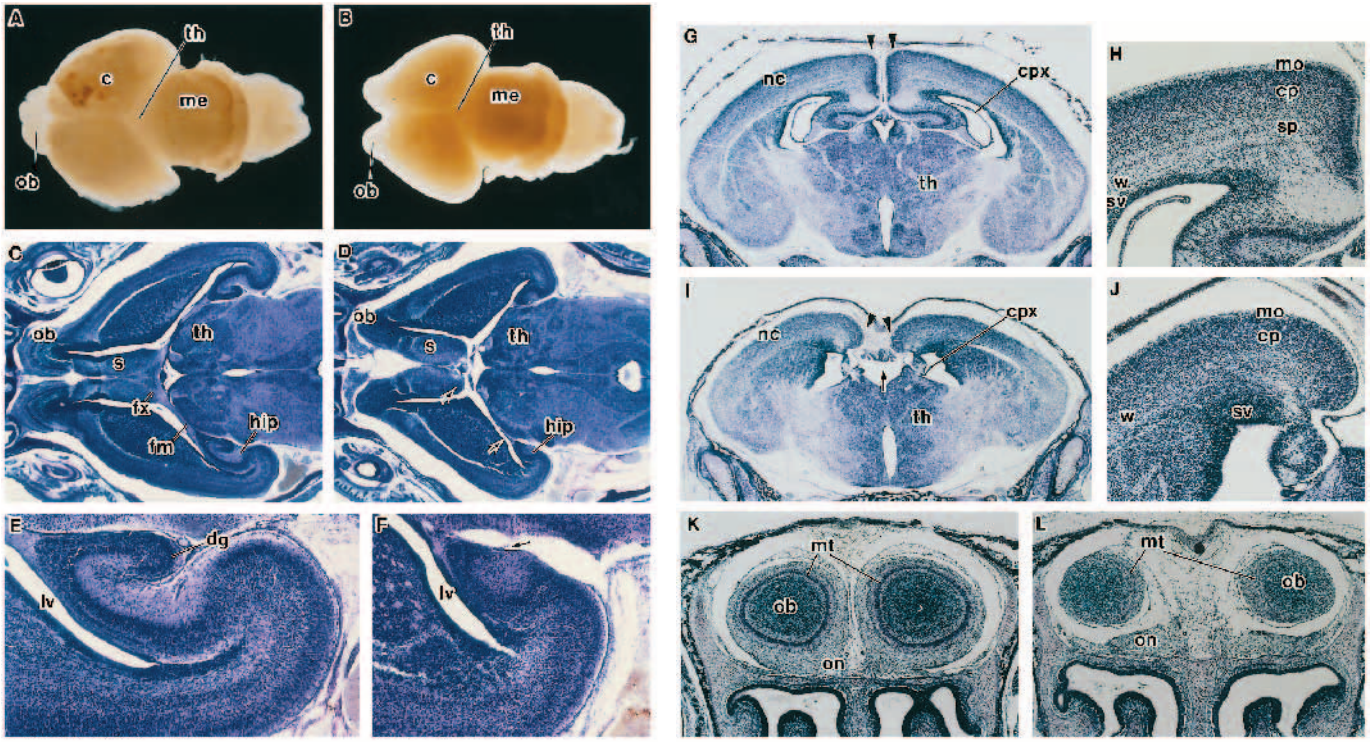

Fig. 3. Architecture of newborn *Emx2*$^{HD/HD}$ brains. (A,C,E,G,H,K) Wild type; (B,D,F,I,J,L) *Emx2*$^{HD/HD}$. (A,B) External morphology of 19.5 dpc brains. In *Emx2*$^{HD/HD}$ brains, cerebral hemisphere (c) and olfactory bulb (ob) are small and located more laterally. (C,D) Horizontal sections of 19.5 dpc brains. (E,F) Highly magnified views of hippocampal regions in C,D. In the *Emx2*$^{HD/HD}$ brain, the reduction of hippocampal region is evident, and dentate gyrus (dg) is missing (arrow in F), but the S-shaped curvature of the pyramidal layer is formed. The fascicles of fornix and fimbria are poor (arrows in D). (G,I) Frontal sections of 19.5 dpc brains; (H,J) Highly magnified views of dorsal part of left cerebral hemisphere in G,I. In *Emx2*$^{HD/HD}$ brains, the medial limbic cortex (arrowheads in G,I) is poorly developed, and in the more lateral cortex, the cortical layer is poor (H,J). Abnormality is also seen in the junctional region between cerebral hemisphere and thalamus; choroid plexus (cpx) is greatly reduced in the lateral ventricle but is hyperplastic in the third ventricle, and the dorsal portion of the third ventricle is expanded (an arrow in I). (K,L) Frontal sections at the olfactory bulb level of 19.5 dpc brain. In the *Emx2*$^{HD/HD}$ mutant, most of