Emx2 and Pax6 Function in Cooperation with Otx2 and Otx1 to Develop Caudal Forebrain Primordium That Includes Future Archipallium

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One of the central issues in developmental neurobiology is how the forebrain is organized ontogenetically. The traditional view is that the anterior neuroectoderm first develops into mesencephalic and prosencephalic vesicles; the latter vesicle subsequently develops into the diencephalon and secondary prosencephalon, of which dorsal parts protrude to generate the telencephalon. The diencephalon yields the pretectum, thalamus, and prethalamus, and the telencephalon produces the archipallium, neopallium, and ganglionic eminences. By identifying cell descendants that once expressed Emx2 with use of the Cre knock-in mutant into the Emx2 locus and analyzing phenotypes of double mutants between Emx2 and Otx2/Otx1 and between Emx2 and Pax6, we propose that at the 3–6 somite stage, the anterior neuroectoderm develops into three prominia: midbrain, caudal forebrain, and rostral forebrain. The caudal forebrain primordium generates not only the pretectum, thalamus, and prethalamus but also the archipallium, cortical hem, choroid plexus, choroidal roof, and eminentia thalami. The primordium corresponds to the Emx2- or Pax6-positive region at the 3–6 somite stage that most probably does not include the future neopallium or commissural plate. Otx2 and Otx1 that are expressed in the entire future forebrain and midbrain cooperate with this Emx2 and Pax6 expression in the development of the caudal forebrain primordium; Emx2 and Pax6 functions are redundant. In the embryonic day 9.5 Emx2−/−/Pax6−/− double mutant, the caudal forebrain remained unspecified and subsequently transformed into tectum in a mirror image of the endogenous one.

Key words: Emx2; Pax6; Otx2; Otx1; forebrain; archipallium; diencephalon; tectum

Introduction

The rostral brain comprises a series of structures rostrocaudally and dorsoventrally. It is widely accepted that in front of the midbrain, the forebrain consists caudorostrally of the pretectum (p1), thalamus (dorsal thalamus, p2) and prethalamus (ventral thalamus, p3) (Puelles and Rubenstein, 1993, 2003). However, it is still a matter of dispute how forebrain structures in front of the prethalamus are organized; moreover, it is not yet certain how the forebrain is regionalized into these structures ontogenetically. An initial version of the prosomeric model postulated the archipallium and eminentia thalami as p4 structures rostral to p3 prethalamus and caudal to p5 neopallium (here “caudal forebrain” is used to indicate these p1 to p4 structures). In the traditional view, however, the archipallium is a dorsomedial structure, the neopallium is a dorsolateral structure, and ganglionic eminences are ventral structures of the telencephalon rostrally to the prethalamus (here “telencephalon” is used in this sense).

The initial morphological landmark in the anterior neuroectoderm is the preotic sulcus. In the neural plate rostral to this sulcus, a series of transcriptional factors, Otx2, Gbx2, Pax2, En1, Pax6, Irx3, and Six3, are expressed in a nested pattern, initially partly overlapping each other but being distinctly segregated by the 6–8 somite stage. This, together with the overexpression/ectopic expression studies in avian, led to a proposal that mutually inhibitory interactions among these genes determine the boundaries between each territory in the rostral brain (Kobayashi et al., 2002). The view proposes three divisions in the initial brain regionalization: forebrain rostral to zona limitans intrathalamica (ZLI), that caudal to ZLI, and midbrain. Another view in avian proposes that in the forebrain, the pretectum first differentiates and the thalamus and prethalamus are formed with the ZLI development (Larsen et al., 2002). The view proposes three divisions in the initial brain regionalization: forebrain rostral to zona limitans intrathalamica (ZLI), that caudal to ZLI, and midbrain. Another view in avian proposes that in the forebrain, the pretectum first differentiates and the thalamus and prethalamus are formed with the ZLI development (Larsen et al., 2002). The view proposes three divisions in the initial brain regionalization: forebrain rostral to zona limitans intrathalamica (ZLI), that caudal to ZLI, and midbrain. Another view in avian proposes that in the forebrain, the pretectum first differentiates and the thalamus and prethalamus are formed with the ZLI development (Larsen et al., 2002).
ren and Price, 1997; Schwarz et al., 1999; Martinez-Barbera et al., 2000; Liu and Joyner, 2001; Lagutin et al., 2003). However, the details of the defects, their processes, and the primary limits remain for future studies to synthesize ontology of forebrain structures.

Previously, we reported that in the Emx2+/−/Otx2+/− mutant, the commissure region of the pretectum develops but the non-commissure region of the pretectum, prethalamus, and thalamus are lost (Suda et al., 2001). In contrast, the ectopic Emx2 expression over the entire forebrain and midbrain in the Otx2 locus (Otx2+/Emx2) is specifically incompatible with the development of the commissure region of the pretectum. Emx2 is not expressed in the pretectum or thalamus when they are formed. Moreover, the Otx2 expression is not unique to the caudal forebrain; How is the caudal forebrain specified in the archipallium defects in the E12.5 mutant? This situation can be explained simply by postulating a third gene, X, the expression of which overlaps and is functionally redundant with the Emx2 expression. Here, we propose that X is the Pax6 gene and that the caudal forebrain primordium spanning from the future pretectum to the archipallium is established against rostral forebrain and midbrain primordia at the 3–6 somite stage through the cooperation of Emx2 and Pax6 with Otx2 and Otx1.