the olfactory nerves (on) fail to project to the olfactory bulb, and the mitral cell layer (mt) in the bulb is disorganized (L). c, cerebral hemisphere; cp, cortical plate; cpx, choroid plexus; dg, dentate gyrus; fm, fimbria; fx, fornix; hip, hippocampus; lv, lateral ventricle; me, mesencephalon; mo, molecular layer; mt, mitral cell layer; nc, neocortex; ob, olfactory bulb; on, olfactory nerve; s, septum; sp, cortical subplate; sv, subventricular zone; th, thalamus; w, white matter.

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*$^{HD/HD}$ 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*$^{HD/HD}$ telencephalon, 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. 5G). In the *Emx2*$^{HD/HD}$ mutants, it ended just at the sulcus (Fig. 5H). Consistently in the *Emx2*$^{HD/HD}$ mutant, *Wnt-3a*, a homologue of *Drosophila wingless* which encodes a secreted molecule (Roelink and Nusse, 1991) was expressed in the expanded roof, and the *Wnt-3a*-positive region in the telencephalic evagination was remarkably reduced (Fig. 5I,J). *noggin*, which codes for a secreted molecule is normally expressed in the non-evaginated roof, but not in the pallium. In the *Emx2*$^{HD/HD}$ mutant, it was expressed in the entire expanded roof, though its intensity was somewhat lower (Fig. 5K,L). Thus, in terms of analyses with

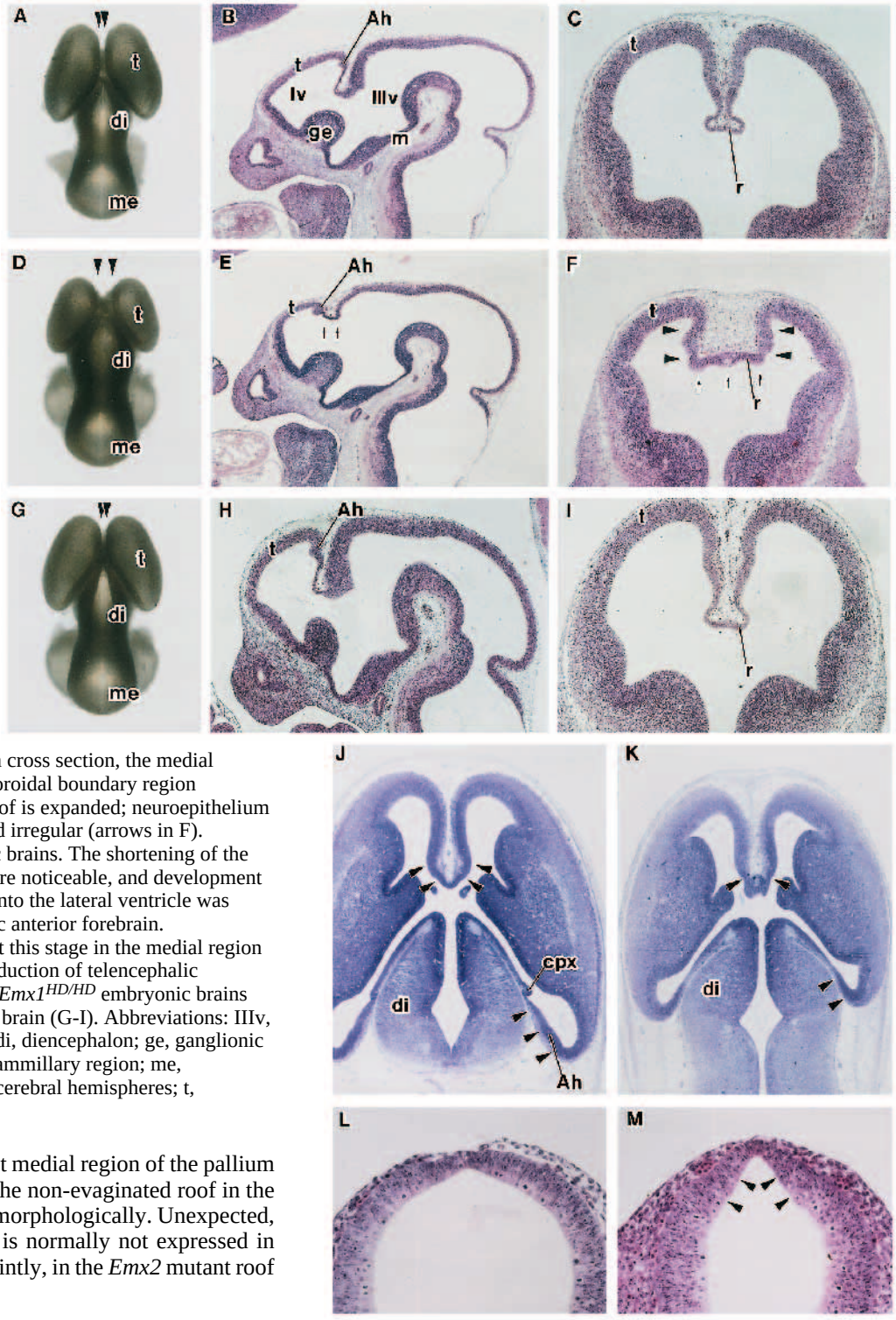

Fig. 4. Architecture of *Emx1*$^{HD/HD}$ and *Emx2*$^{HD/HD}$ embryonic brains. (A-C,J,L) Wild type; (D-F,K,M) *Emx2*$^{HD/HD}$; (G-I) *Emx1*$^{HD/HD}$. (A,D,G) External appearance of 11.5 dpc embryonic brains. In *Emx2*$^{HD/HD}$ mutants, the telencephalon (t), the anlages of cerebral hemisphere are significantly smaller than normal and are separated from each other. Instead, the roof between the two hemispheres is exposed (arrowheads in A,D,G). (B,E,H) Sagittal and (C,F,I) cross sections of 11.5 dpc brains. Anomalies are seen in the dorsal portion of the *Emx2*$^{HD/HD}$ forebrain corresponding to the telen-diencephalic boundary, while the ventral structures such as ganglionic eminence (ge) and mammillary region (m) are apparently normal. In *Emx2* mutants Ammon's horn (Ah) is protruding toward the lateral ventricle, but the neuroepithelium between the caudal portion of this protrusion and the diencephalon is strikingly shortened (arrows in E). In cross section, the medial pallium is shortened in the pallio-choroidal boundary region (arrowheads in F). In contrast, the roof is expanded; neuroepithelium in the expanded roof is thickened and irregular (arrows in F). (J,K) Horizontal sections of 13.5 dpc brains. The shortening of the medial region of the pallium was more noticeable, and development of the protrusion of Ammon's horn into the lateral ventricle was poor. (L,M) Cross sections of 9.0 dpc anterior forebrain. Abnormalities are already detected at this stage in the medial region of the dorsal forebrain such as the reduction of telencephalic evagination (arrowheads in M). The *Emx1*$^{HD/HD}$ embryonic brains are indistinguishable from wild-type brain (G-I). Abbreviations: IIIv, third ventricle; Ah, Ammon's horn; di, diencephalon; ge, ganglionic eminence; lv, lateral ventricle; m, mammillary region; me, mesencephalon; r, the roof between cerebral hemispheres; t, telencephalic evagination.