Materials and Methods

Mutant mice. Emx2, Emx1 (Yoshida et al., 1997), Otx2 (Matsuo et al., 1995), and Otx1 (Suda et al., 1997) mutant mice were generated as described. The sources of Sey and ROSA26R mice are as described previously (Hill et al., 1991; Soriano, 1999). Mice were housed in environmentally controlled rooms under the institute guidelines for animal and recombinant DNA experiments.

Generation of Cre knock-in mutant into Emx2 locus. To construct the targeting vector, the neomycin-resistant gene directed by the PGK gene promoter and polyadenylation (polyA) signal (neo) was flanked withloxP sequences; moreover, this was conjugated to the Cre recombinase gene (Cre) that lacks the polyA signal, generating the Cre-neo cassette. A DNA fragment encompassing 6.8 kb 5’ upstream to 2.3 kb 3’ downstream of the translation initiation site of the Emx2 gene was isolated from C57BL/6 genomic DNAs. The Cre-neo cassette was inserted into the translational initiation codon of this fragment; ATG of the Cre gene corresponds to ATG of the Emx2 gene (supplemental Fig. 2, available at www.j-neurosci.org as supplemental material). The diphtheria toxin-A fragment gene, driven by the MC1 promoter, was used for negative selection of homologous recombinants as described previously (Yagi et al., 1993b). Details of the vector construction will be provided on request. The vector was linearized with NotI digestion, homologous recombinants were isolated with TT2 embryonic stem cells, and mutant mice (Emx2+/Cre-neo−) were generated as described previously (Yagi et al., 1993a). The mice were mated with LeflyCre mice (Yamamoto et al., 2001) to excise neo, generating Emx2+/Cre+ mice.

Genotyping of mice. Genotypes of mice and embryos were determined routinely by PCR; genomic DNAs were obtained from tails or yolk sacs. The primers used to identify Emx2, Emx1, Otx2, Otx1, Pax6, and ROSA26R wild-type and mutant alleles were as described previously (Grindley et al., 1995; Matsuo et al., 1995; Suda et al., 1997; Yoshida et al., 1997; Soriano, 1999). Those primers used to detect the Cre-neo knock-in allele in the Emx2 locus were the 5’ primer (5’-GCCCTGCTGGCGAATATCATGGTGGAAAAT-3’) in the neo gene and the 3’ primer (5’-GACGGAAATTGGGCTAGTGATGTG-3’) in the first exon of the Emx2 gene; the primers used to detect the Cre knock-in allele were the 5’ primer (5’-AAGAAGGCAACACTCTCATGGATTGTC-3’) and the 3’ primer (5’-CGAACATCTTTCAAGGGCCG-3’) in the Cre gene.

Histochemical analysis. β-Galactosidase (βGal) staining and histolog-
views of the hippocampal region. The Otx2 double mutants (m, and anterior pretectum are lost in the double mutant; however, the analysis was incomplete as to the lateral/dorsal limit of the medial pallium loss in the double mutant (Fig. 1B, D, F) (data not shown). The Wnt8b and Left1 expression was residual in the medial pallium (Fig. 1H) (data not shown), and Wnt8b-positive eminentia thalami were lost (Fig. 1J). In contrast, the Wnt8b-negative and Ngn2- and Pax6-positive neopallium developed laterally; the Dlx1-positive subpallium was formed almost normally (Fig. 1L, N, P).

Morphologically, the midline structure between Wnt8b-positive remnants in the double mutant (the structure between two arrowheads in Fig. 1) was the roof; however, it was not the telencephalic roof. In the wild-type dorsal telencephalon, Msx1 is intense in the choroid plexus and ventral cortical hem and weak in the roof (Fig. 1Q). The Msx1 expression was intense in the double mutant roof (Fig. 1R). The wild-type choroidal roof expresses Wnt8b (Fig. 1G) and Lhx5 (Fig. 15); the double mutant roof expressed neither of them (Fig. 1H, T). The midbrain roof expresses Msx1 intensively (Fig. 1U), whereas it does not express Wnt8b (Fig. 1V) or Lhx5 (Fig. 1W).

Thus, we conclude that in the Emx2−/−Otx2+−/− double mutant, in addition to the prethalamus, thalamus, and anterior pretectum we described previously (Suda et al., 2001), the archipallium, cortical hem, choroid plexus, choroidal roof, and eminentia thalami fail to develop. The rostral forebrain territory of ganglionic eminences, neopallium, and the Fgβ8-positive commissural plate, however, develop. A question remains as to the lateral/dorsal limit of the medial pallium loss in the double mutant. As discussed below, the intensity of the Emx2 expression is discontinuous at a boundary between the archipallium and neopallium (see Fig. 3Bb). The limit of the medial pallium defect probably corresponds to this boundary. Histologically, hippocampal structures were entirely absent at E15.5 (Suda et al., 2001), and Ephb1- and Prox1-positive regions were completely missing. We speculate that the Wnt8b and Left1 expressions extend into the most medial neopallium or the cingulate/retrosplenial neopallium (Shinozaki et al., 2004); the residual Wnt8b- and Left1-positive regions in the double mutant may represent this most medial neopallium. However, no data exist that demonstrates the structures to which the dorsalmost/lateralmost aspect of the Wnt8b- and Left1-positive regions actually correspond.