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-diencephalon 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

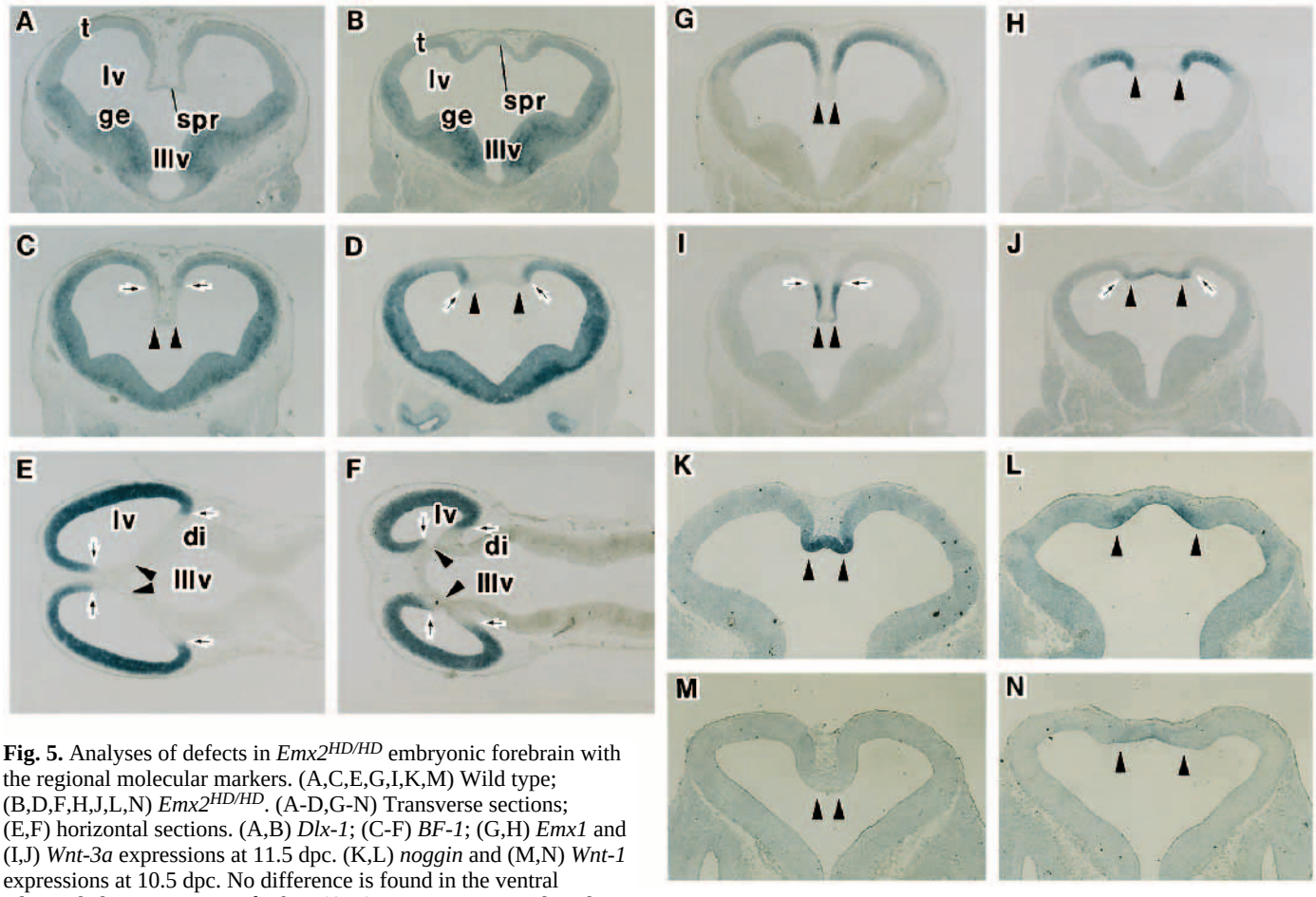

Fig. 5. Analyses of defects in *Emx2*$^{HD/HD}$ embryonic forebrain with the regional molecular markers. (A,C,E,G,I,K,M) Wild type; (B,D,F,H,J,L,N) *Emx2*$^{HD/HD}$. (A-D,G-N) Transverse sections; (E,F) horizontal sections. (A,B) *Dlx-1*; (C-F) *BF-1*; (G,H) *Emx1* and (I,J) *Wnt-3a* expressions at 11.5 dpc. (K,L) *noggin* and (M,N) *Wnt-1* expressions at 10.5 dpc. No difference is found in the ventral telencephalon expression of *Dlx-1* (A,B). *BF-1* is expressed in almost the entire wild-type telencephalon except the dorsal-medial region (indicated by arrows and arrowheads in C and E). In *Emx2*$^{HD/HD}$, the *BF-1*-positive region is unchanged, but the negative region in the telencephalon is remarkably reduced (D,F). *Emx1* expression normally extends beyond the *BF-1*-positive margin to part of the dorsal-medial region of the telencephalon, but does not extend to the sulcus or the roof. In *Emx2*$^{HD/HD}$, the *Emx1* expression ends at the sulcus that delineates the pallio-choroidal boundary (arrowheads in H) and is not found in the expanded roof (H). *Wnt-3a* is expressed in the *BF-1* negative region of the dorsal-medial pallium (small arrows in I) and the roof in wild type. In *Emx2*$^{HD/HD}$, *Wnt-3a* is expressed in the expanded roof, but its positive domain in the pallium is markedly reduced (J); its end roughly corresponds to the margin of the *BF-1*-positive domain (D). In wild type, *noggin* is expressed in the roof between hemispheres, but not in the pallium. In *Emx2*$^{HD/HD}$, *noggin* is expressed in the entire expanded roof, though its intensity is somewhat lower (K,L).*Wnt1* is normally not expressed in the roof, but its expression is detected, though faintly, in the *Emx2* mutant roof (M,N). Abbreviations as in Fig. 4 legend.

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,b; 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

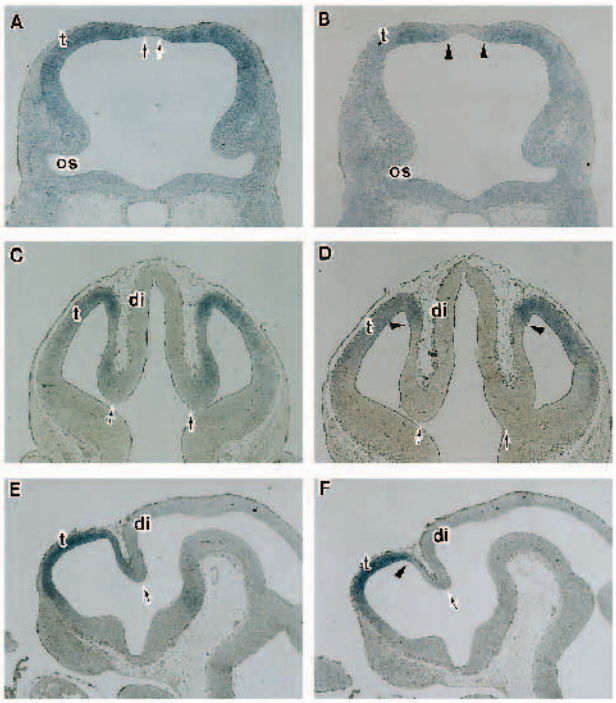

Fig. 6. *Emx1* and *Emx2* expressios in dorsal forebrain at 9.5-11.5 dpc. (A,C,E) *Emx2* expression; (B,D,F) *Emx1* expression. Arrows indicate the medial boundary of *Emx2* expression, and arrowheads that of *Emx1* expression. (A,B) Cross sections at 9.5 dpc. Both genes are expressed in the telencephalic evagination from the secondary prosencephalon, however, they are not expressed in the dorsal-medial 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 telen-diencephalic 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 tempospatially 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 $Emx2^{HD/HD}$ 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