Results

Archipallium, choroid plexus, and eminentia thalami are also lost in Emx2−/−Otx2+−/− double mutant

Our previous study demonstrated that the prethalamus, thalamus, and anterior pretectum are lost in the Emx2−/−Otx2+−/− double mutant; however, the analysis was incomplete as to the defects in more anterior regions (Suda et al., 2001). The double mutant does not develop beyond embryonic day 15.5 (E15.5) (Suda et al., 2001). In this telencephalon, the neopallium was reduced with a disorganized laminar structure; the cortical plate was hardly visible. Ganglionic eminences were hyperplastic. However, these regions were present whereas neither the CA

fields, dentate gyrus, fimbria, nor choroid plexus was formed at E15.5, and Ammon’s horn was not apparent at E12.5. To confirm this telencephalic phenotype, analyses were made with molecular markers. In the most medial pallium adjacent to the roof, transtherein (TTR)-positive choroid plexus develops (Fig. 1A); it is adjacent to the Wnt3a-, Wnt5a-, and Wnt2b-positive fimbria or cortical hem (Fig. 1C) (Grove et al., 1998). Ephb1 and Prox1 expressions cover the hippocampal field (Fig. 1E) (data not shown), and Wnt8b and Left1 expressions cover the entire medial pallium (Fig. 1G, I) (data not shown). Neither the TTR-, Wnt3a-, Wnt5a-, Wnt2b-, Ephb1-, nor Prox1-positive structure was apparent in the Emx2/Otx2 double mutant (Fig. 1, A, B, D, F) (data not shown). The Wnt8b and Left1 expression was residual in the medial pallium (Fig. 1H) (data not shown), and Wnt8b-positive eminentia thalami were lost (Fig. 1J). In contrast, the Wnt8b-negative and Ngn2- and Pax6-positive neopallium developed laterally; the Dlx1-positive subpallium was formed almost normally (Fig. 1L, N, P).

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Figure 2. Emx2/Otx1, Emx1/Otx2, and Emx1/Otx1 double mutant phenotypes. Sagittal sections of E18.5 embryos stained with cresyl violet (A, B) and E12.5 embryos stained with hematoxylin and eosin (C–F) are shown. The insets in A and B show enlarged views of the hippocampal region. The Emx2/Otx1 double mutant exhibits forebrain defects similar to but milder than the Emx2/Otx2 double mutants (B, D). Defects were not apparent in either the Emx1/Otx2 (E) or the Emx1/Otx1 (F) double mutant. AH, Ammon’s horn; DG, dentate gyrus; f, fimbria; h, hippocampus; Pt, pretectum; PTh, prethalamus; Th, thalamus. Scale bars: A, C, 500 μm; A, inset, 250 μm.

RNA in situ hybridization. Section and whole-mount in situ hybridization were performed using digoxigenin-probes as described previously (Wilkinson, 1993). The probes used were as follows: Bf1 (Tao and Lai, 1992), Dlx1 (Bulfone et al., 1993), Dmbx1 (Miyamoto et al., 2002), Ebf1 (Garel et al., 1997), Emx2 and Emx1 (Yoshida et al., 1997), En2 (Davis and Joyner, 1988), EphrinA2 (Flenniken et al., 1996), Ephb1 (IMAGE clone AA058194), Fgβ8 (Crosley et al., 1996), Gbx2 (Bulfone et al., 1993), Irx1 (Bosse et al., 1997), Lhx2 (Porter et al., 1997), Lhx5 (Sheng et al., 1997), Lim1 (Fujii et al., 1994), Msx1 (Hill et al., 1989), Ngn2 (Sommer et al., 1996), Otx2 (Matuo et al., 1995), Pax2 (Dressler et al., 1990), Pax6 (Stoykova et al., 1996), Shh (Echelard et al., 1993), Six3 (Oliver et al., 1995), Tcf4 (Korinek et al., 1998), TTR (Wakasugi et al., 1985), Wnt3a (Roelink and Nusse, 1991), Wnt7b (Parr et al., 1993), and Wnt8b (IMAGE clone AA170920).

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Otx1 cooperates with Emx2, but Emx1 does not cooperate with Otx2 or Otx1, in forebrain development

Otx2 and Emx2 have cognates, Otx1 and Emx1, respectively. It was then examined whether these cognates also participate in forebrain development. Histologically, development of the prethalamus, thalamus, and pretectum was meager in the E18.5 and E12.5 Emx2−/−Otx1−/− double mutant (Fig. 2B, D). The telencephalon was smaller, but the choroid plexus developed in the double mutant. The hippocampal field developed poorly at E18.5 (Fig. 2B, inset), and at E12.5, Ammon’s horn was markedly deformed (Fig. 2B, inset), and at E12.5, Pretectum cells remained in a more limited region of the forebrain, whereas it is faint in the pallium, whereas it is faint in the pallium. At E12.5, the expression is persistent in the pallium, whereas it is faint in the eminentia thalami and others (Fig. 3A).

Origin of the thalamus and pretectum cells

The Emx2 mRNA expression is not found at E9.5 in the region posterior to ZLI (Fig. 3Ac) (Simeone et al., 1992; Suda et al., 2001). At E12.5, the expression is persistent in the pallium, whereas it is faint in eminentia thalami and others (Fig. 3Ad). Obviously, the Emx2 expression narrows with the forebrain development. To determine cell descendants that once expressed Emx2, mice were generated in which the Cre recombinase gene was knocked-in into the Emx2 locus (supplemental Fig. 2, available at www.jneurosci.org as supplemental material) and mated with ROSA26R mice (Soriano, 1999). The analysis has inherent problems of incomplete and ectopic Cre-mediated recombination, but the βGal expression in embryos that harbor both Cre in the Emx2 locus and ROSA26R clearly demonstrated the cells that once expressed Emx2. At E9.5, the βGal-positive region was found in the intermediate mesoderm, eyes, nose, and
The anterior pretectum are lost in the forebrain, as expected (Fig. 3A). The βGal-positive region in the forebrain was, however, more caudally expanded than the endogenous Emx2 mRNA expression at this stage (Fig. 3C) and almost overlapped with Pax6 mRNA expression (Fig. 3D). In the E12.5 brain, the βGal expression was evident not only in the archipallium, eminentia thalami, and prethalamus but also in the thalamus and pretectum (Fig. 3E). Furthermore, it developed in a mirror image toward the normal one. Normally, the tectum displays a distinct rostrocaudal gradient of cytoarchitectonic maturation (Fig. 4A); rostrally superior colliculus and caudally inferior colliculus are formed. The duplicated tectum displayed the rostrocaudally opposite cytoarchitecture (Fig. 4B–H). Duplication of the cerebellum did not occur. The pallium was reduced greatly; it was lost in the medial portion of the telencephalon (Fig. 4I). Gliogenic eminences were present and rather hyperplastic (Fig. 4A,B, I, J); the duplication of the tegmentum was not apparent.

Normally, En2 is expressed in the posterior midbrain at E15.5 (Fig. 5A) (Davis and Joyner, 1988); in the double mutant, an En2-positive structure was also present in the anterior part of the “duplicated tectum” (Fig. 5B). EphrinA2 is also expressed in the caudal part of the normal midbrain (Fig. 5C) (Flenniken et al., 1996), and the duplicated tectum expressed EphrinA2 (Fig. 5D). Thus, the mirror image nature of the tectum duplication was confirmed molecularly.
At E15.5, the duplicated tectum was less pronounced than at E18.5, and at E12.5, it was even less distinct (Fig. 6B). The ectopic En2 and EphrinA2 expressions were, however, present in the E12.5 Emx2/Pax6 double mutant (Fig. 5F, H). The Fgf8 expression in the isthmus was unaffected. However, that in the commissural plate was caudally expanded by the Emx2−/− single mutation (Fig. 5O) (Fukuchi-Shimogori and Grove, 2003; Shinozaki et al., 2004); the expansion was more marked in the Emx2−/−/Pax6−/− double mutant (Fig. 5N). Consequently, the expression fused to the Fgf8 expression in the roof of the prethalamus. At E12.5, the ectopic En2 and EphrinA2 expressions (Fig. 5F, H) were juxtaposed to this Fgf8 expression (Fig. 5J). At E10.5, no ectopic En2 expression was apparent (Fig. 5L).

**Emx2 cooperates with Pax6 in forebrain development**

In E12.5 Emx2/Pax6 double mutants, histologically an amorphous structure was present at the place where normally the thalamus/pretectum develops (Fig. 6B, arrow); otherwise, diencephalic structures were not apparent, and Ammon’s horn also did not develop. In the Pax6−/− single mutant, diencephalic structures are poor but present; Ammon’s horn is also apparent (supplemental Fig. 3, available at www.jneurosci.org as supplemental material) (Stoykova et al., 1996; Warren and Price, 1997). In contrast, neither the Pax6- and Dlx1-positive prethalamus, Gbx2- and Tcf4-positive thalamus, and Tcf4- and Ebf1-positive pre-tectum are present (supplemental Fig. 3, available at www.jneurosci.org as supplemental material) (Stoykova et al., 1996). The Ebf1-negative and Pax6-/Lim1-positive commissure region of the pretectum scarcely exists. Thus, Pax6 was previously concluded to function in a fine-tuning aspect of diencephalon development by regulating cell growth, but not in determination of its territory (Stoykova et al., 1996; Warren and Price, 1997). In contrast, neither the Pax6-/Dlx1-/Lim1-positive prethalamus (Fig. 6D, F, N), Gbx2-/Tcf4-positive thalamus (Fig. 6H, J), Ebf1-positive non-commissure region of the pretectum (Fig. 6L), nor Lim1-positive commissure region of the pretectum (Fig. 6N) was apparent at all in the Emx2−/−/Pax6−/− double mutant. The Dlx1-negative supraoptic-paraventricular area was also lost (Fig. 6F). The amorphous structure did not express any of the diencephalic markers and was surrounded by the Tcf4 expression (Fig. 6J); the structure might correspond to the most rostral midbrain structure, the griseum tectalis.

The Shh expression demarcates the presumptive ZLI that terminates dorsally in the Fgf8-positive prethalamus roof; this *Shh* expression was present in the double mutant (Fig. 6P), although morphologically, ZLI was never formed. Thus, the mechanism that initiates the Shh expression is independent of forebrain development under *Emx2* and *Pax6*. The ectopic En2 and EphrinA2 expressions (Fig. 5F, H) juxtaposed posteriorly to the dorsal end of this Shh-positive stripe, suggesting that structures rostral to the stripe are not involved in the duplication. The prethalamic region between this Shh stripe and the Bf1-positive cerebrum (Fig. 6O, arrowhead) was absent in the *Emx2/Pax6* double mutant at E12.5.