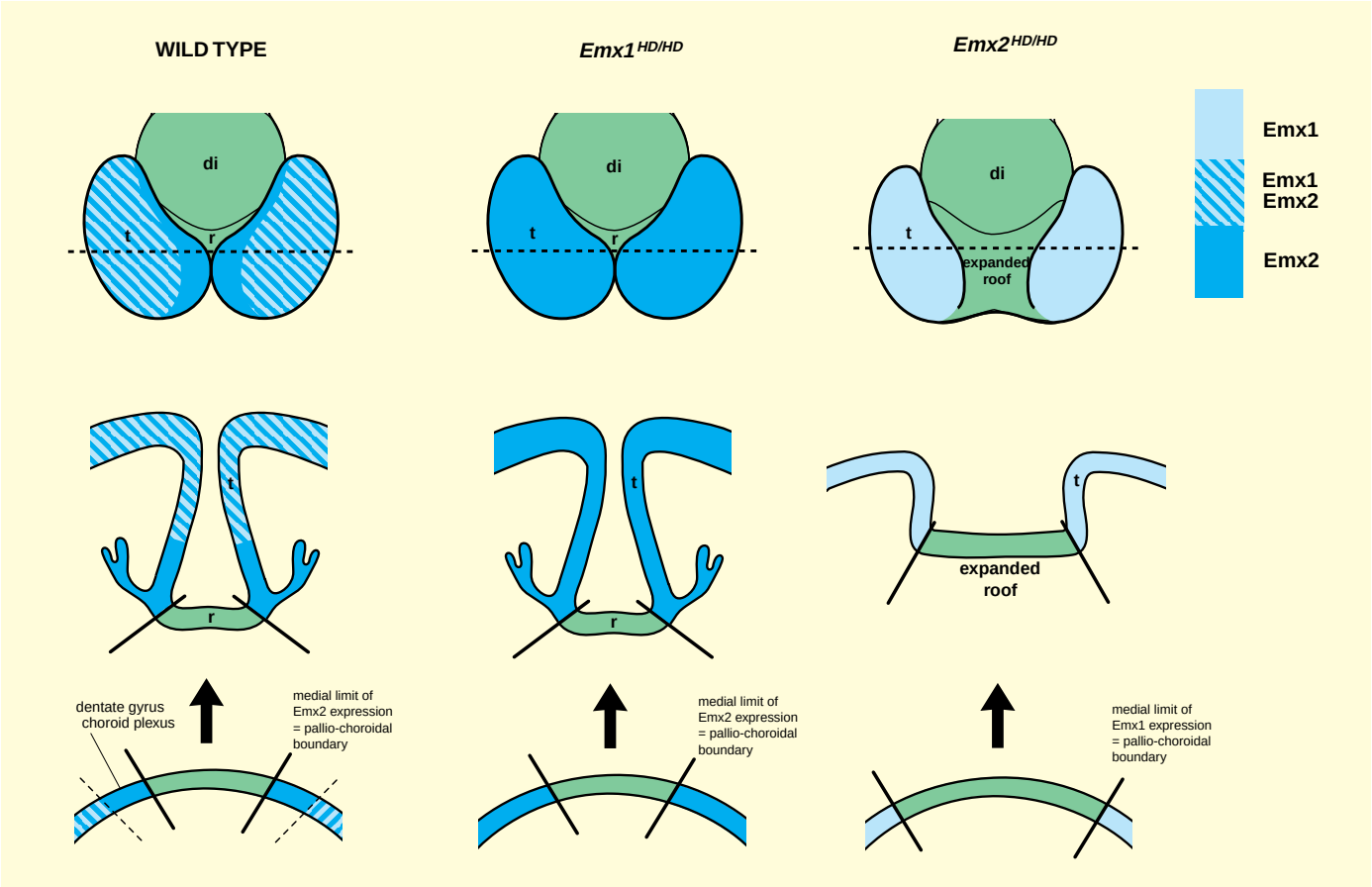

Fig. 7. Schematic representation of *Emx* gene functions upon dorsal telencephalon patterning. There is an *Emx2*-positive and an *Emx1*-negative region in early dorsomedial forebrain (indicated by blue in the left). *Emx2* defines the pallio-choroidal boundary, and its disruption shifts the boundary laterally to the site of *Emx1* expression (right). Dentate gyrus, choroid plexus in lateral ventricles, hippocampus and medial limbic cortex are lost completely or partially by this shift of the boundary; they originate from the medial pallium that was transformed into the roof derivatives in the *Emx2* mutant. In contrast, *Emx2*-deficiency is compensated by *Emx1* and vice versa in the more lateral region where both are expressed.

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 (Schimmang 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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