**Figure 5.** Marker analysis of tectum duplication in *Emx2−/−/Pax6−/−* double mutant. *En2* expression (A, B, E, F, K, L), EphrinA2 expression (C, D, G, H), and *Fgf8* expression (J, M–O) at E15.5 (A–D), E12.5 (E–J), and E10.5 (K–O), respectively, in embryos of the genotype indicated at the top are shown. Mid-sagittal (A–J), whole-mount lateral (K, L), and whole-mount dorsal (M–O) views are shown. The arrows indicate ectopic *En2* (B, F), ectopic EphrinA2 (D, H), and *Fgf8* expression rostrally juxtaposed to the ectopic *En2* and EphrinA2 expression (J), respectively. Cr, Cerebrum; Mb, Midbrain. Scale bars: (in A, E) A–J, 500 μm; (in K) K–O, 250 μm.
Figure 6. Marker analysis of Emx2<sup>–/–</sup> Pax6<sup>–/–</sup> double mutant defects in the E12.5 diencephalon. A–N show parasagittal views. A, B, Histological sections stained with hematoxylin and eosin. C–N, The expression of each marker is indicated to the left in embryos of the genotype indicated at the top. D and P show the medial views of hemisected brains, with the BFI expression in the ganglionic eminences and pallium in orange and the Shh expression in dark blue; dotted lines contour the dorsal limits of diencephalon. The arrows in the double mutant panels indicate the ectopic structure that does not express any of the diencephalic markers indicated. The arrowhead in G indicates the prethalamic region that is absent in the Emx2/Pax6 double mutant (P). In the Pax6 single mutant, the Pax6<sup>–/–</sup>, Dlx1<sup>–/–</sup>, and Wnt6<sup>–/–</sup>-positive eminentia thalami, the Pax6<sup>–/–</sup>, Dlx1<sup>–/–</sup>, and Lim1<sup>–/–</sup>-positive prethalamus, the Gsx2<sup>–/–</sup> and Tcf4<sup>–/–</sup>-positive thalamus, and the Emx1<sup>–/–</sup> and Tcf4<sup>–/–</sup>-positive anterior pretectum are reduced but present; the Pax6<sup>–/–</sup> and Lim1<sup>–/–</sup>-positive posterior pretectum scarcely exists (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). The Emx2<sup>–/–</sup> PAX6<sup>–/–</sup> double mutant (data not shown), and the double mutant was the same as the single mutant (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). The Emx2<sup>–/–</sup> PAX6<sup>–/–</sup> double mutant defects in the E12.5 diencephalon. The entire pallium was, however, positive to Lhx2 and BF1 that are never found in the cortical hem (Fig. 7U,V) (Muzio et al., 2002) (data not shown). The Emx2<sup>–/–</sup> Pax6<sup>–/–</sup> double mutant roof at the telencephalic level (the medial part between the arrowheads in Fig. 7) did not express choroidal roof markers: neither Wnt8b nor Lhx5 (Fig. 7H, J). Instead, the Msx1 expression was intense (Fig. 7L). This was also the case in the Emx2<sup>–/–</sup> Otx2<sup>–/–</sup> double mutant roof at the telencephalic level (Fig. 1). In these telencephalic phenotypes, the Emx1<sup>–/–</sup> Pax6<sup>–/–</sup> double mutant was the same as the Pax6<sup>–/–</sup> single mutant, and the Emx1<sup>–/–</sup> Emx2<sup>–/–</sup> Pax6<sup>–/–</sup> triple mutant was the same as the Emx2<sup>–/–</sup> Pax6<sup>–/–</sup> double mutant (supplemental Fig. 4, available at www.jneurosci.org as supplemental material).

Onset of Emx2<sup>–/–</sup> Pax6<sup>–/–</sup> defects
At E10.5, the Emx2 expression in the caudal midbrain is somewhat expanded, and the Emx2-negative rostral brain is reduced in the
Emx2−/− Pax6−/− double mutant (Fig. 5L). The Shh-positive ZLI was also present at this stage (Fig. 8Ab). Of note is that at this stage a region was present between the Shh-positive stripe and BF1-positive cerebral hemispheres that corresponds to prethalamus and eminentia thalami in wild-type embryos (Fig. 8Ab, arrows); it was not apparent at E12.5 (Fig. 6P). At this stage, BF1-positive cerebral hemispheres were almost normal rostrally but somewhat reduced caudally. Pax6 and Dlx1 expressions were more properly segregated into the pallium and subpallium, respectively, at E10.5 than at E12.5 (data not shown) (Muzio et al., 2002).

At E9.5, the Fgf8 expression in the isthmic region was normally found in the Emx2/Pax6 double mutant (Fig. 8Ac,Ad); however, the En2 expression was somewhat expanded, and the En2-negative rostral brain was slightly reduced (Fig. 8Ac,Af). In wild-type embryos, Dmxb1 is expressed in the mesencephalon and a part of the presumptive anterior diencephalon was normal. The presumptive rostral diencephalon was expanded, and the Emx2/Pax6/Pax6 double mutant region was normally found in the diencephalic region and subregionalization of the brain was slightly reduced (Fig. 8Ag); the Dmxb1-positive region was slightly enlarged and the BF1- and Dmxb1-negative presumptive anterior diencephalon was reduced, but obviously present, in the Emx2/Pax6 double mutant (Fig. 8Aa). BF1-positive cerebral hemispheres were somewhat reduced. E9.5 is the stage when several markers start to be expressed in the diencephalic region and subregionalization occurs at this region. One such marker is Tcf4, which marks the future thalamus and pretectum (Fig. 8Aa); the Tcf4 expression was never observed in the Emx2−/− Pax6−/− double mutant (Fig. 8Aa). Another marker examined was Wnt7b that normally marks the future archipallium, eminentia thalami, prethalamus, and a part of the thalamus (Fig. 8Aa); the Wnt7b expression was also not found in the double mutant (Fig. 8Aa).

Finally, the analysis was conducted at the 6 somite stage for the initial defects. The anterior neuroectoderm expresses Otx2 in the region that corresponds to the future forebrain and midbrain (Fig. 8Ba), Six3 in the most anterior region (Fig. 8Bc), and Irx1 complementarily in the caudal aspect of the Otx2-positive neuroectoderm (Fig. 8Be). Pax6 is expressed in the caudal forebrain primordium (Fig. 8Bg), and Pax2 is expressed in the midbrain and eye primordium (Fig. 8Bi). No changes were apparent in any area positive to these markers in the Emx2/Pax6 double mutant (Fig. 8Bb,Bd,Bf,Bh,Bi). Thus, the prospective caudal forebrain region must be almost normally present in the Emx2−/− Pax6−/− double mutant at the 6 somite stage.

**Discussion**

**Otx2/Otx1/Emx2/Pax6 and caudal forebrain**

Previously, we have identified the enhancer responsible for the Otx2 expression in the anterior neuroectoderm (AN enhancer) and reported that the Emx2−/− Otx2ΔAN/ΔAN mutant that specifically lacks the Otx2 expression under this enhancer exhibits the same defect in caudal forebrain development (Kurokawa et al., 2004a). The Emx2 expression occurs around the 3 somite stage, and the AN enhancer activity is lost beyond the 6 somite stage. This indicates that the defect occurs around the 3–6 somite stage. The Cre knock-in mutant into the Emx2 locus demonstrated that the Emx2 and Pax6 expressions initially mainly overlap and that the double mutant defects may correspond to these Emx2 and Pax6 expressions at the 3–6 somite stage. We propose that in the anterior neuroectoderm maintained by the Otx2 expression under the AN enhancer (Kurokawa et al., 2004a), Emx2 and Pax6 establish the caudal forebrain primordium in cooperation with Otx2 and Otx1 at the 3–6 somite stage (Fig. 9). In this establishment, the Emx2 and Pax6 functions are redundant, as suggested by their single mutant phenotype; in the absence of both genes, the caudal forebrain remains unspecified even at E9.5. The caudal forebrain primordium comprises not only the future preceptum, thalamus, and prethalamus but also the eminentia thalami, archipallium, cortical hem, and choroid plexus. The choroid plexus is inherently associated with the choroidal roof; both choroid plexus cells and choroidal roof cells originated from the Emx2-positive region. Coincidentally, the marker analysis indicated that the choroidal roof was also lost in both the Emx2/Otx2 and Emx2/Pax6
scale bars, 250 μm.

**Figure B.** Onset of Emx2−/−/Pax6−/− double mutant defect. Analyses with the indicated markers at E10.5 (Aa–Ab), E9.5 (Ac–Al) and the 6 somite stage (Ba–Bj) are shown. All images are lateral views; anterior is to the left. The arrows in Ab indicate a region between BF1-positive cerebral hemispheres (orange) and the Shh-positive stripe (dark blue); the white arrowheads in Aa and Ab indicate the stripe. The arrow in Ab indicates the BF1 and Dmox1-negative diencephalic region. The asterisks in A indicate optic vesicles that are not converted into the optic cup by the Pax6 mutation (Grindley et al., 1995). The arrows in B1 and B2 indicate the expression in the eyes. Scale bars, 250 μm.

double mutants. We assume that their roofs at the telencephalic level are the midbrain roofs. In contrast, not only the midbrain but also the ganglionic eminences, neopallium, and commissural plate were formed in both Emx2/Otx2 and Emx2/Pax6 double mutants. Defects were also not apparent in ventral structures of the forebrain.

We previously reported the loss of the archipallium, cortical hem, and choroid plexus but not the thalamic structures in the Emx1/2 double mutant (Shinozaki et al., 2004). It occurs around E9.5 when the neural tube closes at the forebrain level and when the Emx1 expression takes place. In contrast, the loss of the archipallium together with thalamic structures in Emx2/Otx2 and Emx2/Pax6 double mutants is an event at the 3–6 somite stage. In the Emx1/2 double mutant, the archipallium is transformed into the choroidal roof and the roof is expanded; we consider that this is a defect in the later dorso(roof)/ventral(alar) patterning within the p4 prosomere.

**Emx2/Pax6 functions in the neopallium**

Emx2 and Pax6 are also expressed in the neopallium, and at late stages of gestation, the neopallium was abortive in both Emx2−/−/Otx2−/− and Emx2−/−/Pax6−/− double mutants (Suda et al., 2001) (Fig. 4J). One might propose that the telencephalic defects are graded, the dorsocaudally archipallium being more severely disrupted. However, we favor the view that the defects are discontinuous between the neopallium and archipallium. The archipallium territory was not formed at all, whereas the neopallium developed relatively normally at E10.5 in both Emx2−/−/Otx2−/− and Emx2−/−/Pax6−/− double mutants. The intensity of the Emx2 expression was discontinuous at a boundary between the neopallium and archipallium (Fig. 3B). Of note is the fact that cis sequences for the Emx2 and Pax6 expression in the caudal forebrain primordium at the 3–6 somite stage probably do not have the activity in the neopallium (Kimura et al., 1999; Theil et al., 2002; Kleinjan et al., 2004; our unpublished result). In addition, the enhancer of the Emx2 expression in the neopallium does not reflect the early phase of Emx2 expression with the onset later than the 7 somite stage. The enhancer of the Pax6 expression in the caudal forebrain also exists separately from that in the neopallium. We assume that the Emx2 and Pax6 expressions in the neopallium do not function in establishing a territory; rather, these genes may have been recruited with caudorostral and mediolateral gradient of their expressions for subsequent events of neopallial development such as growth and differentiation of radial glial cells, laminar development, pallial–subpallial patterning, regulation of the influx of interneurons, and cortical arealization (Chapouton et al., 1999; Bishop et al., 2000; Mallamaci et al., 2000; Stoykova et al., 2000; Heins et al., 2002; Shinozaki et al., 2002). At the 3–6 somite stage, Emx2 is also not expressed in the future ventral part (midline) of the caudal forebrain or prospective region of ganglionic eminences (Fig. 3Aa). Enhancers of the later Emx2 expression in these regions are also different from the early enhancer (Theil et al., 2002; our unpublished result); among once Emx2-positive regions, Emx2−/−/Otx2−/− and Emx2−/−/Pax6−/− double mutants developed the neopallium, ganglionic eminences, and ventral diencephalon.

The Emx2/Pax6 double mutant phenotype in the telencephalon was also analyzed by Muzio et al. (2002). We agree with their interpretation in their major issue that the neopallial territory is once formed but later respecified into a subpallial character; at E12.5, the pallium is on the way to respecification. However, Muzio et al. (2002) did not consider that the Emx2/Pax6 double mutant defect is principally the defect in the initial brain regionalization at the 3–6 somite stage and neglected the ectopic duplication of the tectum, where normally caudal forebrain is formed. The discrepancy between our view and theirs centers on the cortical hem development. By the Msx1, Otx2, and Id3 expression, they propose that the cortical hem fate also spread into the pallial field; Emx2 was believed by them to cooperate with a low level of Pax6 dorsomedially to protect the pallium against the cortical hem fate. We do not agree with this view. The cortical hem is rather reduced in the Emx2 single mutant and lost in the Emx1/2
The presumptive caudal forebrain region developed normally at the 6 somite stage in the Emx2/Pax6 double mutant (Fig. 8B); however, the region was not specified as the caudal forebrain. Instead, it transformed into an ectopic tectum in a mirror image of the original one. FGF8 is expressed in the roof of the prethalamus later at E9.5, and by the Emx2 mutation, the FGF8 expression in the commissural plate expanded caudally. Most probably this FGF8 signaling has caused the transformation, because the region is unspecified, in the double mutant. In avian, a transplantation of FGF8-soaked beads in a diencephalic region caudal but not rostral to ZLI is known to generate an ectopic midbrain in a mirror image (Crossley et al., 1996; Martinez et al., 1999). The transplantation also duplicated the tegmentum, whereas the Pax6/Emx2 double mutant did not. This is probably because the ventral region of the caudal forebrain is specified by a different genetic code.

In the Emx2/Pax6 double mutant, the caudal forebrain cells rostral to ZLI were probably not involved in the formation of the ectopic tectum. The anterior end of the ectopic En2 and EphrinA2 double mutant (Yoshida et al., 1997; Shinozaki et al., 2004). The Msx1, Otx2, and Id3 expressions are not unique to the cortical hem but also are found in the choroid plexus and roof (Fig. 7K) (Jen et al., 1997; Kurokawa et al., 2004a,b; Shinozaki et al., 2004). We consider that the incorrect Msx1, Otx2, and Id3 expression in the double mutant pallium rather represents roof character and conclude that the archipallium, choroid plexus, and cortical hem are lost in the double mutant. Lhx5 is a marker unique to the choroidal roof (Fig. 7I) (Shinozaki et al., 2004) and was also expressed in the double mutant pallium (Fig. 7J). Neither the Wnt3a-, Wnt5a-, nor Wnt2b-positive structure was formed, although Muzio et al. (2002) reported no changes in the expression of these markers. They also reported no change in the Wnt8b expression. In our double mutant, the Wnt8b expression was residual. The entire double mutant pallium expressed Lhx2 and BF1; the cortical hem never expresses Lhx2 or BF1.

**/Figure 9. Schematic representation of initial regionalization of rostral brain proposed by this study. (A) At the 2 somite stage, the Otx2 expression (orange) in the anterior neuroectoderm that covers the entire future forebrain and midbrain is regulated by the AN enhancer; its caudal limit is obscured in front of the prootic sulcus (PS) (Kurokawa et al., 2004a). This Otx2 expression protects the anterior neuroectoderm against posteriorizing signals; its loss results in the transformation of the ectoderm to the Gbx2-positive metencephalon. B, Otx2, Emx2, and Pax6 expressions occur around the 2–4 somite stage. We propose that at the 3–6 somite stage, the anterior neuroectoderm rostral to the PS first differentiates into the three primordia: rostral forebrain, caudal forebrain, and midbrain. The caudal forebrain primordium corresponds to the Emx2- or Pax6-positive domain (dark green) at this stage; their expression mainly overlaps, but the Pax6 expression extends caudally beyond the Emx2 expression (light blue). To develop the caudal forebrain, Emx2 and Pax6 function redundantly, in cooperation with Otx2 and Otx1. With the loss of both Emx2 and Pax6, the caudal forebrain territory remains unspecified and secondarily transforms into the tectum, as demonstrated by this study. The loss of Emx2 coupled with the hemizygous loss of Otx2 results in the loss of the caudal forebrain, except the posterior (commissure region of) pretectum (PPT) (Suda et al., 2001). The Pax6/Otx2 double mutant phenotype remains to be examined to confirm our proposal. C, The Pax6-positive and Emx2-negative cells generate the posterior pretectum; the Emx2 expression is incompatible with the development of these cells as indicated by the Otx2-\textsuperscript{Emx2} knock-in mutation (Suda et al., 2001). The thalamus (Th) and anterior (non-commissure region of) pretectum (APt) cells are characterized by the loss of the Emx2 expression at E9.5 (light green); however, the continuation of the Emx2 expression is compatible with the development of the rostral part of the pretectum (dark green). Most dorsomedially, the caudal forebrain extends from the pretectum roof to the cortical hem, choroid plexus, and choroidal roof. The Otx2 expression (orange) at this stage is governed by forebrain and midbrain enhancers (FM and FM2) that do not have activity in the rostral forebrain (yellow); they are active in the archipallium (Kurokawa et al., 2004b). The rostral forebrain generates the double mutant. (Shinozaki et al., 2004). The Msx1, Otx2, and Id3 expressions are not unique to the cortical hem but also are found in the choroid plexus and roof (Fig. 7K) (Jen et al., 1997; Kurokawa et al., 2004a,b; Shinozaki et al., 2004). We consider that the incorrect Msx1, Otx2, and Id3 expression in the double mutant pallium rather represents roof character and conclude that the archipallium, choroid plexus, and cortical hem are lost in the double mutant. Lhx5 is a marker unique to the choroidal roof (Fig. 7I) (Shinozaki et al., 2004) and was also expressed in the double mutant pallium (Fig. 7J). Neither the Wnt3a-, Wnt5a-, nor Wnt2b-positive structure was formed, although Muzio et al. (2002) reported no changes in the expression of these markers. They also reported no change in the Wnt8b expression. In our double mutant, the Wnt8b expression was residual. The entire double mutant pallium expressed Lhx2 and BF1; the cortical hem never expresses Lhx2 or BF1.

**/Preoptectum development**

In the pretectum, the anterior (non-commissure) region consisted mostly of the cells that once expressed Emx2, but the posterior (commissure) region consisted mostly of the cells that never expressed Emx2. In light of the role of Pax6 in the development of the commissure region of the pretectum (Stoykova et al., 1996; Schwarz et al., 1999) and cell lineage analysis with vital dye that indicates no cell influx from midbrain after the 5 somite stage (Inoue et al., 2000), it is most likely that the majority of cells in this region are Pax6-positive cells. Thus, it is probable that at the 3–6 somite stage, the Pax6 expression caudally extends beyond the end of the Emx2 expression, and this population of Emx2-negative and Pax6-positive cells mainly generates the commissure region of the pretectum (Fig. 9); these cells also contribute, although less extensively, to the anterior pretectum. Thus, the Emx2 knock-in mutant into the Otx2 locus (Suda et al., 2001) may indicate that the Emx2 expression is incompatible with the development of these cells.

**/Tectum duplication in a mirror image**

The presumptive caudal forebrain region developed normally at the 6 somite stage in the Emx2/Pax6 double mutant (Fig. 8B); however, the region was not specified as the caudal forebrain. Instead, it transformed into an ectopic tectum in a mirror image of the original one. FGF8 is expressed in the roof of the prethalamus later at E9.5, and by the Emx2 mutation, the FGF8 expression in the commissural plate expanded caudally. Most probably this FGF8 signaling has caused the transformation, because the region is unspecified, in the double mutant. In avian, a transplantation of FGF8-soaked beads in a diencephalic region caudal but not rostral to ZLI is known to generate an ectopic midbrain in a mirror image (Crossley et al., 1996; Martinez et al., 1999). The transplantation also duplicated the tegmentum, whereas the Emx2/Pax6 double mutation did not. This is probably because the ventral region of the caudal forebrain is specified by a different genetic code.

In the Emx2/Pax6 double mutant, the caudal forebrain cells rostral to ZLI were probably not involved in the formation of the ectopic tectum. The anterior end of the ectopic En2 and EphrinA2
expression was juxtaposed to the caudal end of the Fgf8 expression where the Shh-positive stripe ended dorsally. At E10.5, a region existed between this Shh-positive stripe and Bfgl-positive cerebral hemispheres, whereas this region was not identifiable by E12.5 (Figs. 6P, 8Ab). The cause of the loss of this region remains for future studies; preliminary bro-modeoxyuridine uptake and terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling assays suggested no marked decrease in cell proliferation or increase in cell apoptosis in this region at E10.5 (data not shown). The region might be lost by posteriorization with the anterior shift of the Shh-positive stripe.

References
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